Pregnancy influences the selection of appropriate reference genes in mouse tissue: Determination of appropriate reference genes for quantitative reverse transcription PCR studies in tissues from the female mouse reproductive axis

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Title: Pregnancy influences the selection of appropriate reference genes in mouse tissue: Determination of appropriate reference genes for quantitative reverse transcription PCR studies in tissues from the female mouse reproductive axis.

Abstract

Selecting stably expressed reference genes which are not affected by physiological or pathophysiological conditions is crucial for reliable quantification in gene expression studies. This study examined the expression stability of a panel of twelve reference genes in tissues from the female mouse reproductive axis and the uterus. Gene expression studies were carried out using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). cDNA was synthesised from RNA extracted from hypothalami, pituitaries, ovaries and uteri of female mice at ages representing weaning, puberty and adulthood as well as pregnancy (13 ± 1 days post-coitus) (n = a minimum of 3 at each age and at pregnancy). The reference genes examined included *18s*, *Actb*, *Atp5b*, *B2m*, *Canx*, *Cyc1*, *Eif4a2*, *Gapdh*, *Rpl13a*, *Sdha*, *Ubc* and *Ywhaz*. The RT-qPCR raw data were imported into the qBASE+ software to analyse the expression stability using GeNorm. These data were also subsequently analysed using other software packages (Delta CT, Normfinder, BestKeeper). A comprehensive ranking was conducted considering all stability rankings generated from the different software analyses. *B2m* and *Eif4a2* deviated from the acceptable range for amplification efficiency and therefore were excluded from the further analyses. The stability of the reference genes is influenced by the software used for the analysis with BestKeeper providing markedly different results than the other analyses. GeNorm analysis of tissues taken at different ages but not including pregnant animals, indicated that the expression of the reference genes is tissue specific with the most stable genes being: in the hypothalamus, *Canx* and *Actb*; in the pituitary, *Sdha* and *Cyc1*; in the ovary, *18s, Sdha* and *Ubc*; and in the uterus, *Ywhaz, Cyc1, Atp5b*, *18s* and *Rpl13a*. The optimal number of reference genes to be used was determined to be 2 in the first three tissues while in the uterus, the V-score generated by the GeNorm analysis was higher

than 0.15 suggesting that 3 or more genes should be used for normalisation. Inclusion of tissues from pregnant mice changed the reference genes identified as being the most stable: *Ubc* and *Sdha* were the most stable genes in the hypothalamus, pituitary and the ovary. The addition of pregnant tissue had no effect on the stability of the genes in uterus (*Ywhaz, Cyc1, Atp5b*, *18s* and *Rpl13a*). Identification of these stable reference genes will be of use to those interested in studying female fertility and researchers should be alert to the effects of pregnancy on reference gene stability. This study also signifies the importance of re-examining reference gene stability if the experimental conditions are changed as shown with the introduction of pregnancy as a new factor in this research.

Introduction

Mammalian female fertility requires the co-ordinated activity of the reproductive axis (the hypothalamus, pituitary and ovaries hence also called the hypothalamo-pituitary-ovarian axis (HPO axis)) and the uterus. Co-ordination between these four organs is predominately hormonal but also includes regulation by both the nervous and immune systems. The regulation is dynamic: it changes both over the life cycle (prepubertal, puberty, adulthood, pregnancy, lactation and senescence) and over the reproductive cycle (humans = menstrual cycle; most other mammals = oestrous cycle). Effective communication between the hypothalamus, pituitary and ovary ensure that an oocyte/oocytes (the female gamete) is/are ovulated in each reproductive cycle, that the female is receptive to a male at the ideal time to maximize the chances of fertilization and that the uterus is prepared to receive and nurture any resulting embryo/embryos. In order to achieve all these functions both the ovary and the uterus undergo cycles of tissue remodelling. It is therefore likely that within each of the four organs there are significant changes in gene expression dependent on the reproductive status of the female. Analysis of gene expression in the female reproductive axis can be determined by reverse transcription real-time quantitative PCR (RT-qPCR), however interpretation of these results requires the identification of stable reference genes.

The most commonly used reference genes to normalise expression include 18S and 28S ribosomal RNA subunits (rRNA), *18s* and *28S*, glyceraldehyde-3-phosphate dehydrogenase, *GAPDH,* and actin-beta, *ACTB* (Suzuki, et al., 2000). A significant portion of total RNA extracted from tissue is rRNA, transcription of which is affected by chromatin remodelling and influenced by biological factors, therefore the reliability of rRNA genes for normalisation has been questioned (Vandesompele, et al., 2002). Expression of both *GAPDH* and *ACTB* have been reported to vary in human adult tissues (Warrington, et al., 2000) and are altered by mitogenic stimuli (Radonić, et al., 2004). Gene expression stability may also be influenced by physiological conditions: in a comparison of mouse brain regions, it was observed that there was a significant down-regulation of candidate reference genes in adult compared to day 7 mice (Boda, et al., 2009). Reliance on a single reference gene may lead to erroneous gene normalisation and hence the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines recommend evaluating whether two, three or more reference genes are required for normalisation (Vandesompele, et al., 2002; Bustin, et al., 2009).

To determine reference gene stability the most commonly used analytical tools include: GeNorm (Vandesompele, et al., 2002), NormFinder (Andersen, et al., 2004), BestKeeper (Pfaffl, et al., 2004), Delta CT and comprehensive gene stability. Here we have assessed twelve reference genes by RT-qPCR using each of the above analyses to check gene stability in the female mouse hypothalamus, pituitary, ovary and uterus at stages in the reproductive life cycle (pre-weaning, puberty, adult) and during pregnancy.

Materials and Methods:

Animals

Female C57BL/6 mice were housed in rooms with a 12:12 hour light: dark cycle (lights on at 0700) at 21 ± 1°C. *Ad libitum* access to both standard laboratory chow and water were provided for all

animals. Ethics approval for this study was granted by the University of Westminster (Ethics Approval VRE1415-0209). These studies were done in full accordance with the Home Office's Animal (Scientific Procedures) Act (1986).

Animals were killed by dislocation of the neck followed immediately by decapitation to allow both the brain and pituitary to be dissected free as soon as possible. Likewise, both the ovaries and the uterus were dissected free from the abdomen immediately after death. Hypothalami, pituitaries, ovaries and uteri were removed from virgin female mice aged 2, 6, 9/10 and 14 weeks and pregnant mice (9 weeks at mating and approximately 13 days *post-coitus* (dpc) of gestation based on detection of mating plug). Tissues from at least 3 mice for each age group and pregnancy were used. All tissues were immediately placed in RNA*Later*® and stored at -80°C until RNA isolation commenced.

RNA Isolation and cDNA Synthesis

RNA was extracted using TRIzol (Invitrogen™, ThermoScientific, UK) and then purified using a RNeasy micro kit (Qiagen, Germany). Total RNA quality and concentration were measured using a Nanodrop 2000® (a 260:280 nm ratio > 1.8 was deemed acceptable quality). cDNA was synthesised using SuperScript II Reverse Transcriptase (200U), approximately 300 ng RNA and random hexamer primers (150ng, Invitrogen™, ThermoScientific, UK). The cDNA samples were stored at -20°C until required.

Determination of reference gene amplification efficiency

The PCR amplification efficiencies and R^2 coefficients were determined for all reference genes included in this study. A standard curve for each tissue (hypothalamus, pituitary gland, ovary and uterus) was generated using pooled cDNA from all age groups plus pregnant samples. The pooled cDNA (starting concentration 1 μg) was used to create 1:10 serial dilutions spanning 5 orders of magnitude and each concentration was assessed in triplicate. The primer efficiencies were

generated using the CFX96 C1000 touch real time qPCR machine and CFX Maestro software (BIO-RAD, UK).

The GeNorm reference gene panel and RT-qPCR

The GeNorm kit, which contains a panel of 12 genes (*18s*, *Actb, Atp5b, B2m, Canx, Cyc1, Eif4a2, Gapdh, Rpl13a, Sdha, Ubc* and *Ywhaz*; Table 1), was used to select the reference genes (PrimerDesign, UK). Each RT-qPCR mix consisted of: master mix (premixed with SYBR green) (10X PrecisionPLUS^{TM}, UK), reference primers (reference gene details listed in Table 1) (6 pmol) and 25 ng of cDNA. Reactions, done in duplicate, were placed in a thermocycler (CFX96 C1000 touch, BIO-RAD, UK) and underwent the following cycle: initial activation at 95°C for 2 minutes; followed by 40 cycles of denaturing at 95°C for 15 seconds and annealing and extension at 60°C for 30 seconds; and ending with a dissociation melt curve (60-95°C). The size of each amplicon for each reaction was determined by electrophoresis on a 2 % agarose gel (Table 1).

Determination and validation of reference gene stability

GeNorm calculates a normalisation factor based on multiple reference genes. GeNorm software hypothesises that two or more normalisers (reference genes) should have the same expression ratio across all samples and experimental conditions. A measure, “M”, which reflects the reference gene stability is established through the standard deviation of the logarithmically transformed expression cycle threshold (CT) ratios. The most stable genes will have the lowest “M” values. The normalisation factor is then calculated using the geometric mean from expression levels of the most stable reference genes. This software recommends the use of at least 2-3 of the most stable reference genes to calculate the normalisation factor as adding a fourth gene does not significantly contribute to the expression stability analysis (Vandesompele, et al., 2002).

NormFinder is a mathematical model describing the RT-qPCR log CT values, estimation of inter- and intra-group variations, sub-group analyses and, finally, the calculation of stability values of the reference genes examined. The reference genes are then ranked according to their stability value

with the most stable gene having the lowest stability value (Andersen, et al., 2004; Palombella, et al., 2017).

BestKeeper was developed on the basis that the most stable reference genes show the least variability in their CT values across the samples examined (Pfaffl, et al., 2004). The software algorithm utilises the reference gene's CT data calculating the geometric mean, the arithmetic mean, and standard deviation (St. Dev); represented as minimum CT, maximum CT and CT St. Dev. Reference genes with a CT St. Dev greater than one are considered unstable. To be able to choose more than one stable gene, a pair-wise correlation analysis is done to estimate the inter-gene relations of all possible pairs: a BestKeeper index combines the most stably expressed reference genes. Then, the Pearson correlation coefficient, the coefficient of determination and the p-value statistically describe the correlation between this index and the reference genes (Pfaffl, et al., 2004; Palombella, et al., 2017).

Delta-CT is based on a process of exclusion: the relative expression of a pair of genes is compared in different samples using the delta CT and St. Dev. Stable genes would not show any variability in the different samples. This procedure is used with more than one pair and various conditions and pairs that show variable expression among samples, and hence have fluctuating expression stability, are excluded.

Comprehensive ranking. In addition to these four algorithms, RefFinder (web-based tool) was used to generate an overall comprehensive ranking for the reference genes. The RefFinder algorithm assigns an appropriate weight for each gene and by incorporating the ranking numbers from GeNorm, NormFinder, BestKeeper and DeltaCT, calculating the geometric mean of the weights for the overall final ranking of genes (Xie, et al., 2012).

Results:

Reference gene amplication efficiency

Amplification efficiencies were determined for the GeNorm panel and ten of the reference genes (*18s, Actb, Atp5b, Canx, Cyc1, Gapdh, Rpl13a, Sdha, Ubc* and *Ywhaz*) had amplification efficiencies in the acceptable range of 90-100% (Table 2). Two of the twelve reference genes, *B2m* and *Eif4a2,* were found to have amplification efficiencies deviating from the accepted range, despite numerous repetitions and troubleshooting, hence they were excluded from the subsequent GeNorm analyses.

GeNorm Analyses

RT-qPCR amplification of the reference genes (*18s, Actb, Atp5b, Canx, Cyc1, Gapdh, Rpl13a, Sdha, Ubc* and *Ywhaz*) was carried out on tissues from the female reproductive axis in pre-weaned (2 week), pubertal (6 week), adult (9, 10 and 14 week) and pregnant mice (9 weeks 13 dpc) with three animals in each group. All genes amplified before 28 cycles and the minimum and maximum CT values for each reference gene are presented in Figure 1A. To determine the optimal number of genes for the RT-qPCR GeNorm analysis, as advised in MIQE guidelines, a pair-wise variation (V-score) was calculated using the GeNorm software (Figure 2). The V-score was below 0.15 for the hypothalamus, pituitary and ovary (Figure 2 A-C) and the mean pairwise variation indicated that two genes should be used for normalisation; however, the V-score was higher than 0.15 for the uterus (Figure 2 D) suggesting that 3 or more genes should be used for normalisation.

Effect of pregnancy on reference gene stability

The RT-qPCR data were organised into two sets: A) age groups alone (excluding the pregnant samples) and B) all the samples (age groups including pregnant samples). Using the GeNorm tool, inclusion of pregnant samples changed the stability values of the reference genes in the hypothalamus, pituitary and ovary (Figure 3).

In the female mouse hypothalamus, the non-pregnant data set analysed by GeNorm identified that *Canx, Actb, Atp5b* and *Ywhaz* (listed in order of stability: highest to lowest; all lists that follow are presented in the same way) were the most stable genes while *Cyc1* and *18s* were the least stable genes (Figure 3: A1). When the pregnant samples were included, *Cyc1* and *18s* remained the least stable genes while *Ubc/Sdha, Ywhaz and Canx* became the most stable genes in the hypothalamus (Figure 3: B1).

In the pituitary gland, *Sdha*, *Cyc1, Ubc and Atp5b* were identified by GeNorm as the most stable genes in the non-pregnant data set (Figure 3: A2) whilst *Ubc, Sdha, Atp5b* and *18s* were the most stable genes when the pregnant samples were included in the analysis (Figure 3: B2). The least stable genes for the pituitary were *Canx* and *Ywhaz* for both data sets. The remaining genes showed a slight fluctuation in the expression.

In the GeNorm analysis of the ovary, *18s/Sdha, Ubc* and *Ywhaz* were identified as the most stable genes in the non-pregnant data set (Figure 3: A3). When pregnant samples were included *Sdha*, *Ubc*, *Ywhaz* and *Atp5b* were identified as the most stable genes (Figure 3: B3). *Actb* and *Cyc1* were both identified as the least stable genes in the female mouse ovary in both the non-pregnant and with pregnant data sets.

In the uterus, fewer genes had stability values less than 1 compared to the other tissues. There was no difference in the ranking for the two data sets (Figure 3: A4 and B4). The most stably expressed genes identified by the GeNorm analysis were *Ywhaz, Cyc1*, *Atp5b and 18s* for both data sets (Figure 3: A4 and B4). *Sdha* and *Ubc* were the least stable genes in both data sets in the uterus (Figure 3: A4 and B4).

Comparison of gene stability using GeNorm NormFinder, BestKeeper, Delta CT and Comprehensive ranking

Data from the RT-qPCR of the panel of genes (*18s, Actb, Atp5b, Canx, Cyc1, Gapdh, Rpl13a, Sdha, Ubc* and *Ywhaz*) in the hypothalamus, pituitary gland, ovary, and uterus were also analysed using

NormFinder, BestKeeper, Delta CT and Comprehensive ranking and are shown below for the two data sets: the non-pregnant samples only (- pregnant) and the non-pregnant and pregnant samples (+ pregnant).

Summary of the most stable genes in each tissue using GeNorm, NormFinder, BestKeeper, Delta CT and Comprehensive ranking analyses:

Hypothalamus

GeNorm:	*Canx > Actb > Atp5b > Ywhaz > Ubc*	- Pregnant
	Ubc > Sdha > Ywhaz > Canx > Actb	+ Pregnant
NormFinder:	*Ywhaz > Canx > Atp5b > Actb > Sdha*	- Pregnant
	Canx > Atp5b > Ywhaz > Sdha > Ubc	+ Pregnant
BestKeeper:	*Atp5b > Canx > Actb > Ywhaz > Sdha*	- Pregnant
	Atp5b > Canx > Actb > Ubc > Ywhaz	+ Pregnant
Delta CT:	*Ywhaz > Canx > Atp5b > Sdha > Actb*	- Pregnant
	Canx > Ywhaz > Atp5b > Ubc > Sdha	+ Pregnant
Comprehensive:	*Canx > Ywhaz > Atp5b > Actb > Sdha*	- Pregnant
	Canx > Atp5b > Ubc > Ywhaz > Sdha	+ Pregnant

Pituitary:

GeNorm:	*Sdha > Cyc1 > Ubc > Atp5b > Actb*	- Pregnant
	Ubc > Sdha > Atp5b > 18s > Actb	+ Pregnant
NormFinder:	*Atp5b > Actb > Cyc1 > 18s > Sdha*	- Pregnant
	Atp5b > Actb > Cyc1 > Ubc > Sdha	+ Pregnant
BestKeeper:	*Ywhaz > Canx > Actb > Atp5b > 18s*	- Pregnant
	Ywhaz > Canx > Actb > Atp5b > 18s	+ Pregnant
Delta CT:	*Atp5b > Cyc1 > Sdha > Actb > Ubc*	- Pregnant
	Atp5b > Cyc1 > Ubc > Actb > Sdha	+ Pregnant
Comprehensive:	*Atp5b > Ubc > Actb > Cyc1 > Sdha*	- Pregnant
	Atp5b > Cyc1 > Sdha > Actb > Ubc	+ Pregnant

Ovary:

GeNorm:	*18s = Sdha > Ubc > Ywhaz > Atp5b*	- Pregnant
	Sdha > Ubc > Ywhaz > Atp5b_ > 18s	+ Pregnant
NormFinder:	*Ywhaz > 18s > Canx > Sdha > Ubc*	- Pregnant
	Atp5b > Gapdh > Ywhaz > Canx > Rpl13a	+ Pregnant
BestKeeper:	*18s > Atp5b > Ubc > Gapdh > Canx*	- Pregnant
	18s > Gapdh > Atp5b > Ywhaz > Actb	+ Pregnant
Delta CT:	*18s > Canx > Ywhaz > Gapdh > Atp5b*	- Pregnant
	Ywhaz > Atp5b > Gapdh > Rpl13a > Canx	+ Pregnant
Comprehensive:	*18s > Atp5b > Canx > Ywhaz > Gapdh*	- Pregnant
	Atp5b > Gapdh > Ywhaz > Canx > Rpl13a	+ Pregnant

Uterus:

GeNorm:	*Ywhaz > Cyc1 > Atp5b > 18s > Rpl13a*	- Pregnant
	Ywhaz > Cyc1 > Atp5b > 18s > Rpl13a	+ Pregnant
NormFinder:	*Actb > 18s > Atp5b > Canx > Gapdh*	- Pregnant
	Actb > Atp5b > 18s > Canx > Gapdh	+ Pregnant
BestKeeper:	*Cyc1 > Rpl13a > Atp5b > 18s > Actb*	- Pregnant
	Ywhaz > Cyc1 > Rpl13a > Atp5b > 18s	+ Pregnant
Delta CT:	*Actb > 18s > Atp5b > Canx > Gapdh*	- Pregnant
	Atp5b > 18s > Actb > Canx > Ywhaz	+ Pregnant
Comprehensive:	*Actb > 18s > Atp5b > Cyc1 > Rpl13a*	- Pregnant
	Atp5b > Ywhaz > Cyc1 > 18s > Actb	+ Pregnant

In the hypothalamus for the non-pregnant data set, the other tools all identified four of the same genes as the GeNorm tool had as being one of the five most stable genes. Whereas the GeNorm analysis had identified *Ubc* as also being stable, the other tools all identified *Sdha* in its place. The rankings did differ between the tools but all identified *Canx* as one of two most stably expressed

genes. When the pregnant samples were included the ranking of the five most stable genes changed and *Atp5b* was replaced by *Sdha* by the GeNorm tool. The two most stably expressed genes were different to the two most stably expressed genes in the non-pregnant data set using GeNorm. By contrast, both the BestKeeper and Delta CT identified the same pair of genes as being the most stable for both data sets and the other two tools both identified *Canx* as being one of the two most stable genes in both data sets.

In the pituitary for the non-pregnant data set, both the Delta CT and Comprehensive tools identified the same five most stable genes as the GeNorm tool although the rank order did differ. The GeNorm analysis had identified *Sdha* and *Cyc1* as being the two most stable genes, but only the Delta CT tool also gave *Cyc1* in its top two. Three of the tools identified *Atp5b* as the most stably expressed gene. When the pregnant samples were included the ranking of the five most stable genes changed and *Cyc1* was replaced by *18S* using GeNorm. The two most stably expressed genes had one in common (*Sdha*) and *Ubc* replaced *Cyc1* as the two most stably expressed genes. There was variability in the selection of genes and their rank order between the other tools compared to the GeNorm analysis. If only the top two genes are considered all the other tools identified the same genes for both data sets (except the Comprehensive tool which only had one gene in common) unlike the GeNorm analysis but there were differences between the tools as to which two genes were identified.

In the ovary for the non-pregnant data set, the other tools identified three to four of the same genes as the GeNorm tool amongst the five most stable genes. The other tools all identified *Canx* as one of the top five stably expressed genes but the GeNorm analysis did not. The rankings did differ between the tools but all identified *18s* as one of two most stably expressed genes. When the pregnant samples were included, the genes identified were the same but the ranking of the five most stable genes changed using GeNorm. The two most stably expressed genes were *Sdha* and *Ubc* compared to *18s* and *Sdha* as the most stably expressed genes in the non-pregnant data set. Using the other tools with respect to the two most stably expressed genes, the BestKeeper and

Comprehensive tools both identified the same gene in both data sets (but not the same gene and different from that identified by GeNorm analysis). Both NormFinder and Delta CT had no genes in common between the non-pregnant and with pregnant data sets for the two most stably expressed genes.

In the uterus for the non-pregnant data set, there was variability in the genes identified by each tool. The GeNorm analysis identified *Ywhaz* as being the most stably expressed but it was not identified as one of the five most stably expressed by any of the other tools. All tools identified *Atp5b* as the third most stably expressed gene and all included *18s* in their top five ranking. When the pregnant samples were included the ranking of the five most stable genes was unaltered using GeNorm. The two most stably expressed genes were different to the two most stably expressed genes in the non-pregnant data set. The other tools differed with up to two genes being replaced by others and the rank order changing. The NormFinder, BestKeeper and Delta CT tools all identified one of the two genes most stably expressed as being the same (albeit each a different gene) in both the non-pregnant and with pregnant data sets.

Discussion:

According to the criteria recommended by the MIQE guidelines, adding more than one reference gene for data normalisation reduces the variability of the RT-qPCR data (Bustin, et al., 2009). The GeNorm analyses for the hypothalamus, pituitary and ovary studied herein confirm that optimally two or more genes should be used instead of a single gene. The V values for the uterus were above 0.15 hence in this tissue, the use of more than two reference genes would be recommended. The reference gene selection assay included a panel of 12 murine housekeeping genes, however, the amplification efficiencies for *B2m* and *Eif4a2* deviated from the accepted range so were not included in these analyses. Ten murine housekeeping genes (*18s, Actb, Atp5b, Canx, Cyc1, Gapdh, Rpl13a, Sdha, Ubc* and *Ywhaz*) were therefore investigated for their stability in tissues from the female reproductive axis in pre-weaned, pubertal, adult and pregnant mice.

The reference genes, *18s, Gapdh* and *Actb*, have been widely used as reference genes for these tissues however in the hypothalamus, *18s and Gapdh* were amongst the least stable reference genes. In the pituitary, the *18s* stability ranking was affected once pregnant samples were included by GeNorm analysis. In the ovary, *Actb* was the least stable reference gene identified by GeNorm analysis. These findings that *18s, Gapdh* and *Actb* are potentially pernicious reference genes are consistent with reports in human brain, kidneys and heart as well as rat fetal brain (Vandesompele, et al., 2002; Al-Bader, & Al-Sarraf, 2005 respectively) . Furthermore, the ranking of expression stability of the reference genes differs in each tissue; for example, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide, *Ywhaz,* was one of the most stable genes in the hypothalamus, ovary and uterus whilst in the pituitary gland it was the least stable gene. Whilst ranked amongst the 5 most stable genes in the hypothalamus (*Sdha* was ranked 6th most stable in the non-pregnant data set), pituitary and ovary using the GeNorm tool, both *Ubc* and *Sdha* were unstable in the uterus. This highlights the importance of checking the reference gene stability in the context of each experiment as an essential step in quantitative RT-PCR (Bustin, et al., 2009).

All of the reference genes' gene products are known to be involved in essential intracellular functions hence they are expressed in most cells. Their relative expression may be different in some tissues and/or altered by physiological status. Except for the ranking obtained from the BestKeeper tool using the non-pregnant data set, *Ywhaz* was identified as being one of the most stable genes in the ovaries in both the non-pregnant and with pregnant data sets. *Ywhaz* encodes for a molecule that forms a hub for several signalling pathways within cells (Sluchanko, 2018). No information on the pattern of *Ywhaz* expression or its function, and therefore regulation, within the mouse ovary is known. Although *Ywhaz* (using *Gapdh* as a reference gene) has been identified as being differentially expressed within the corpus luteum, a transient, hormone-secreting structure of the bovine ovary (Goravanahally, et al., 2009), when tissue was sampled from different areas of the ovary (so with and without tissue of the corpus luteum) and at different times of the oestrous cycle or in pregnancy, the NormFinder and BestKeeper tools but not GeNorm, identified *Ywhaz* as one of

the top four most stably expressed genes (Schoen, et al., 2015). In this study we extracted mRNA from entire mouse ovaries taken at different times of the oestrous cycle. The bovine ovary is approximately 500 fold greater in mass than the mouse ovary hence researchers will tend to sample the tissue rather than extracting mRNA from the entire organ. Great care is therefore required when sampling a tissue as heterogeneous as the ovary to ensure it is representative of tissue being investigated. Another example of the differences between tissue and the effects of physiological status effects is *Sdha*, which produces a subunit of the mitochondrial enzyme, succinate dehydrogenase. Succinate dehydrogenase participates in both the electron transport chain and the citric acid cycle. Although succinate dehydrogenase has essential intracellular functions, its expression will vary since it is well established that the number of mitochondria per cell varies between cell types and that within a cell type can be affected by cell cycle stage and stress (Cole, 2016). Using the GeNorm tool, *Sdha* was detected as one of the stably expressed genes in the hypothalamus, pituitary gland and the ovary in both the non-pregnant and pregnant data sets. By contrast, it was not regarded as being stably expressed in the uterus in either the non-pregnant or pregnant data sets. The uterus undergoes regular tissue re-modelling in response to hormone-induced changes over the oestrous cycle in the non-pregnant animal as well as during pregnancy. Others have demonstrated that uterine expression of *18s*, *Actb* and *Gapdh* are altered by sex steroids (Durrer, et al., 2005; Craythorn, et al., 2009; Schroder, et al., 2009). Oestrogen receptor 2 (beta), *Esr2*, has been detected in the mitochondria of the rabbit uterus so it is possible that *Sdha* uterine expression could be modulated by oestrogen (Monje, & Boland, 2001).

Other factors that may affect the stability of reference gene expression within a tissue include age and gender. In this study, tissues from pre-weaned (2 week), pubertal (6 week) and adult (10 week and 14 week) animals were included in the GeNorm analyses. Only the BestKeeper tool identified *Ywhaz*, as one of the top 5 most stable genes in the pituitary. The GeNorm tool identified it as the least stable of the 10 genes in both the non-pregnant and pregnant data sets. This is in stark contrast to a study aimed to identify the most stable genes in both healthy and tumorous pituitaries from humans, mice and dogs (Van Rijn, et al., 2014). Using both the GeNorm and NormFinder tools,

YWHAZ/Ywhaz was identified as one of the most stable genes in all 3 species and in both healthy and tumorous pituitaries. No information on the gender and age of the subjects for all 3 species, or the breed of the dogs, is provided. These examples all illustrate the care required in providing full descriptions of the samples being compared, even when following the MIQE guidelines, to identify the best experiment-specific reference genes to use.

Although differences have been reported in the mouse brain between day 7 mice and adults (Boda, et al., 2009), we did not observe differences in reference gene stability between pre-weaned and adult mice in either the hypothalamus or pituitary. However, when pregnant adults were also considered, the ranking of gene stability was affected in the hypothalamus, pituitary and ovary with reference genes in the uterus not altered when using the GeNorm analysis. Using the GeNorm tool, adding the pregnant samples to the analysis changed the two most stable genes identified in all the tissues except the uterus. Pregnancy causes substantial gene changes in different systems and axes since the maternal body has to make a myriad of physiological adaptations to maintain the pregnancy. Evidence for these substantial gene changes is supported by RNA-seq and microarray findings: the reference genes overlap many different systemic pathways including inflammatory, metabolic, genetic processing and cellular responses (Mittal, et al., 2015; Knight, et al., 2018). Taken together these findings support the RT-qPCR analyses reported here that pregnancy influences the stability and expression of commonly used reference genes in the hypothalamus, pituitary and ovarian tissue.

There was no apparent effect of pregnancy on the stability of the reference genes in the mouse uterus. Consistent with our findings, reference genes expression analysis of the swine uterus showed similar stability ranking across different reproductive stages except in the later stages of pregnancy (Martínez-Giner, et al., 2013). This study considered mid-pregnancy (day 10), a time when the myometrium of the uterus is quiescent and steroid production by both the ovaries and placentae relative constant. As described above, others have demonstrated that the expression of some commonly used reference genes are altered in the uterus in response to sex steroids, in

particular oestrogen (Durrer, et al., 2005; Craythorn, et al., 2009; Schroder, et al., 2009). It is anticipated therefore that there may be differences in reference gene expression in early pregnancy, when the endometrium of the uterus undergoes decidualization to provide support to the newly implanted embryos whilst the placentae are establishing, and then again at the end of the pregnancy when the myometrium undergoes many changes to enable it to act as a syncytium during parturition. At both these stages there are significant changes from the normal hormonal milieu.

Another criterion investigated in this study were the different algorithms that investigate reference gene expression stability. There are various applications and software packages that are designed for this purpose and each one of them might produce different patterns of stability depending on the calculation method used (Kozera, & Rapacz, 2013). In the present study, we have used the GeNorm analysis, which is based on the delta delta CT method, as our reference tool. This analysis has been criticised due to the influence of PCR efficiencies where GeNorm data can be skewed if these efficiencies vary from 100% (Robledo, et al., 2014). However, GeNorm analysis has been the most commonly adopted tool by researchers as it also enables the determination of the optimum number of reference genes to be selected based on a pairwise variation of n versus n+1 reference genes (De Spiegelaere, et al., 2015). The MIQE guideline criteria emphasise the use of more than one reference gene for data normalisation and GeNorm software is the only software that includes this criterion in the reference genes stability analysis. The addition of pregnant samples to the analysis altered the gene pair to be used as references when using the GeNorm analysis for the hypothalamus, pituitary and ovary but the same effect was only detected using the Bestkeeper or Delta CT tools with the ovary. For each of the tissues the least concurrence in the genes found to be the five most stable were those identified by the GeNorm analysis. Other studies have also observed differences in reference gene stability using the BestKeeper analysis (Petriccione, et al., 2015; Garson, et al., 2019) perhaps because the BestKeeper analysis relies on the use of non-corrected raw Cq values, which is a completely different approach to the expression stability analysis

compared to the other software (De Spiegelaere, et al., 2015). It is therefore unclear which is the ideal tool to use and further research is required.

From this study, we conclude that it is necessary to examine the stability of the reference genes before conducting any gene expression analyses using RT-qPCR. Another essential aspect to consider is the examination of the expression stability of reference genes according to the experimental conditions of any research; if the experimental conditions are to be changed the expression stability needs to be checked again to ensure there are no fluctuations in the expression of the stable genes. Once a gene is found to be stable in a tissue, investigators should be alert to the influences of physiological status on the reference genes; we have found that inclusion of pregnant samples affects the ranking of gene stability in the hypothalamus, pituitary and ovary but not uterus. This is likely due to the significant changes required to enable the maternal physiological adaptations to pregnancy and further investigation is merited to determine whether the stage of pregnancy elicits different changes in reference gene stability.

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Table 1: Reference genes with their accession numbers (GeNorm kit, PrimerDesign), reference genes names, function, accession numbers, anchor nucleotide and product length. PCR amplicon sizes were confirmed independently with 2% agarose gel electrophoresis.

Gene	Full name of the gene name	Function	Accession number	Anchor nucleotide	Amplicon size (bp)
18s	18S ribosomal RNA	RNA constituent	NR_003278.3	134	93
Actb	β-actin	Nucleosome binding –cytoskeleton component	NM_007393.3	597	84
Atp5b	ATP synthase	ATP biosynthesis	NM_016774.3	1115	112
B2m	β-2-microglobulin	Glycoprotein binding	NM_009735.3	202	120
Canx	Calnexin	Ca^{+} binding activity	NM_007597.3	2827	105
Cyc1	Cytochrome c isoform 1	Electron activity	NM_025567.2	514	141
Eif4a2	eukaryotic translation initiation factor 4A2	ATPase and helicase activity	NM_013506.2	876	152
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	Metabolism	NM_008084.2	793	127
Rpl13a	ribosomal protein L13a	RNA constituent	NM_009438.5	691	130
Sdha	succinate dehydrogenase complex flavoprotein subunit A	Mitochondrial electron transport	NM_023281.1	2018	133
Ubc	ubiquitin C	Protease binding	NM_019639.4	2225	129
Ywhaz	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta	Cell signalling	NM_011740.3	1045	141

Table 2: The PCR efficiencies and R^2 coefficients values for the reference genes in the female (pregnant + age groups) mouse hypothalamus, pituitary gland, ovary and uterus. The PCR efficiency was conducted using standard curves (1:10 dilution covering 5 orders of magnitude) with a starting concentration of the cDNA (1 µg). The cDNA was pooled from all age groups and pregnant samples for each tissue separately. *: genes with poor efficiency that is deviate from the accepted range.

HYPOTHALAMUS			
Reference gene	PCR efficiency (%)	R^2 Coefficient	M (Slope)
18S	97	0.9876	-3.39
ACTB	95	0.9906	-3.45
ATP5B	99	0.9902	-3.34
B2M*	73	0.8591	-4.2
CANX	96.45	0.986	-3.41
CYC1	105	0.9905	-3.2
EIF4A2*	135	0.8267	-2.7
GAPDH	108.20	0.9694	-3.14
RPL13A	98	0.9911	-3.37
SDHA	101	0.9911	-3.28
UBC	100	0.9908	-3.32
YWHAZ	98	0.9875	-3.36

PITUITARY			
Reference gene	PCR efficiency (%)	R^2 Coefficient	M (Slope)
18S	104	0.9943	-3.22
ACTB	98	0.9765	-3.371
ATP5B	102	0.9907	-3.27
B2M*	83.9	0.9409	-3.78
CANX	95	0.9902	-3.44
CYC1	98.96	0.99	-3.347
EIF4A2*	145.8	0.7646	-2.56
GAPDH	101.8	0.9899	-3.28
RPL13A	93	0.9776	-3.5
SDHA	95	0.9854	-3.45
UBC	97	0.9907	-3.389
YWHAZ	100	0.9913	-3.319

OVARY			
Reference gene	PCR efficiency (%)	R^2 Coefficient	M (Slope)
18S	99	0.9894	-3.342
ACTB	90	0.99	-3.578
ATP5B	100	0.9906	-3.298
B2M*	86.64	0.9691	-3.69
CANX	100	0.99	-3.323
CYC1	93	0.9743	-3.51
EIF4A2*	121.8	0.943	-2.891
GAPDH	98.82	0.9922	-3.352
RPL13A	96	0.9961	-3.42
SDHA	100.97	0.9878	-3.299
UBC	100	0.99	-3.317
YWHAZ	100	0.9759	-3.32

UTERUS			
Reference gene	PCR efficiency (%)	R^2 Coefficient	M (Slope)
18S	100	0.9934	-3.316
ACTB	98.39	0.9917	-3.361
ATP5B	95	0.9794	-3.45
B2M*	78.34	0.8795	-3.98
CANX	99.66	0.9834	-3.33
CYC1	100	0.9956	-3.322
EIF4A2*	137.65	0.9306	-2.66
GAPDH	97	0.9929	-3.389
RPL13A	100	0.986	-3.318
SDHA	93	0.9991	-3.53
UBC	101.78	0.9762	-3.28
YWHAZ	100	0.9947	-3.324

Figure 1: Box and whiskers plot showing the mean CT distribution of reference genes in the female mouse hypothalamus, pituitary gland, ovary and uterus with tissues from ages 2, 6, 9, 10 and 14 weeks where n=3. The mean CT is represented by (+) within the box for each reference gene, the line in the box represents the median value and the whiskers at each end of the box represent the minimum and maximum values.

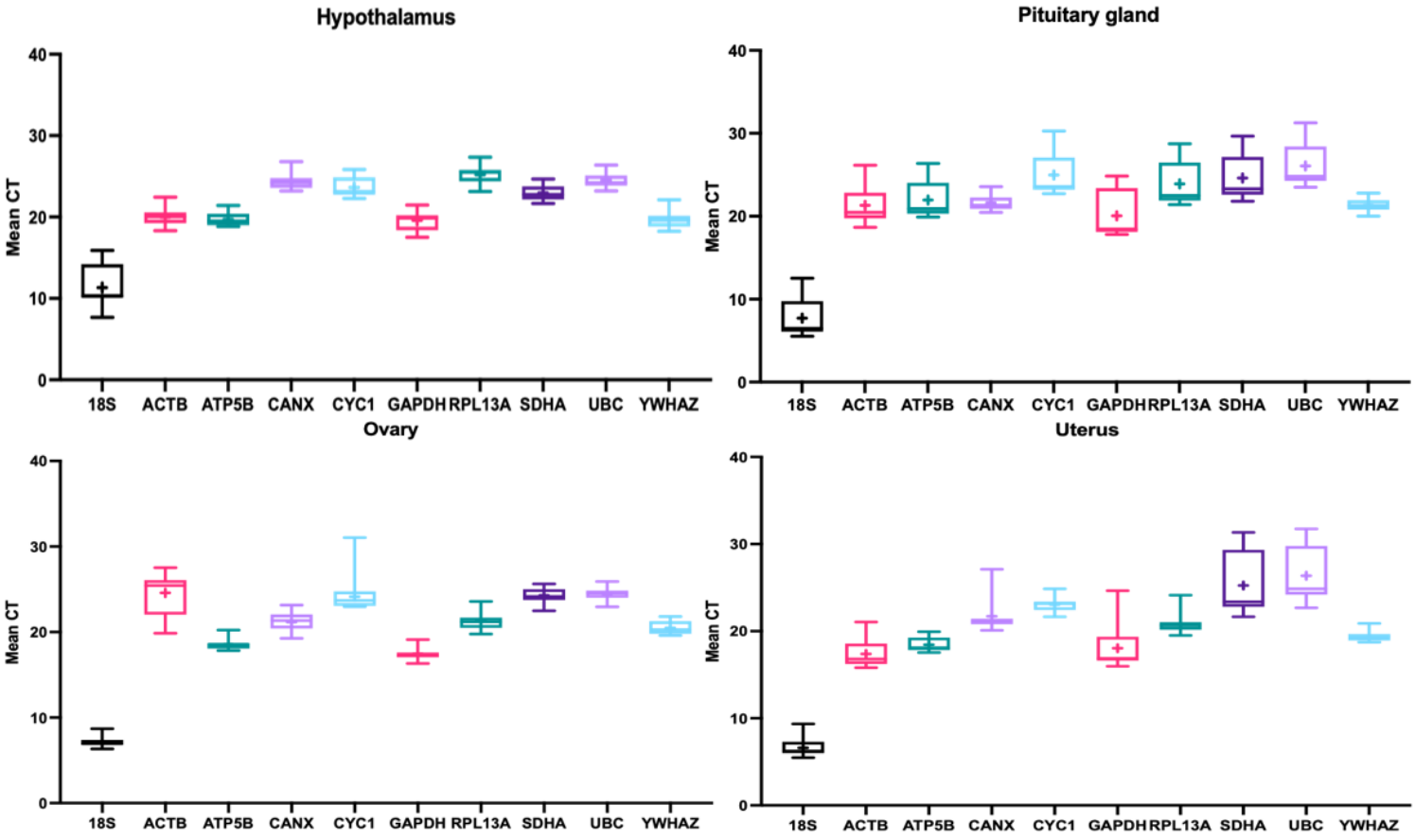


Figure 2: Determination of the optimal number of stable reference genes to be used in normalisation using a variation score (V- score) by GeNorm. V- score below 0.15 indicates insignificance of adding more reference genes for normalisation. The optimal number of reference genes with the age group only and the ages plus the pregnant samples did not change; data below is shown for the ages only. A comparison between two consecutive candidate reference genes (n vs. n+1) for the non-pregnant: hypothalamus, pituitary, ovary and uterus. This is plotted as the variation score or the V value, which was obtained automatically by the GeNorm, qBase+ software.

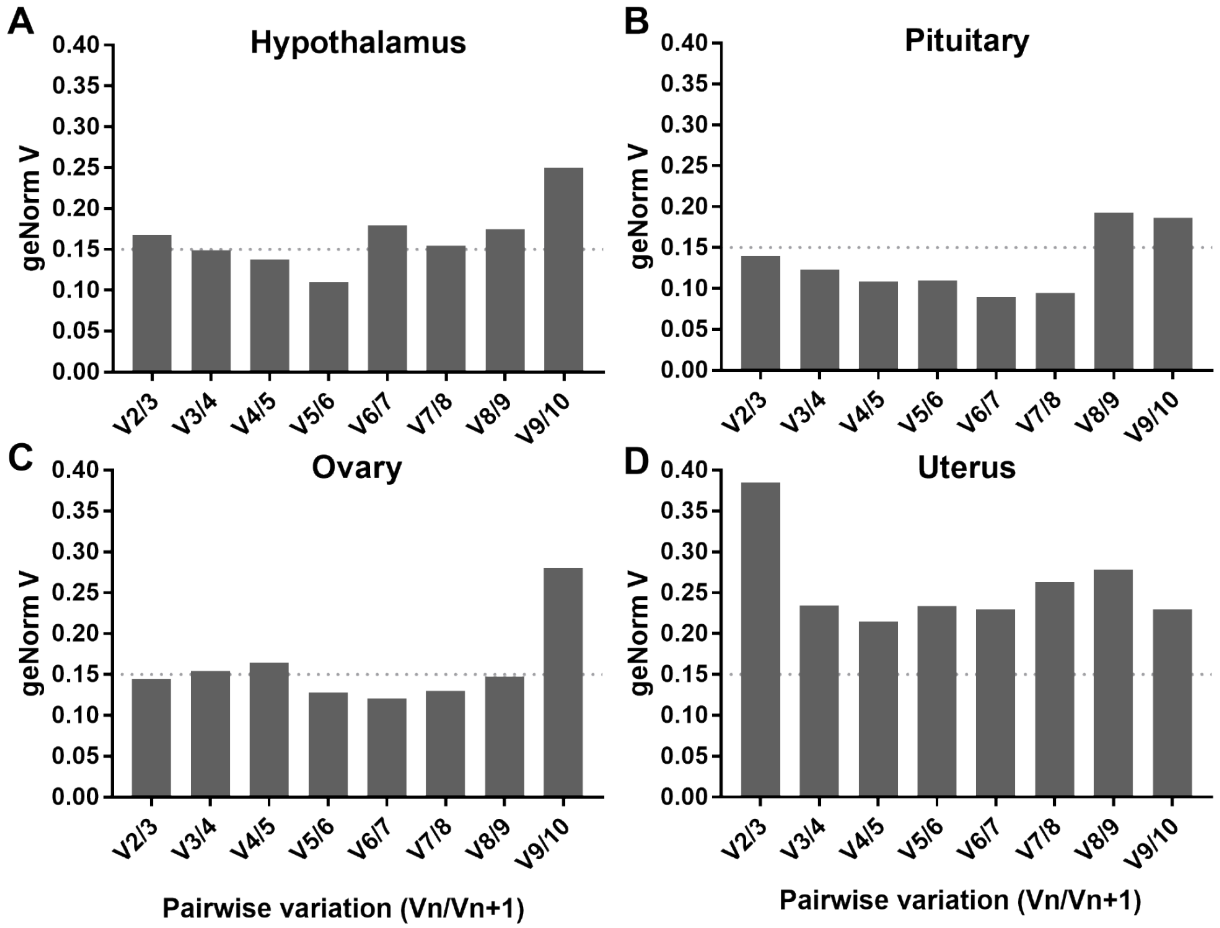
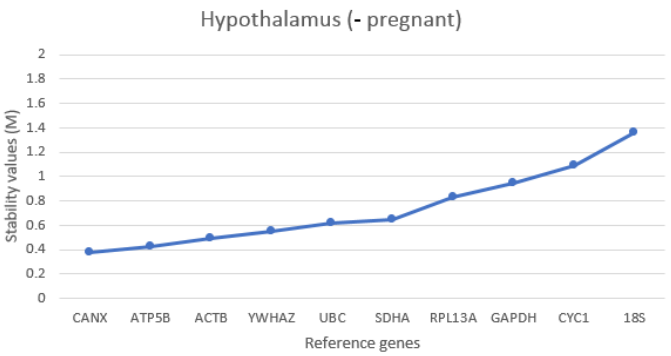
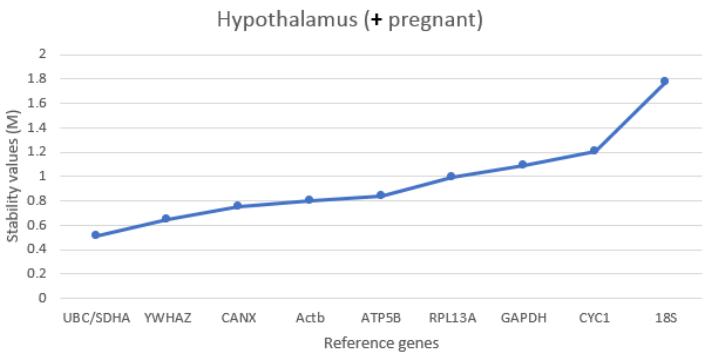


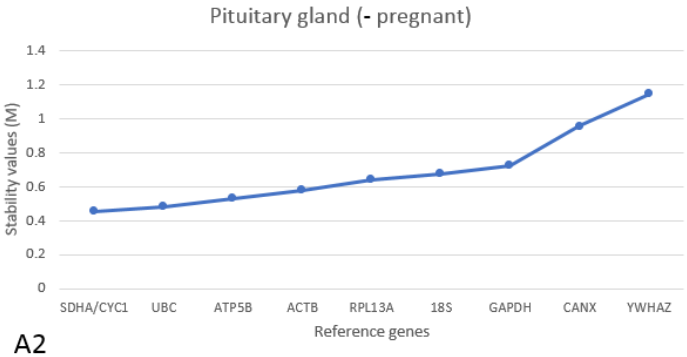
Figure 3: GeNorm analysis. *18s, Actb, Atp5b, Canx, Cyc1, Gapdh, Rpl13a, Sdha, Ubc* and *Ywhaz* expression stabilities were investigated in the female mouse hypothalamus (A1, B1), pituitary (A2, B2), ovary (A3, B3) and uterus (A4, B4). The samples were divided in to two groups: the age group which included samples from mice aged 2, 6, 9, 10 and 14 weeks (A1-4) and a second group (+ pregnant) which included these ages plus 13 dpc pregnant mice (B1-4). The data were analysed using GeNorm in qBase^{+} (Biogazelle, Belgium). The most stably expressed genes are displayed on the left hand side of each figure (A1-B4) with the least stably expressed genes on the right hand side.



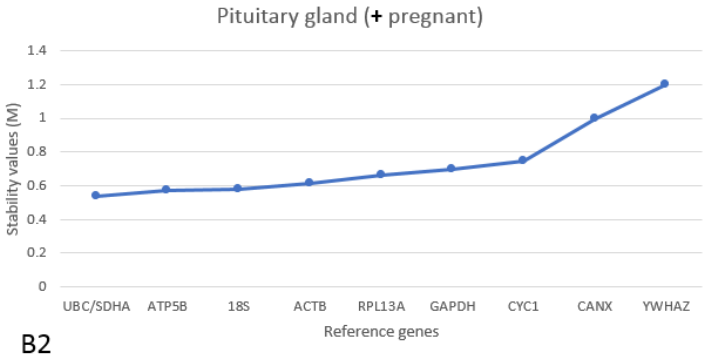
A1



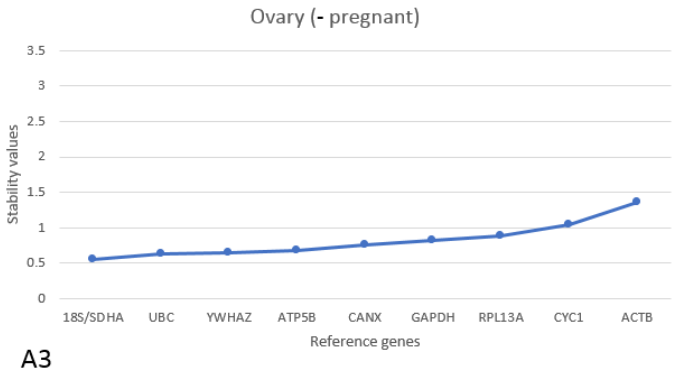
B1



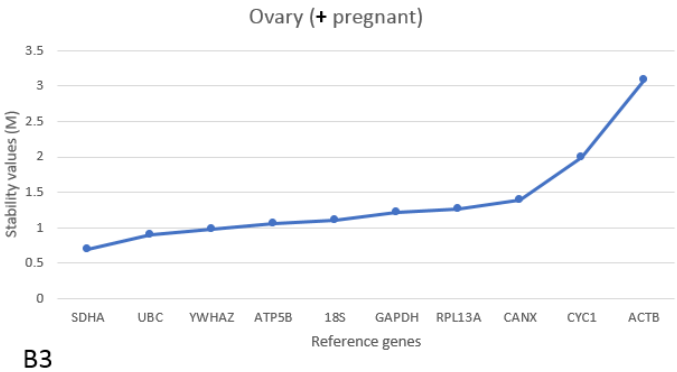
A2



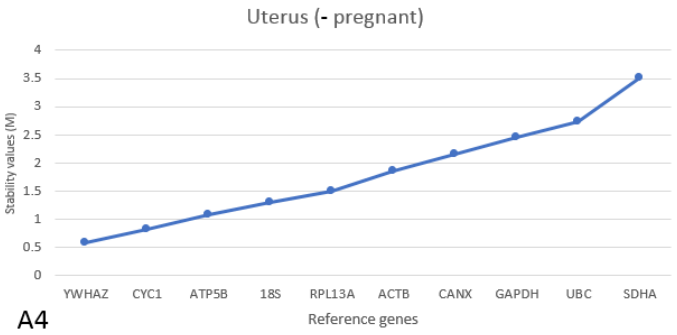
B2



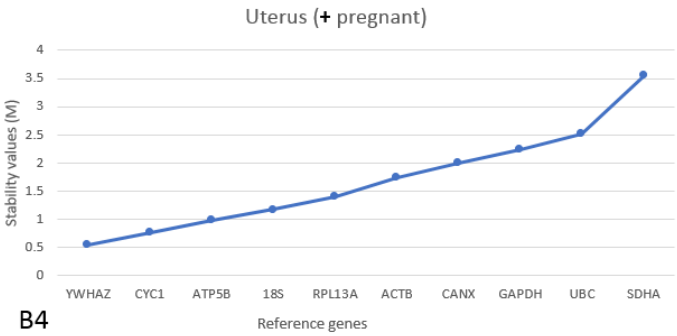
A3



B3



A4



B4

MOST STABLE LEAST STABLE

MOST STABLE LEAST STABLE