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Title

Identification of reference genes for RT-qPCR in ovine mammary tissue during late-pregnancy, lactation and in response to maternal nutritional programming.

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Running head: Ovine mammary reference genes

Abstract

The mammary gland is a complex tissue consisting of multiple cell types which, over the lifetime of an animal, go through repeated cycles of development associated with pregnancy, lactation and involution. The mammary gland is also known to be sensitive to maternal programming by environmental stimuli such as nutrition. The molecular basis of these adaptations is of significant interest, but requires robust methods to measure gene expression. Reverse transcription quantitative PCR (RT-qPCR) is commonly used to measure gene expression, and is currently the method of choice for validating genome-wide expression studies. RT-qPCR requires the selection of reference genes that are stably expressed over physiological states and treatments. In this study we identify suitable reference genes to normalize RT-qPCR data for the ovine mammary gland in two physiological states; late pregnancy and lactation. Biopsies were collected from offspring of ewes that had been subjected to different nutritional paradigms during pregnancy to examine effects of maternal programming on the mammary gland of the offspring. We evaluated eight candidate reference genes and found that two reference genes (*PRPF3* and *CUL1*) are required for normalising RT-qPCR data from pooled RNA samples, but five reference genes are required for analysing gene expression in individual animals (*SENP2, EIF6, MRPL39, ATP1A1, CUL1*). Using these stable reference genes, we showed that *TET1,* a key regulator of DNA methylation, is responsive to maternal programming and physiological state. The identification of these novel reference genes will be of utility to future studies of gene expression in the ovine mammary gland.

Keywords: ovine, mammary gland, nutritional programming, RT-qPCR, reference gene.

Introduction

The mammary gland is a dynamic organ that undergoes repeated cycles of development during the physiological stages of pregnancy, lactation and involution. Dramatic developmental changes and metabolic adaptations occur in the mammary gland during the transition from late pregnancy to lactation, in order to synthesise and secrete milk. These processes are carefully regulated by complex signalling networks, involving hormones of the endocrine system and local factors, and are influenced by the health and nutritional status of the animal (11, 17, 31). Development and function of the mammary gland may also be programmed by experiences *in-utero*, including the level of nutrition of the dam (6, 16, 28, 32, 40). In sheep, *ad libitum* nutrition of the dam has been shown to reduce the size of the fetal mammary gland and reduce the amount of milk produced during the first lactation of adult offspring (32, 40). In rodents, a maternal diet high in fat has been linked to increased breast cancer risk in offspring (16). Understanding the molecular mechanisms that underpin maternal programming will benefit animal production, and is of the utmost importance in human and animal health research.

The use of high-throughput sequencing (HTS) technologies, such as RNA-seq, has enabled analysis of the mammary transcriptome, providing insights into the patterns of gene expression involved in mammary gland development and function (12). Transcriptomic tools allow for further exploration into molecular mechanisms that may modulate effects in the mammary gland from external influences. To ensure accuracy of results, HTS data must be validated. This is typically done by correlation with expression data generated by RT-qPCR (reverse transcription quantitative PCR), a highly sensitive and specific technique for measuring gene expression (8). RT-qPCR is considered to be the gold standard for gene expression analysis as it is able to specifically detect transcript expression over a wide dynamic range (39). RT-qPCR is, however, subject to technical variation introduced during RNA extraction, cDNA synthesis or during reverse-transcriptase reactions. To combat this, internal controls, such as reference genes, must be used to normalize data (41). Ideal reference genes are expressed at levels similar to the gene(s) of interest, and are stably-expressed across all samples. Fluctuations in reference gene expression across physiological states can significantly skew the measurement of target gene expression (10).

Selection of appropriate reference genes for studies of mammary gland development during late pregnancy and lactation may be difficult as changes in cell numbers, differences in ratios of cell types, as well as changes in cell metabolism and biological processes leads to variation in the expression of genes (5). Potential modulation of gene expression through maternal nutritional programming may also contribute to variation in expression of reference genes. While studies in other species have identified reference genes for bovine and porcine mammary tissue during pregnancy and lactation (4, 37), there are no studies, to date, for the ovine mammary gland, and no studies investigating stability of reference genes in offspring of maternal nutritional programming studies.

In this study we identify, in a non-biased way, candidate reference genes for normalising RT-qPCR data in the ovine mammary gland during late pregnancy and lactation and in response to maternal nutritional programming.

Material and methods

Animals and sampling

Ovine mammary gland tissue was sampled from a sub-set of twin-bearing, twin-born ewe-offspring of a previously published maternal nutritional programming study (22, 32). Briefly, Romney ewes (G0 dams) were fed a sub-maintenance (Sm_{P21-50}), maintenance (M_{P21-50}) or *ad-libitum* (Ad_{P21-50}) pasture allowance during early gestation (P21-50), and reallocated to either a maintenance ($M_{P50-140}$) or ad libitum ($Ad_{P50-140}$) pasture allowance during mid-to-late gestation (P50-140) (Fig. 1A). The ewe offspring generated were utilised as the experimental animals of the present study, and were therefore from one of six dam nutritional treatment groups: SmM, SmAd, MM, MAd, AdM, and AdAd (Fig.1B, Table 1). All ewe offspring (G1 offspring) were managed under the same New Zealand commercial pastoral farming conditions and received the same level of nutrition (average intakes). Mammary parenchymal tissue (30 - 50 mg) was sampled from 10 ewes per treatment (n=60) via needle biopsy (Bard® Magnum® reusable core biopsy gun and 12G, 10cm core biopsy needles, Bard Biopsy Systems) during late pregnancy (135 ± 2.4 SD days of gestation) and again during lactation (15 ± 1.27 SD days post partum). Tissue samples were immediately

frozen in liquid nitrogen, then stored at -80°C until RNA extraction. Ewes were approximately 2 years of age at the time of the study. Late pregnancy biopsies were collected in September 2011 (ewe age 733.9 ± 1.66 (SD)) and lactation biopsies were collected in October 2011 (ewe age 761.0 ± 2.11 (SD)). The study was conducted at the Massey University Keeble Sheep and Beef farm, 5 km south of Palmerston North, New Zealand. The study was approved by the Massey University Animal Ethics Committee, Palmerston North, New Zealand.

RNA extraction and cDNA synthesis

Total RNA was isolated from mammary tissue samples using Trizol (Invitrogen) and purified using RNeasy mini kit (Qiagen). Genomic DNA contamination was eliminated via on-column digestion with DNase (Qiagen), as per the manufacturer's protocol. The concentration and quality of RNA was measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop) and integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). Only RNA with RNA integrity numbers (RINs) above 7 was use in this study. 1 µg of total RNA was used as template to perform cDNA synthesis using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) as per the manufacturer's protocol. Controls with no reverse transcriptase were used to assess the possibility of genomic DNA contamination in both RT-PCR and RT-qPCR.

Pooling of samples

One of the aims of this study was to identify candidate reference genes that could be used to validate RNA-seq data (Paten et al., *unpublished data*) by RT-qPCR. For RNA-sequencing we attempted to minimise individual variation between animals within the treatments by pooling RNA from multiple individuals (20, 21, 23). RNA from samples within the same treatment group was pooled separately for the two time points, late pregnancy and lactation. 2 µg of RNA, subsampled from three randomly selected animals per treatment, was incorporated into pools (Fig. 1C). Three pools per treatment were generated for late pregnancy samples and two pools were generated per treatment for lactation samples. The pools were: Late pregnancy; SmM, MM, and AdM (n = 3 for each treatment, total samples n = 9), and lactation; SmM, MM, and AdM (n = 2 for each treatments, total samples n = 6). To assess variation in expression of candidate genes between individuals, RT-qPCR analysis was also carried out on a subset of samples from individual animals from all

six treatment groups; SmM, MM, AdM, SmAd, MAd, and AdAd (n = 3 for each treatment) (Fig. 1B).

Selection of potential reference genes

Candidate reference genes were selected from RNA-seq data (Paten et al., *unpublished data*) from a study designed to investigate gene expression in the mammary gland, during late pregnancy and lactation, of ewes subjected to maternal nutritional programming. RNA-seq data was generated from pooled RNA (as detailed above) on an Illumina Hi-Seq 2000 (service provided by New Zealand Genomics Limited). Reads were mapped to the *Ovis aries* genome (version 3.2) using CLC Genomics Workbench (CLC Bio). To identify candidate reference genes from the RNA-seq data, genes were initially ranked based on the standard deviation (SD) of total gene reads relative to their overall expression (i.e. SD / total gene reads). This relative SD accounts for the fact that genes with high expression will have a higher SD than genes with low expression. By ranking genes on their relative SD we were attempting to determine the variation in gene expression irrespective of expression level. The genes with the lowest standard deviation (relative to their overall expression: SD% range = 0 – 1.03%) were analyzed for expression stability using geNorm (41) and NormFinder software (3). Genes were allocated a ranking from 1 to 100 for expression stability (1 representing most stable and 100 representing least stable) for each of the three methods for measuring expression stability (SD%, geNorm, and NormFinder). The sum of the ranking numbers were calculated and used to create an overall ranking of expression stability (with lower numbers representing less variable genes). Genes which ranked well for high expression stability, and which had low to medium expression based on the RNA-seq data (total gene reads approximating the mean), were chosen for evaluation as reference genes via RT-qPCR (refer to Table 2 for genes and expression stability rankings). Four genes were selected from the RNA-seq data; *CUL1* (part of the E3 ubiquitin ligase complex), *IPO9* (nuclear transport receptor), *PRP3* (U4/U6 small nuclear ribonucleoprotein) and *SF1* (RNA splicing). Two additional candidate reference genes (*MRPL39*, *EIF6*), which were stably expressed in the RNA-seq data, were selected from the literature (4, 37) and compared with *ATP1A1* (9), which had been previously used as a reference gene in our laboratory. Co-regulation of reference genes is known to bias the calculations for gene expression stability using geNorm (41). Possible co-regulation was detected between CUL1

and EIF6, and CUL1 and ATPA1 (determined using Ingenuity Pathway Analysis software (Ingenuity Systems, www.ingenuity.com)).

Primer design

RT-qPCR Primers were designed using Primer3Plus (38) (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). Where possible primers were designed to span intron / exon boundaries to allow detection of amplification from contaminating genomic DNA. *In silico* specificity of the primers was assessed using primer-BLAST (44)

Primer sequences and their amplicon lengths are listed in Table 3. Primers were highly specific as shown by a single band when PCR product was run on a 2% agarose gel, and a single peak observed in melt curve (data not shown). PCR products were also sequenced to confirm their specificity. The efficiency of primers was calculated from RT-qPCR of a 10 x dilution series of the cDNA**.** The RT-qPCR reaction efficiency was between 90 and 110% for all primer pairs (Table 3).

Quantitative PCR reactions

RT-qPCR reactions were carried out on a Bio-Rad C1000 Thermal cycler (Bio-Rad CFX96 Real-Time System) using SsoFast EvaGreen Supermix (BioRad) with 10 × diluted cDNA template and 300 nM of oligonucleotide primers. The following PCR program was used: 1 min initial incubation at 95°C followed by 40 cycles of 5 seconds at 95°C and 30 seconds at 60°C. On completion the reactions were held at 95°C for 10 seconds, reduced to 65°C and incrementally raised by 0.5°C until reaching 95°C for a melt curve analysis. In all cases the Cq measured for no template controls and –RT controls was greater than 40. Reactions were carried out in duplicate for each sample to minimise effects of technical errors, duplicates that differed by more than 0.5 cycles were repeated.

Data analysis

RT-qPCR data was analysed using the Bio-Rad CFX Manager™ software. For the samples tested, raw Cq values were obtained and used to determine gene expression stability with geNorm^{PLUS}. Gene expression stability analysis was carried out using the geNorm algorithm (41) implemented in qbase+ (version 2.6) (15). geNorm calculates the average pairwise variation of a candidate reference gene with all other control genes,

reported as the 'M' value. The lower the M value the more stably expressed the gene. The use of a single reference gene for data normalisation is not recommended (41) and geNorm also performs a pairwise variation analysis (V value), based on the geometric mean of all the candidate reference genes, to identify the optimal number of reference genes required. For analysis of TET1 expression, raw Cq values were obtained using the Bio-Rad CFX Manager™ software and imported into qbase+ (version 2.6) (15). Outliers were identified in RT-qPCR data using Grubbs' test (7) as implemented by the outliers package in R. TET1 expression was normalized by the geometric mean of the relative quantities for the selected reference genes. Differences in TET1 gene expression were determined using ANOVA with a Tukey HSD post-hoc test implemented in R.

Results

Reference gene stability in pooled samples

Our aim was to identify appropriate reference genes for the mammary gland in late pregnancy and lactation that did not change as a result of maternal nutritional programming in order to validate RNA-seq data (Paten et al., *unpublished data*). For the RNA-seq analysis we pooled RNA samples in an attempt to minimise individual variation (20, 21, 23). We therefore examined the expression of our candidate reference genes across our pooled samples, for both late pregnancy and lactation, which were derived from the three maternal nutritional programming groups (SmM, MM and AdM) (Fig. 2A) during late pregnancy and lactation. Expression data derived from RT-qPCR was used to carry out the gene stability analysis with geNorm (Fig. 2B). The gene expression stability measures (M) of these genes indicate that all of the candidate reference genes are stably expressed across physiological time points (lactation and late pregnancy) and amongst the nutritional programming groups (M values < 0.5 is indicative of highly stable expression in homogenous tissue samples (15, 41)). The results showed that *PRP3*, *CUL1* and *SF1*, which were all candidate reference genes selected from the RNA-seq data, had the highest expression stability across pooled samples (M = 0.183, 0.190, 0.195, respectively) (Fig. 2B). *MRPL39*, selected from literature, had an intermediate expression stability ranking (M = 0.234), while the other two candidate genes selected from literature, *EIF6* and *ATP1A1A*, were ranked the least stable (M = 0.308, 0.327,

respectively). The remaining genes, *SENP2* and *IPO9*, selected from RNA-seq, had an intermediate expression stability ranking (M = 0.259, 0.273, respectively). In general, reference genes selected from RNA-seq data were more stably expressed than those chosen from the literature.

Pairwise variation analysis suggests that two genes, *PRP3* and *CUL1*, would be acceptable to accurately normalize expression data (Fig. 2C, V < 0.15 (15, 41)). The addition of a third gene would have no significant effect, as the V2/3 value was less than the suggested cut-off of 0.15 (41).

Reference gene stability in individual animal samples

Our rationale for pooling samples for our RNA-seq analysis was to minimize individual variation between animals within the treatments (20, 21, 23). In order to determine the levels of individual variation in gene expression, and also to extend our search for reference genes to include analyses performed on individual animals, we also performed expression stability of potential reference genes for individual animal samples from within all maternal nutrition treatment groups (SmM, SmAd, MM, MAd, AdM, AdAd). Variation in expression of reference genes was much greater for the individual animal samples compared with the pooled samples (Fig. 3A compared with Fig. 2A) such that no combination of the reference genes could normalize expression data across both late pregnancy and lactation. If a slightly higher cut-off of V<0.2 is used then five reference genes may be used for normalization of RT-qPCR data generated from individuals (*CUL1, ATP1A1, IPO9, EIF6* and *SENP2*). However, because our aim was to identify reliable and robust reference genes within each physiological state (rather than reference genes that were stable over time), the two physiological states were also analyzed separately.

Analyzing all of the individual samples that comprised the pools (Fig. 4) none of the genes had an M value of less than 0.5, which is considered to represent stable expression in a homogenous sample (15, 41). The biopsies were standardised as much as possible for this study but are still likely to comprise of different proportions of cell types. In a heterogeneous sample, such as this, M-values of less than 1 can be considered stable (15, 41) and four of the genes sampled (*CUL1, ATP1A1, IPO9* and *SENP2*) met these criteria.

Extending this analysis to all of the treatment groups during late pregnancy (Fig. 3B) shows 7/8 reference genes have an acceptable stability value (M < 1) (15, 41). At late pregnancy, M values of reference genes were higher compared with the pooled samples, indicating greater variation between individuals. The ranking of reference genes also differed from the pooled samples (Fig. 3C), with the least stable reference gene in the pools (*ATP1A1*) being ranked as most stable amongst the individuals. Analysis of V values (Fig. 3D) indicated that the five most stably expressed reference genes (*SENP2*, *EIF6*, *MRPL39*, *ATP1A1* and *CUL1*) would need to be used for accurate normalisation of expression data of individual animals sampled during late pregnancy. Unlike the pooled samples, the reference genes chosen from RNA-seq data (*CUL1*, *IPO9*, *PRP3* and *SF1*) were less stably expressed than those chosen from literature (*EIF6* and *MRPL39*) and *ATP1A1*, which was a previously used reference gene. The exception to this is that *SENP2*, selected from RNA-seq data, ranked as the most stably expressed gene for individual animal samples for late pregnancy.

Expression stability (M) values of reference genes during lactation were also higher when analyzed for individual animals compared to pooled samples, indicating a higher level of variation. Six of the reference genes had an M value < 1, and can be considered relatively stably expressed (Fig. 3D). Analysis of the V value indicated that the top five most stably expressed reference genes (*MRPL39*, *SENP2*, *EIF6*, *CUL1*, *ATP1A1*) would need to be used to normalize expression data (Fig. 3E).

In both physiological states the least stable genes in this analysis were *SF1* and *PRP3,* which were considered to be highly stable in the analysis of the pooled RNA samples (Fig. 2B). Although, when only the animals that comprised the pools were analyzed (Fig. 4), *PRP3* was considered to be relatively stable in late pregnancy (M = 0.697), but not in lactation (M = 1.242).

Using Ingenuity pathway analysis software possible co-regulation was identified between CUL1 and ATPA1, and CUL1 and EIF6. This has the potential to bias calculations of gene expression stability (41). The correlation coefficients for expression of these genes are relatively low (r = 0.32 – 0.55), with the exception of CUL1 and ATPA1 for the individual animals (r = 0.89, Fig. 3). This indicates, at least for the pooled RNA samples, that there is no evidence for co-regulation amongst these genes. However, this, together with the fact that

five reference genes are required for the normalization of RT-qPCR data from individual animals, may justify selection and testing of additional reference genes in individual animals.

Sensitivity analysis of selected reference genes in RT-qPCR analysis

As there is substantial individual variation in expression of our candidate reference genes (Fig. 3A) we wanted to determine if the candidate genes we determined to be the most stable (*SENP2*, *EIF6*, *MRPL39*, *ATP1A1* and *CUL1*) provided more sensitivity to detect differences in transcript abundance of a gene of interest, compared with two of the less stable reference genes (*SF1* and *PRP3*). For this analysis we examined the expression of *TET1* (Tet methylcytosine dioxygenase 1).

DNA methylation, the addition of a methyl group to cytosine residues, is a well-studied epigenetic mechanism. DNA methylation has been associated with imprinting (reviewed in 1), X-inactivation (43), repression of gene expression (18) and, more recently, repressing intragenic promoter activity (29), alternative splicing (13, 26, 33, 34) and controlling transcriptional elongation (25, 33). The TET enzymes convert 5-methylcytosine to 5-hydroxymethyl cytosine (36), which is then further processed to result in the regeneration of a non-methylated cytosine (14, 27). The biological functions of the derivatives of 5-methylcytosine are unknown, but they may also act as epigenetic marks that recruit transcriptional regulators (35). Loss of 5-hydroxymethyl cytosine has been observed in different cancers, including breast cancer, and is associated with decreased expression of *TET1* (42).

Using stable reference genes (*SENP2*, *EIF6*, *MRPL39*, *ATP1A1* and *CUL1)* expression of *TET1* decreases from late pregnancy to lactation (63% reduction), and using the sub-optimal reference genes (*SF1* and *PRP3*) yields a similar result (60% reduction) (Fig. 5A). Using the sub-optimal reference genes does increase variation in gene expression (range = 0.19 - 3.6 with appropriate reference genes and 0.03 – 7.28 with sub-optimal reference genes). If the difference in *TET1* expression were less marked it would be unlikely to be detected using sub-optimal reference genes.

This is indeed what we see when we compare the effect of late pregnancy maternal nutrition on the expression of *TET1* in the mammary gland of offspring (Fig. 5B). Irrespective of physiological state, *ad libitum* maternal nutrition in late pregnancy results in a decrease of 35% in *TET1* expression in offspring (maintenance = 1.48, *ad libitum* = 0.95) when using appropriate reference genes. If the same data is analyzed with sub-optimal reference genes, no significant difference in gene expression is reported and the mean expression value is higher in offspring from dams fed an *ad libitum* diet during late pregnancy (maintenance = 1.44, *ad libitum* = 2.01).

Discussion

Transition from late pregnancy to lactation requires extensive physiological and metabolic adaptation in the mammary gland. These adaptations are regulated by endocrine hormones and local factors, and may be altered by external environmental events such as maternal nutritional programming. In order to understand the molecular basis of these processes and adaptations we need to accurately and sensitively monitor differences in gene expression. The ability of RT-qPCR to accurately detect changes in gene expression relies upon the selection of stably expressed reference genes. Studies in other species have shown that the expression of commonly used reference genes may vary between physiological and nutritional states and experimental treatments (2, 4, 19, 37). Variation in expression of reference genes may limit the ability to detect and verify changes in expression of target genes, thus reducing the percentage of genes that validate. In a recent study RT-qPCR validation of microarray data was improved by 13% (from 33% to 46%) when less stable reference genes were changed to more stable ones (10). In the present study we also observed a marked difference in the detection of a differentially expressed gene, *TET1*, when analyzed with poor and high quality reference genes (Fig. 5). The use of poor reference genes introduced significant variation in the analysis which masked detection of more subtle gene expression differences. These findings highlight the importance of choosing appropriate internal controls for RT-qPCR studies.

To date there are no studies which compare expression stability of reference genes in the ovine mammary gland. Therefore in the present study candidate reference genes

were selected from RNA-seq expression data (*PRP3*, *CUL1*, *SF1*, *SENP2* and *IPO9*) and from studies conducted in other species (*MRPL39*: bovine (4, 19), porcine (37); *EIF6* (4) and *ATP1A1* (9, 24). These genes were evaluated across pooled and individual RNA samples.

RNA samples may be pooled for gene expression analysis when samples are limited, in order to reduce costs, or in an attempt to reduce the effects of biological variation between individuals, particularly when the focus is on identifying expression patterns across the population (20, 21, 23). Consistent with this, there was considerably less variation in expression of candidate reference genes in the pooled samples (Fig. 2) compared with the individual animal samples (Fig. 3). geNorm analysis indicated that all of the genes tested had high stability in the pooled samples, and that the geometric mean of the two most stable genes (*PRP3* and *CUL1*) could be used to normalize expression data in mammary gland tissue samples, across late pregnancy and lactation, of ewes subjected to maternal nutritional programming.

In contrast to the pooled RNA samples, gene expression was less stable when tested across the individual animal samples, implying that the pooling strategy we have employed is effectively reducing the individual variation in gene expression. When both physiological states (late pregnancy and lactation) were analyzed together no combination of the candidate genes could be used to normalize the RT-qPCR data. Analyzed separately, the same five reference genes were recommended for normalization of RT-qPCR data (*SENP2*, *EIF6*, *MRPL39*, *ATP1A1* and *CUL1*), but the order in which these genes were ranked differs between the physiological states.

We observed high levels of variation in gene expression between individuals (Fig. 3A). This may be, at least partially, attributed to limitations in the sampling method used in this study. Biopsy sites were standardised as much as practical, but the mammary gland is a mixed tissue type (containing mammary epithelial cells, fibroblasts, blood vessels, connective and adipose tissue) and it is likely that individual biopsy samples contained different proportions of these cell types. In addition, gene expression in the mammary gland is known to be patchy, with not all epithelial cells actively expressing genes for milk synthesis and secretion (30). It may be possible to use cell sorting and labelling to obtain more homogenous samples. Increasing sample sizes would also reduce the effect of

individual variation, and it is likely that the relatively small sample sizes in this study were insufficient to account for biological variation arising from the heterogeneous nature of the mammary tissue (30).

Analysis of pooled RNA samples revealed *PRP3* and *CUL1* as the most stable reference genes, but *PRP3* was ranked least stable in the analysis of individual animals and *CUL1* was ranked as moderately stable. It is unknown why genes that ranked highly for stability among the pooled samples ranked so poorly when analyzed in individual animals and *vice versa*. When we compare analysis of pooled samples (Fig. 2, AdM, MM, SmM) with the individual animals that comprised those pools (Fig. 4), *CUL1* is the most stable gene but *PRP3* continues to rank poorly, particularly for lactation. This indicates that *CUL1* (and to a lesser degree *PRP3)* may be more variable amongst the treatments that were not included in the pooled experiment (AdAd, MAd, SmAd). This reinforces the importance of determining appropriate references genes for each tissue and experimental paradigm.

We used TET1, a key gene involved in epigenetic remodelling, to validate the quality of the reference genes identified in this study (Fig. 5). Here we show that when using high quality reference genes the decrease in *TET1* expression between late pregnancy and lactation is able to be accurately detected. When using low quality reference genes we were still able to detect a difference in TET1 expression, however, a greater level of variation was introduced into the analysis. *TET1* expression has been shown to correlate with lower levels of 5-hydroxymethylcytosine (42) and raises the possibility that epigenetic remodelling is required for maturation of the mammary gland prior to lactation. Unexpectedly, when using high quality reference genes, we were also able to detect that the expression of *TET1* is responsive to maternal nutritional programming, as *ad libitum* feeding of dams late in pregnancy results in offspring with significantly lower levels of *TET1* expression in the mammary gland. When low quality reference genes were used this difference could not be detected, highlighting the importance of using high-quality, stably expressed reference genes for data normalisation, particularly for detection of more subtle differences in expression of genes. The physiological significance of TET1 expression in the ovine mammary gland, and the role of 5-hydroxymethylcytosine in maternal programming, is yet to be determined.

Conclusions

This study demonstrates that reference gene expression can vary between physiological states, treatments (such as maternal gestational nutrition) and even between individual samples within the same treatment group and physiological state. We have identified novel reference genes for the mammary gland (i.e. *PRP3* and *CUL1*) and we show that using stable reference genes (*SENP2*, *EIF6*, *MRPL39*, *ATP1A1* and *CUL1*) increases the sensitivity of RT-qPCR analyses using *TET1* as an example. These findings highlight the importance of confirming stability of expression of reference genes, under specific experimental conditions, for RT-qPCR.

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Author contributions:

AMP performed the RNA extractions, cDNA synthesis and RT-qPCR experiments with assistance from EJD. AMP, PKD and EJD designed the reference gene study, analyzed the data and interpreted the results. SJP, SWP, HTP and PRK designed and managed animal experiments. SWP milked ewes before and after lactation biopsies. AMP, SJP, SWP, HTB, and PRK assisted in tissue collection. SJP, HTP, and PRK sourced funding for these experiments. AMP, PKD and EJD drafted the manuscript. All authors edited and approved the final version of the manuscript.

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Figure Captions

Fig. 1: Experimental design and RNA-pooling strategy used for this reference gene study. (A) Maternal-feeding paradigm. Romney ewes (G0) were fed *ad libitum* until day 21 of pregnancy when animals were randomly allocated to a sub-maintenance (Sm), maintenance (M) or *ad libitum* (Ad) diet. At day 50 of pregnancy, ewes were randomly reallocated to either a maintenance (M) or *ad libitum* (Ad) diet until day 140 of pregnancy when all ewes were switched to an *ad libitum* diet. (B) The offspring (G1) exposed to maternal nutritional programming treatments are identified according to the nutrition that their G0 mothers

565 received during pregnancy, i.e., the SmM groups' mothers were allocated a sub-
566 maintenance diet in early gestation and a maintenance diet in mid-late gestation (Sm = sub-
567 maintenance, M = maintenance, Ad = *ad libitum)* as detailed in Table 1. All G1 offspring
568 were fed *ad libitum*. RNA was extracted from G1 mammary biopsies collected during late
569 pregnancy (LP) or lactation (L) and the number of individual RNA samples isolated are
570 indicated in the diagram. For RT-qPCR of individual animals only three RNA samples were
571 used for each group in order to conserve RNA for future experiments. For pooling, RNA
572 samples were randomly allocated to one of three pools for LP and one of two pools for L;
573 each pool consisted of RNA isolated from three individual animals.

574 **Fig. 2:** Expression and stability analysis of the eight candidate genes in pooled RNA samples.
575 (A) Relative quantity of the eight candidate reference genes in pooled RNA samples across
576 the two physiological states (late pregnancy (Lpreg) and lactation (Lact)) and three maternal
577 programming treatment groups, *ad libitum*/maintenance (AdM), maintenance/maintenance
578 (MM), sub-maintenance/maintenance (SmM). (B) geNorm stability analysis (M value) of the
579 candidate reference genes. Low M values indicate more stable expression. All M values <
580 0.5 which is considered highly stable. (C) geNorm pairwise variation analysis (V value) of the
581 candidate reference genes. V < 0.15 (marked by dashed line) is considered as the upper limit
582 for selecting an adequate combination of reference genes, all combinations of pairwise
583 variation meet this criteria and two reference genes are recommended.

584 **Fig. 3:** Expression and stability analysis of the eight candidate genes in individual RNA
585 samples. (A) Relative quantity of the eight candidate reference genes in individual RNA
586 samples across the two physiological states (late pregnancy (Lpreg) and lactation (Lact)) and
587 in the six maternal programming treatment groups, *ad libitum*/maintenance (AdM),
588 maintenance/maintenance (MM), sub- maintenance/maintenance (SmM), *ad libitum*/ *ad*
589 *libitum* (AdAd), maintenance / *ad libitum* (MAd), sub-maintenance/*ad libitum* (SmAd) (B)
590 geNorm stability analysis (M value) of the candidate reference genes in late pregnancy. Low
591 M values indicate more stable expression. All M values, with the exception of *SF1,* are less
592 than 1 which is considered moderately stable. (C) geNorm pairwise variation analysis (V
593 value) of the candidate reference genes in late pregnancy. V < 0.15 (marked by dashed line)
594 is considered as the upper limit for selecting an adequate combination of reference genes
595 and only the inclusion of five reference genes meets this criteria. (D) geNorm stability
596 analysis (M value) of the candidate reference genes in lactation. Low M values indicate
597 more stable expression. All M values, with the exception of *PRP3* and *SF1,* are less than 1
598 which is considered moderately stable. (E) geNorm pairwise variation analysis (V value) of
599 the candidate reference genes in lactation. V < 0.15 (marked by dashed line) is considered
600 as the upper limit for selecting an adequate combination of reference genes and only the
601 inclusion of five reference genes meets this criteria.

602 **Fig. 4:** Expression and stability analysis of the eight candidate genes in the individual RNA
603 samples that were used to constitute the RNA pools. (A) geNorm stability analysis (M value)

of the candidate reference genes in both physiological states. Low M values indicate more stable expression. All M values, with the exception of *SF1* and *PRP3,* are less than 1 which is considered moderately stable. (B) geNorm pairwise variation analysis (V value) of the candidate reference genes in late pregnancy. V < 0.15 (marked by dashed line) is considered as the upper limit for selecting an adequate combination of reference genes and no combination of reference genes satisfied this criteria. (C) geNorm stability analysis (M value) of the candidate reference genes in late pregnancy. Low M values indicate more stable expression. All M values, with the exception of *SF1,* are less than 1 which is considered moderately stable. (D) geNorm pairwise variation analysis (V value) indicates that the most stable five or six genes would be appropriate for normalizing RT-qPCR data. (E) geNorm stability analysis (M value) of the candidate reference genes in lactation. Low M values indicate more stable expression. Only four of the tested genes have moderately stable expression (M < 1). (F) geNorm pairwise variation analysis (V value) indicates that no combination of reference genes can be used for normalizing RT-qPCR data.

Fig. 5: Normalization of *TET1* expression with stable reference gens and sub-optimal reference genes. (A) *TET1* expression differs significantly between late pregnancy and lactation when using stable reference genes (*SENP2*, *EIF6*, *MRPL39*, *ATP1A1* and *CUL1*). (B) When *TET1* expression is normalized to sub-optimal reference genes, a significant difference in gene expression is observed, but there is more variation in the normalized expression values. (C) *TET1* expression is responsive to maternal nutritional programming. *Ad libitum* feeding in late pregnancy results in lower levels of *TET1* expression in the mammary glands of the adult offspring when data is normalized to the expression of stable reference genes. (D) When the same data is normalized to sub-optimal reference genes, no difference in *TET1* expression is observed.

Tables:

Table 1: Summary of maternal nutritional treatments used in this study.

Treatment	Pasture allowance during early gestation (P21-50)	Pasture allowance during mid-late gestation (P50-140)
SmM	Sub-maintenance	Maintenance
SmAd	Sub-maintenance	*Ad-libitum*
MM	Maintenance	Maintenance
MAd	Maintenance	*Ad-libitum*
AdM	*Ad-libitum*	Maintenance
AdAd	*Ad-libitum*	*Ad-libitum*

Table 2: Ranking of candidate reference genes

Gene code	Gene description	SD% rank	geNorm rank	NormFinder rank	Overall rank
SF1	Splicing factor 1 isoform 2	2	4	5	2
SENP2	Sentrin-specific protease 2 isoform 1	6	2	4	3
CUL1	Cullin 1	4	7	3	5

PRPF3	U4/U6 small nuclear ribonucleoprotein *PRP3*	14	17	14	12
IPO9	Importin 9	10	19	6	10
MRPL39	Mitochondrial ribosomal protein L39	From literature			
EIF6	Eukaryotic translation initiation factor 6	From literature			
ATP1A1	ATPase, Na+/K+ transporting, alpha 1 polypeptide	Previously used in laboratory			

633
634
635 **Table 3:** Gene name, primer sequences, amplicon length (bp) and PCR efficiency for
636 reference genes evaluated.

Gene	NCBI accession	Forward Primer 5′ → 3′	Reverse Primer 5′ → 3′	Amplicon length (bp)	PCR efficiency (%)*
MRPL39	XM_004002812.1	CCCTGGAAGTTGAAGCAAAA	GGTTCTGGGATGCCTTCTCT	90	98.1
EIF6	NM_001162563.1	AATTGAGGACCAGGATGAGC	GCACACCAGTCATTCACCAC	114	103.8
ATP1A1	NM_001009360.1	GAGATTGTGTTCGCCAGGAC	CGTCTCCAGTTACAGCCACA	94	95.9
CUL1	XM_004008343.1	AAAAATACAACGCCCTGGTG	CTGAGCCATCTTGGTGACTG	116	95.9
IPO9	XM_004014142.1	ACTACGAGGACGACGAGGAG	GGCAGAGGAAGTCTGTGAGG	93	98.3
PRPF3	XM_004002449.1 XM_004002450.1	ACAGATGATGGAAGCAGCAA	GGTTGGGAGGATGAAGGAGT	105	101.0
SF1	XM_004019657.1	GAGAGTTGGCTCGCTTGAAT	CCCCTCCACACTTGGTACAC	120	99.6
SENP2	XM_004003073.1 XM_004003074.1	GAGGTGTTCAAAGGGGAAAA	TCTTCAGACAGGTCGGGTTC	105	101.0
TET1 (target gene)	XM_004021627.1	TTTCTCTGGGGTCACTGCTT	TGAGCGGTTATCTTCTCGTG	115	100.6

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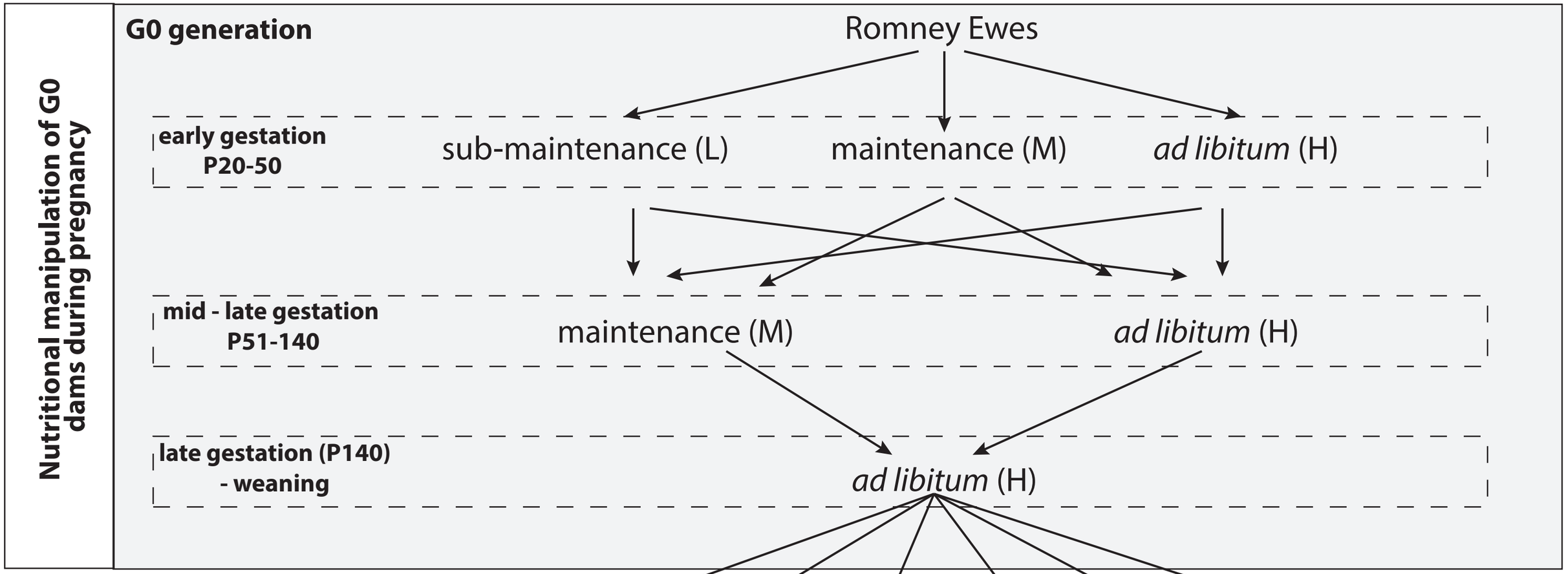
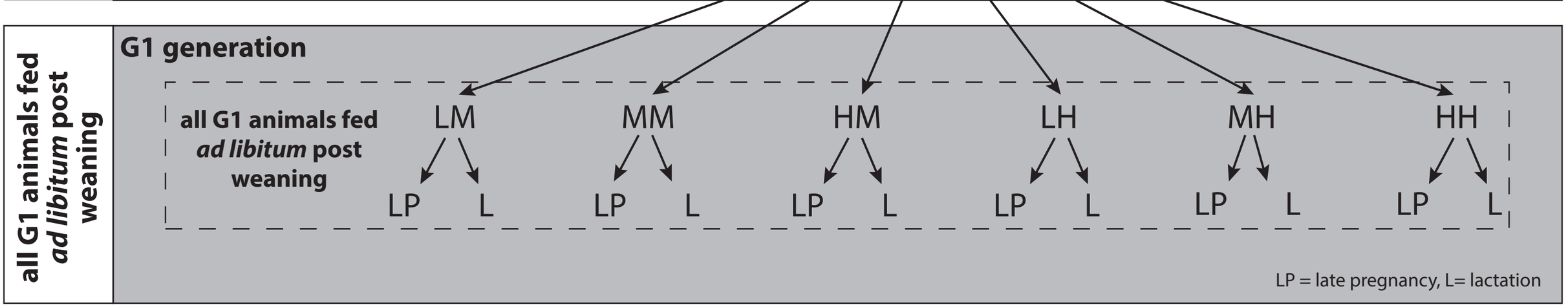
638
639

A.



B.

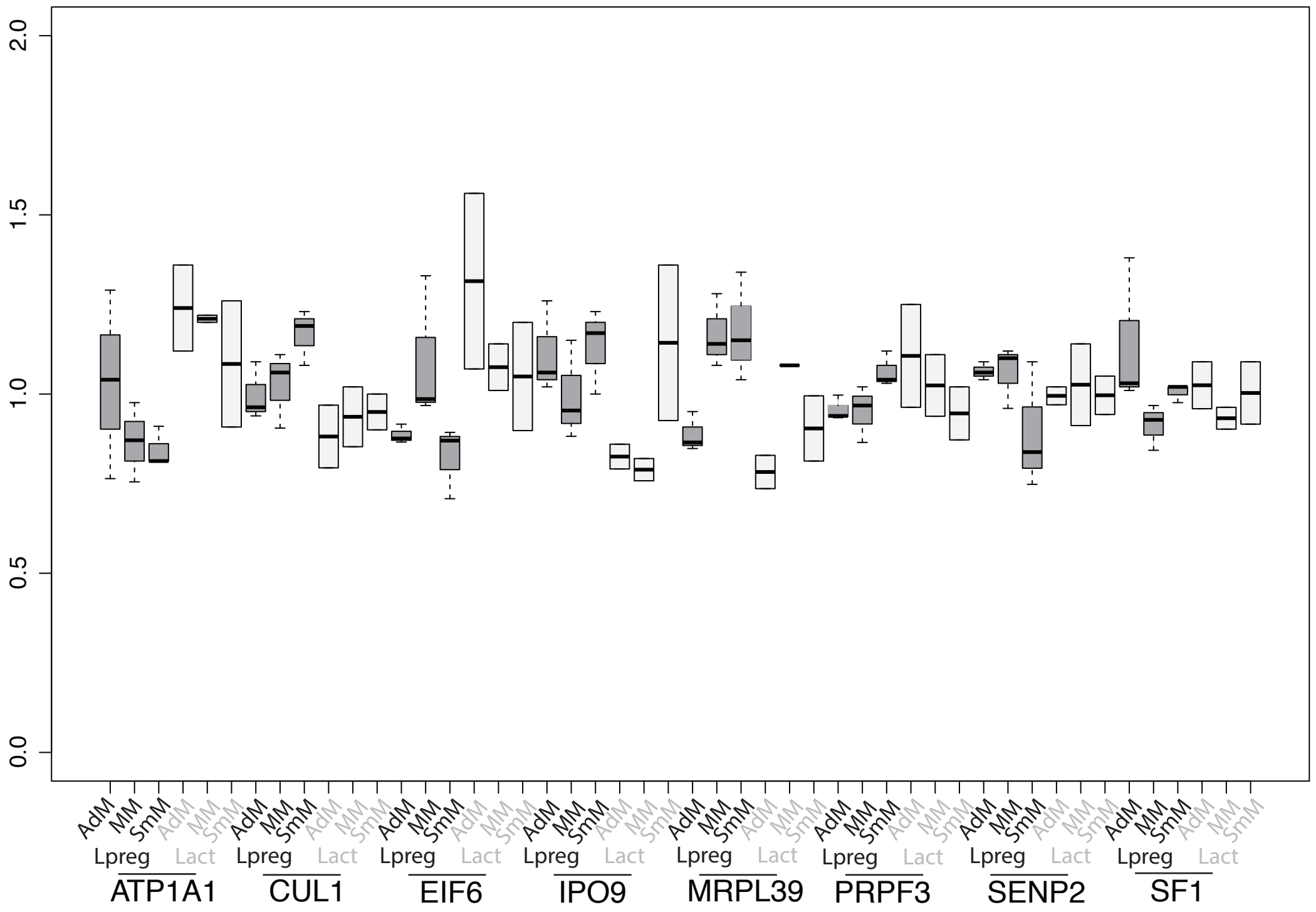


C.

qRT-PCR samples & pooling stragey												
Individual animal samples*	n=9	n=6	n=9	n=6	n=9	n=6	n=8	n=6	n=8	n=6	n=8	n=6
Pooled animal samples n = 3 individual animals per pool	Pool 1 Pool 2 Pool 3	Pool 1 Pool 2	Pool 1 Pool 2 Pool 3	Pool 1 Pool 2	Pool 1 Pool 2 Pool 3	Pool 1 Pool 2						

*** only 3 individual animals were used in this reference gene study (to conserve RNA), but all individual samples were used to generate pools**

A.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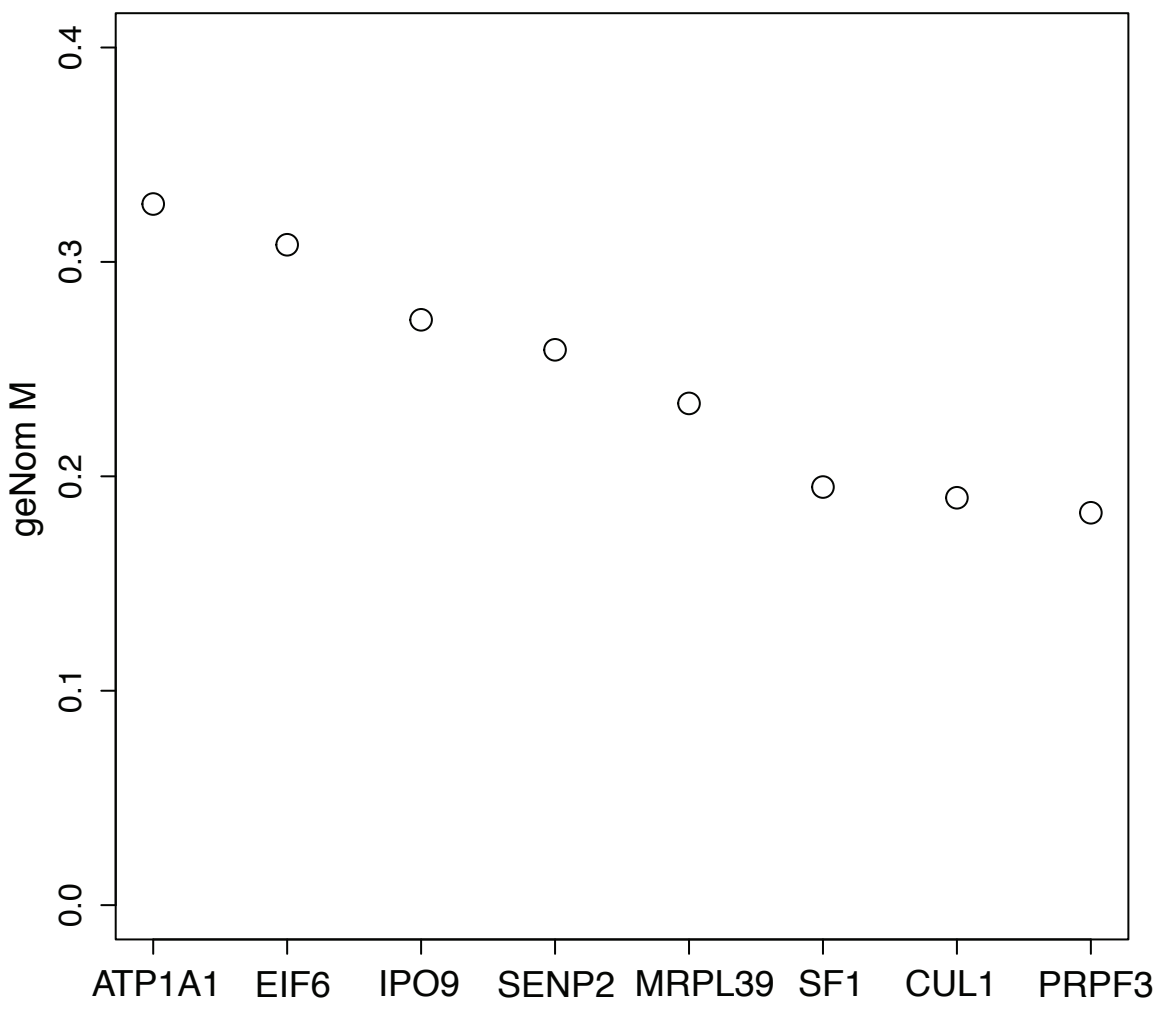
1.5
1.0
0.5
0.0
AdM MM SmM AdM MM SmM AdM MM SmM AdM MM SmM AdM MM SmM AdM MM SmM AdM MM SmM AdM MM SmM AdM MM SmM AdM MM SmM AdM MM SmM AdM MM SmM AdM MM SmM AdM MM SmM AdM MM SmM AdM MM SmM
Lpreg Lact Lpreg Lact Lpreg Lact Lpreg Lact Lpreg Lact Lpreg Lact Lpreg Lact Lpreg Lact
ATP1A1 CUL1 EIF6 IPO9 MRPL39 PRPF3 SENP2 SF1

B.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0.3
0.2
0.1
0.0
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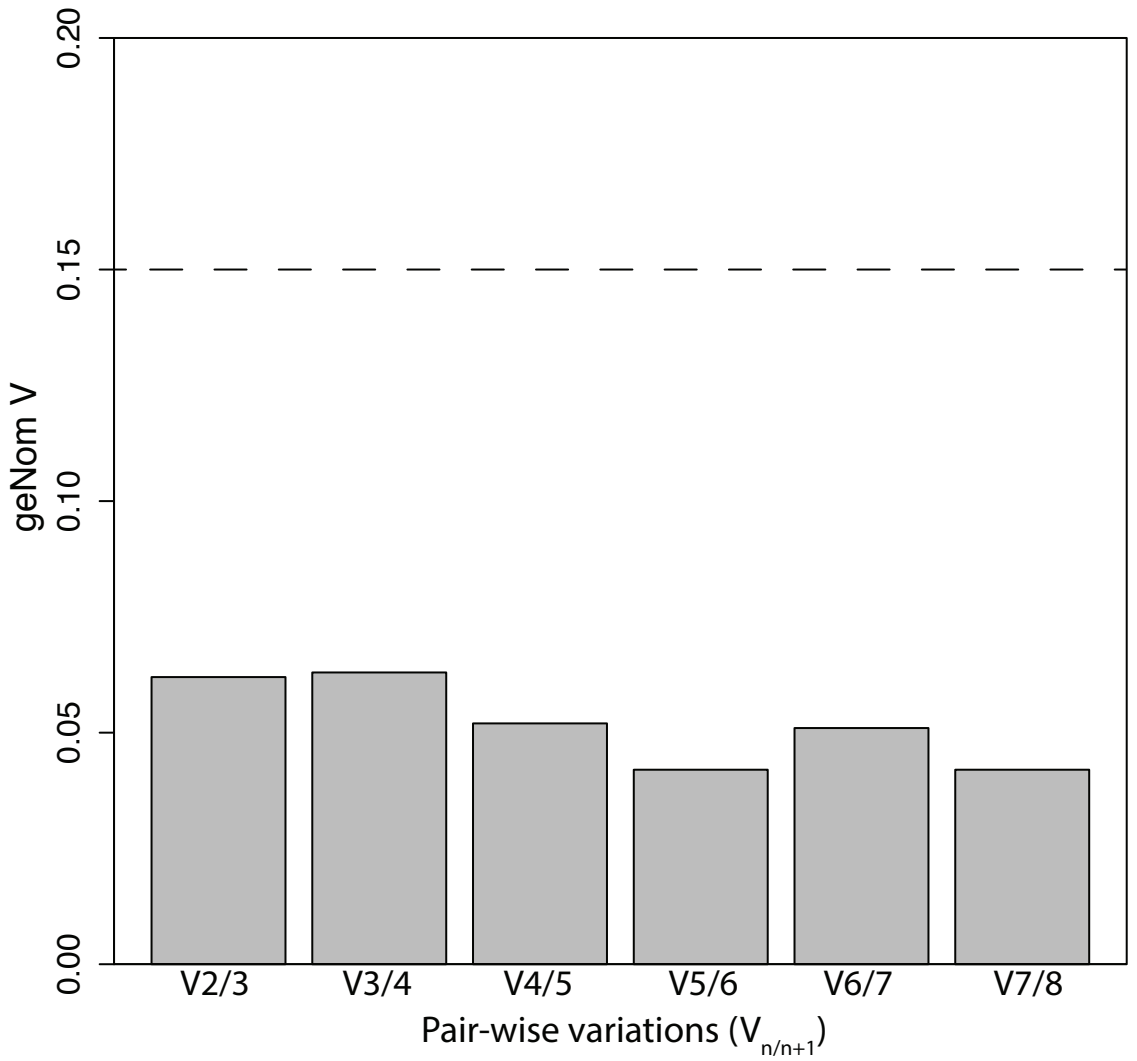
ATP1A1 EIF6 IPO9 SENP2 MRPL39 SF1 CUL1 PRPF3

C.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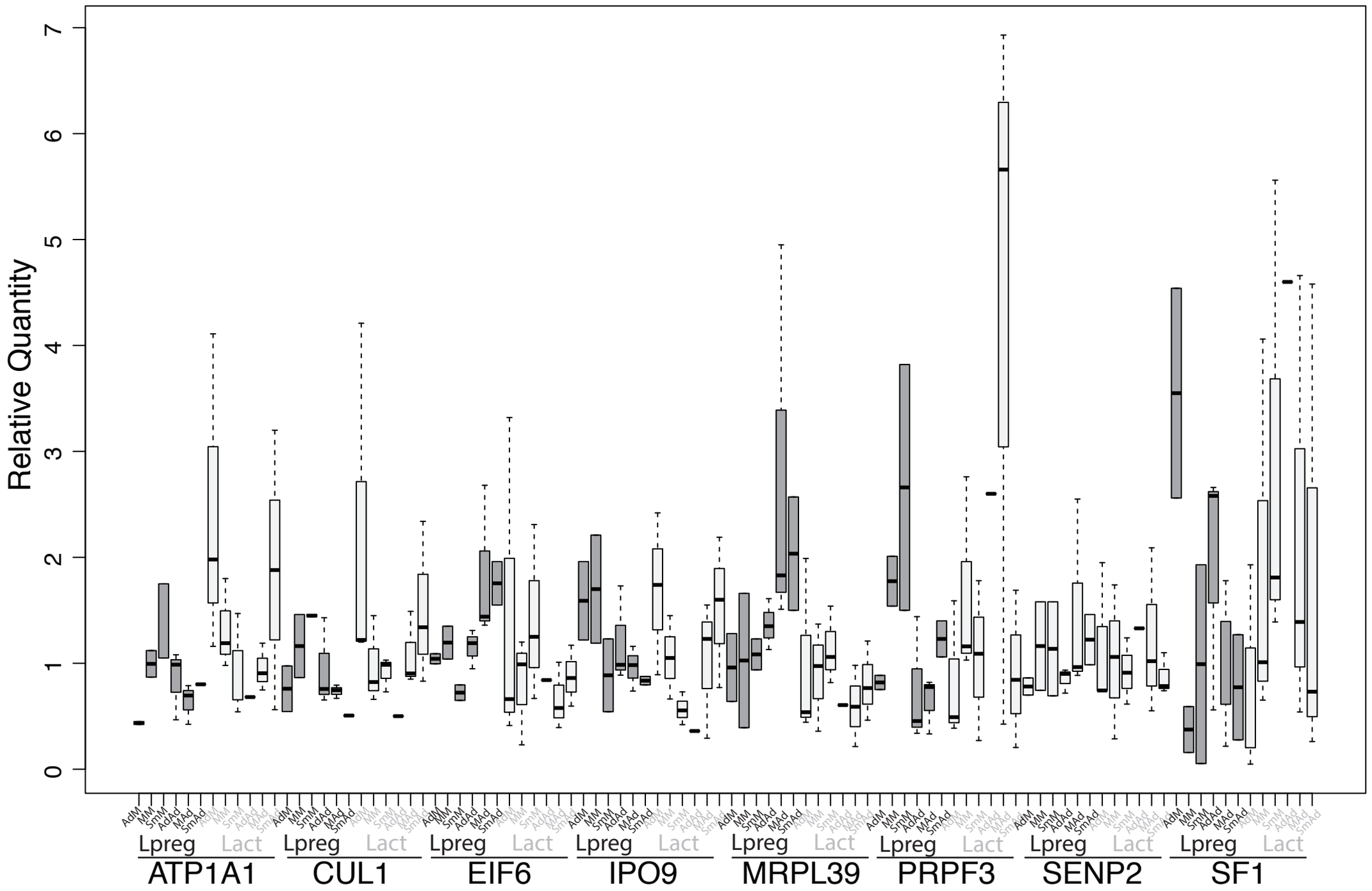
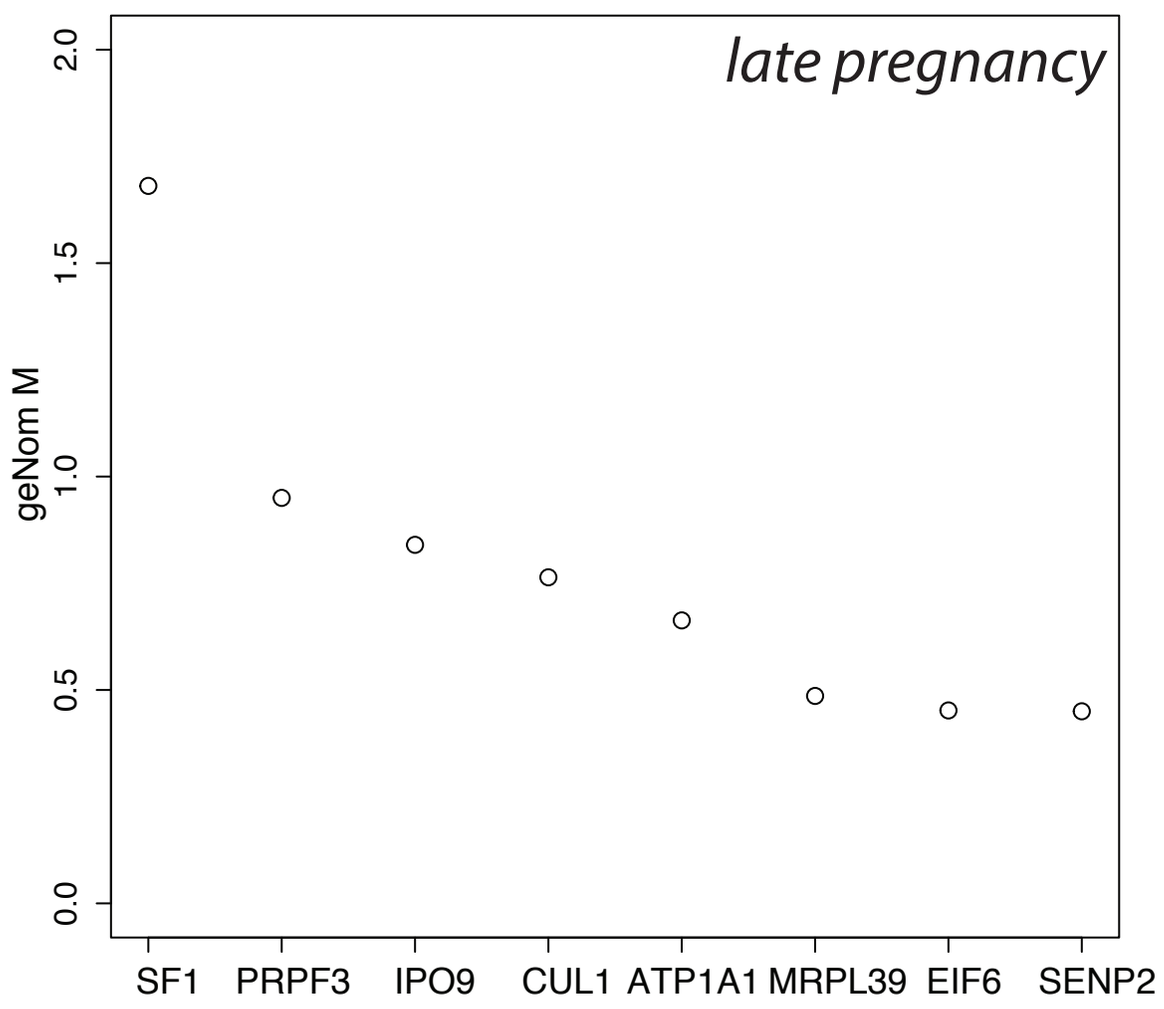
0.15
0.10
0.05
0.00
geNom V
V2/3 V3/4 V4/5 V5/6 V6/7 V7/8
Pair-wise variations (V n/n+1)

A.


Relative Quantity
0
1
2
3
4
5
6
7
AdM MM SmM AdAd MAd SmAd
Lpreg Lact
ATP1A1
CUL1
EIF6
IPO9
MRPL39
PRPF3
SENP2
SF1

B.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0.5
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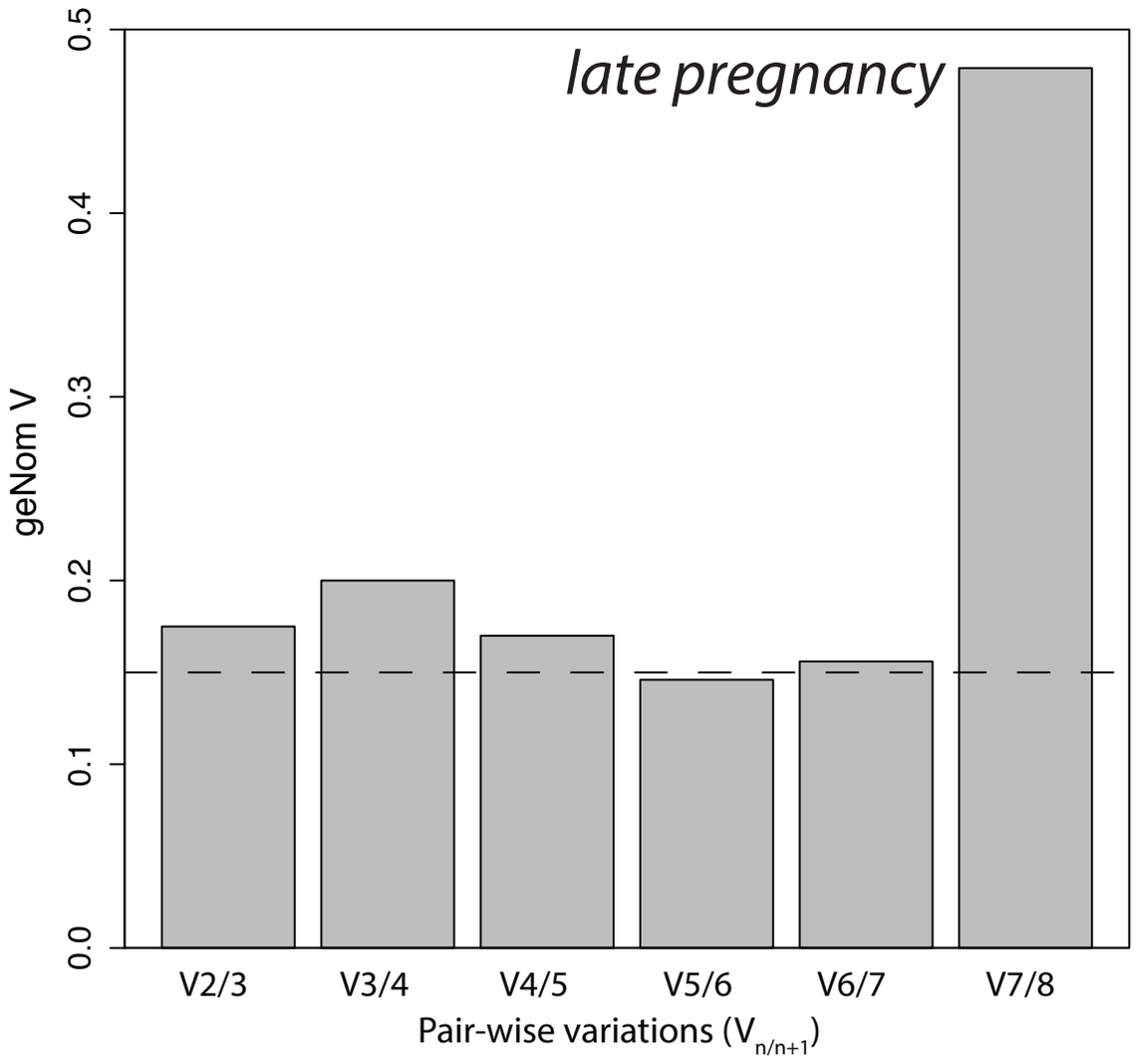
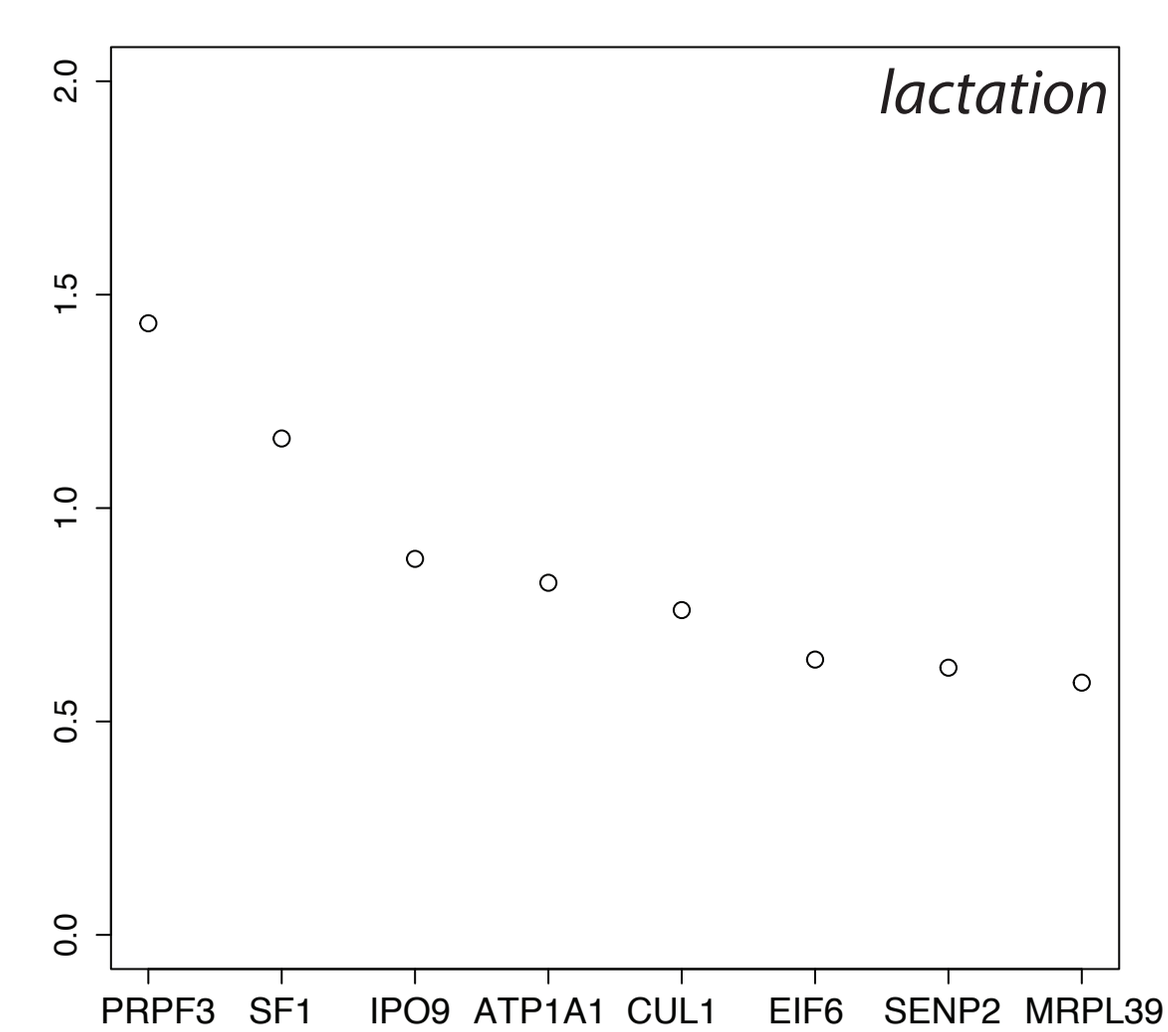
1.5
2.0
SF1 PRPF3 IPO9 CUL1 ATP1A1 MRPL39 EIF6 SENP2

C.


late pregnancy
geNom V
0.0
0.1
0.2
0.3
0.4
0.5
V2/3 V3/4 V4/5 V5/6 V6/7 V7/8
Pair-wise variations (V n/n+1)

D.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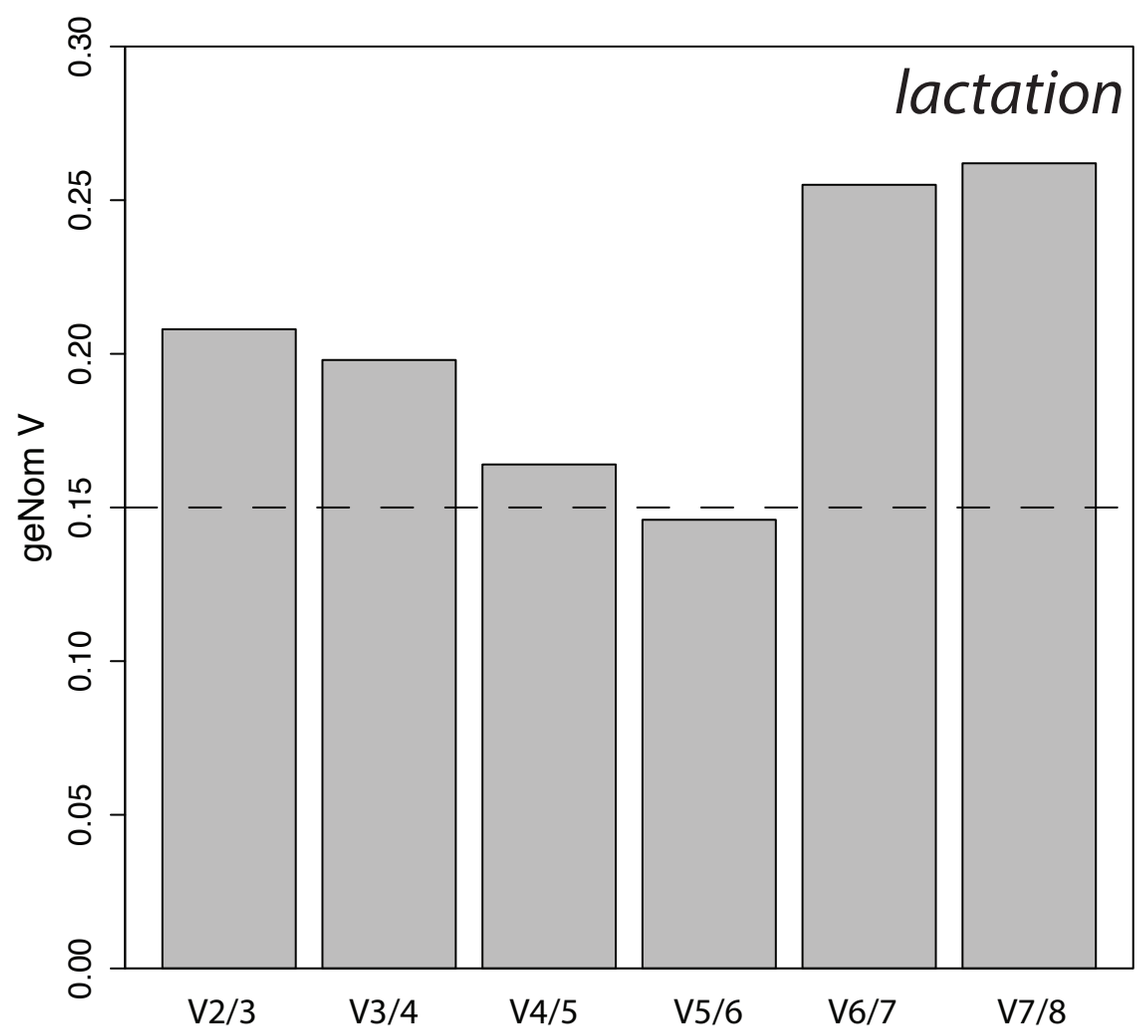
1.0
1.5
2.0
PRPF3 SF1 IPO9 ATP1A1 CUL1 EIF6 SENP2 MRPL39

E.


lactation
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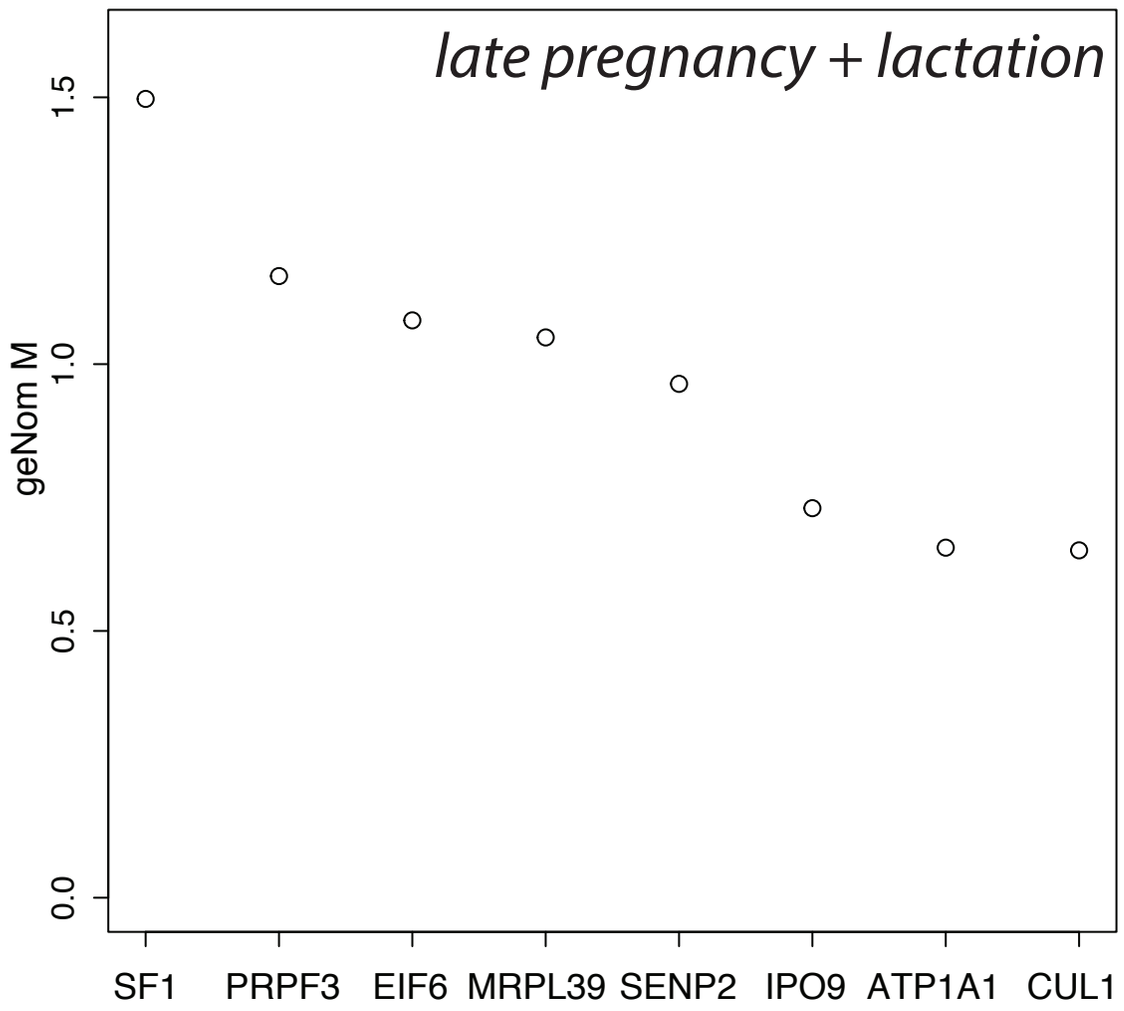
0.10
0.15
0.20
0.25
0.3
V2/3 V3/4 V4/5 V5/6 V6/7 V7/8
Pair-wise variations (V n/n+1)

A.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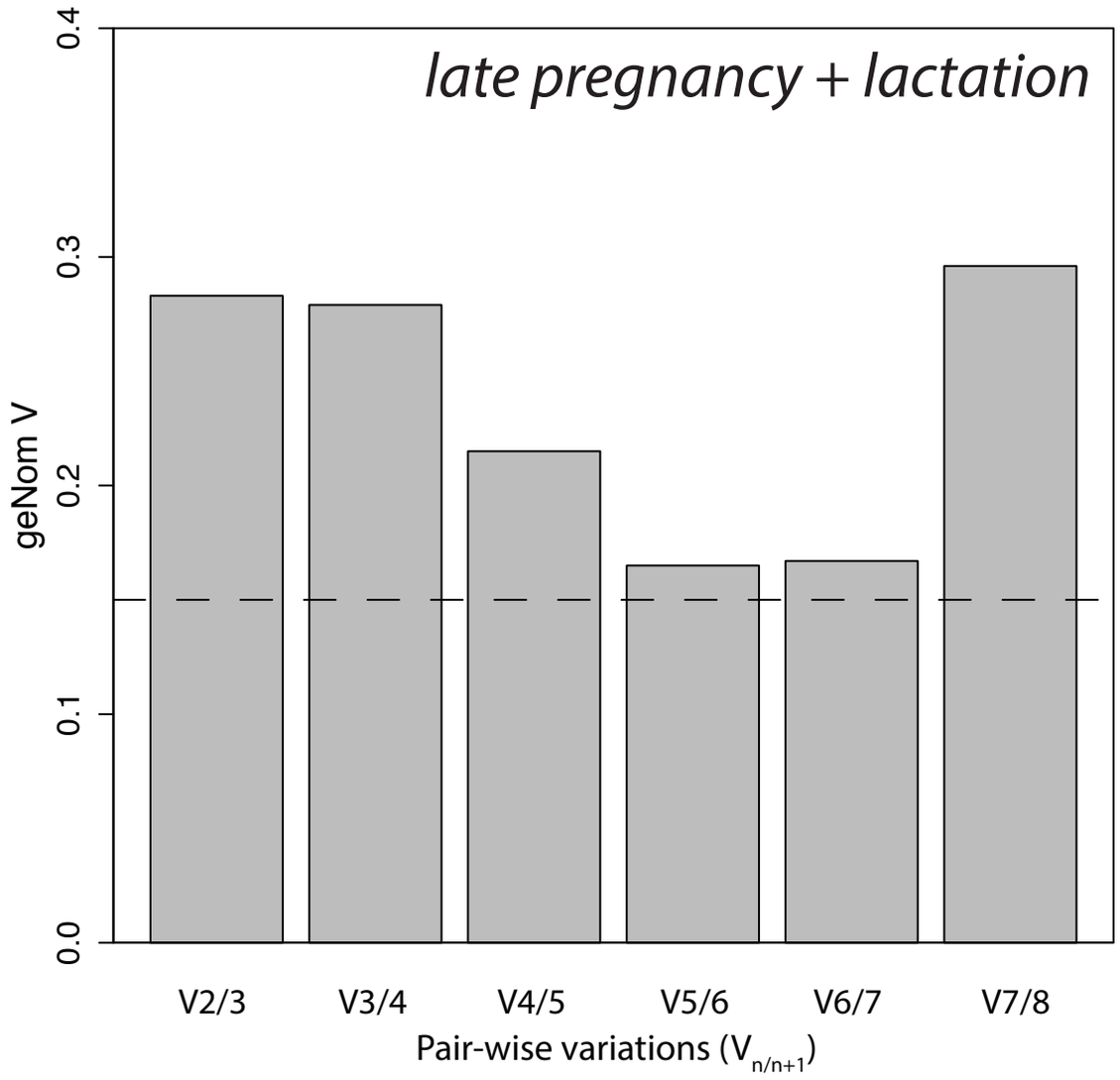
PRPF3
EIF6
MRPL39
SENP2
IPO9
ATP1A1
CUL1

B.


late pregnancy + lactation
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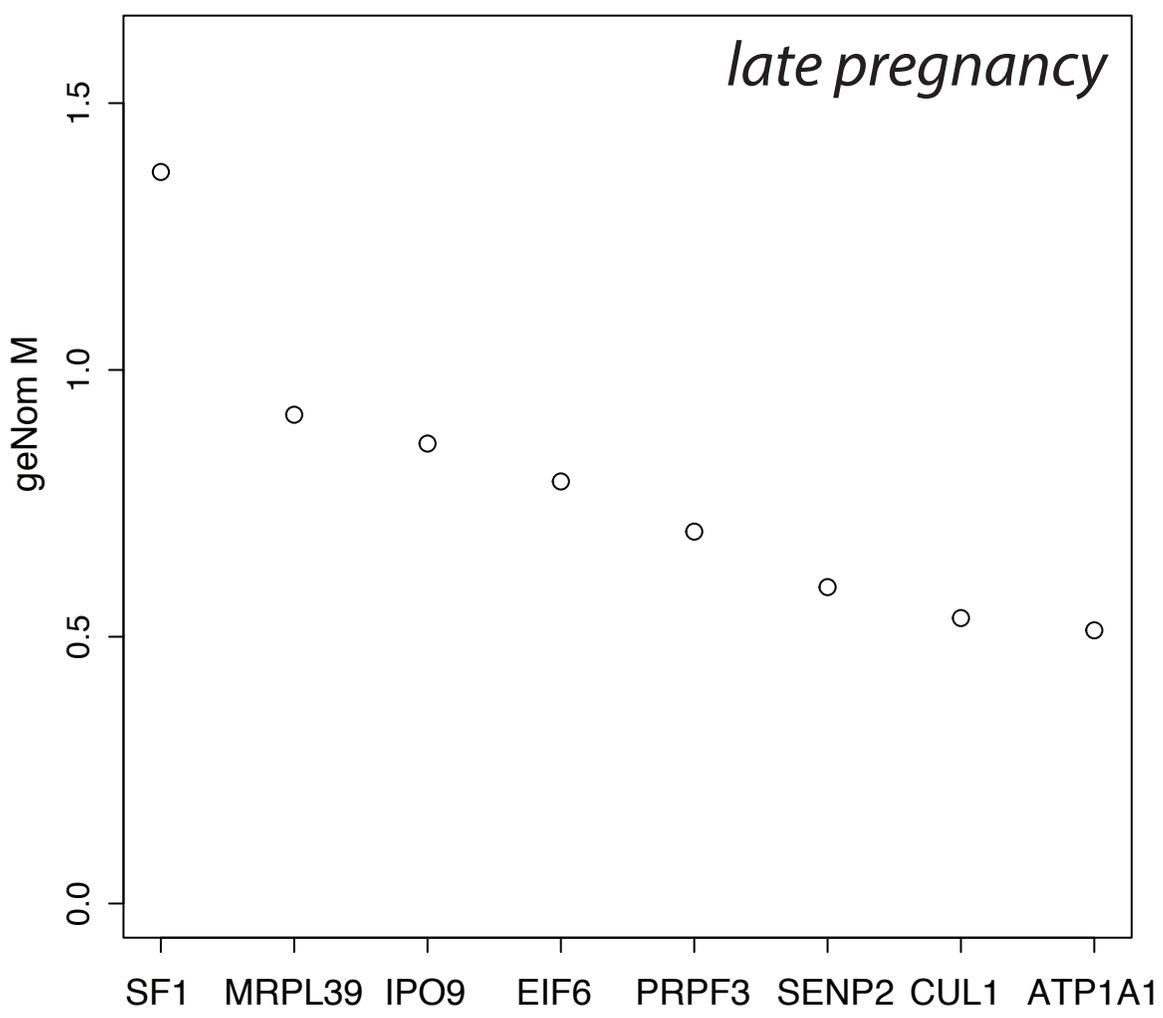
0.1
0.2
0.3
0.4
V2/3
V3/4
V4/5
V5/6
V6/7
V7/8
Pair-wise variations ($V_{n/n+1}$)

C.


late pregnancy
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0.0
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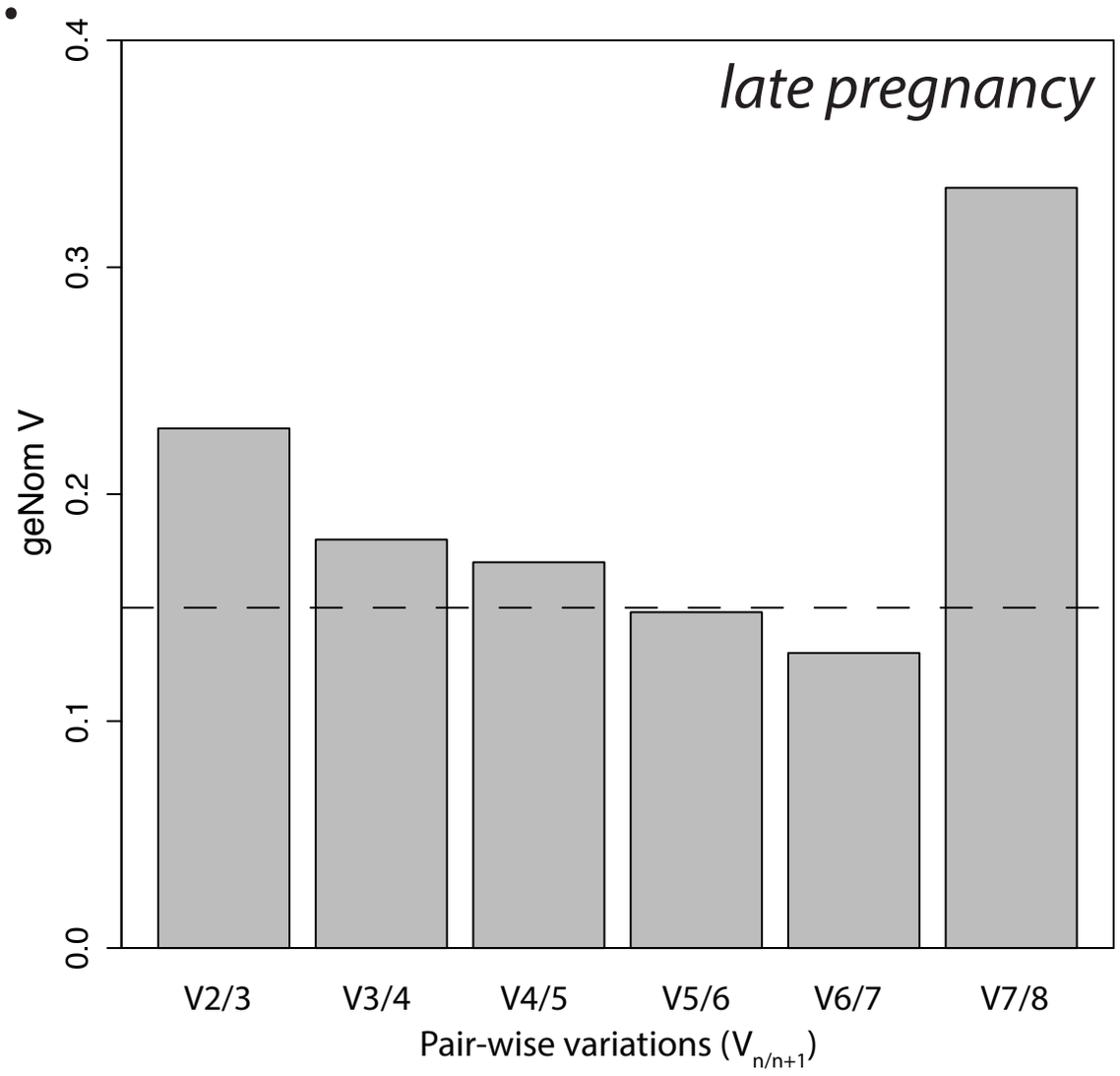
SF1
MRPL39
IPO9
EIF6
PRPF3
SENP2
CUL1
ATP1A1

D.


late pregnancy
geNom V
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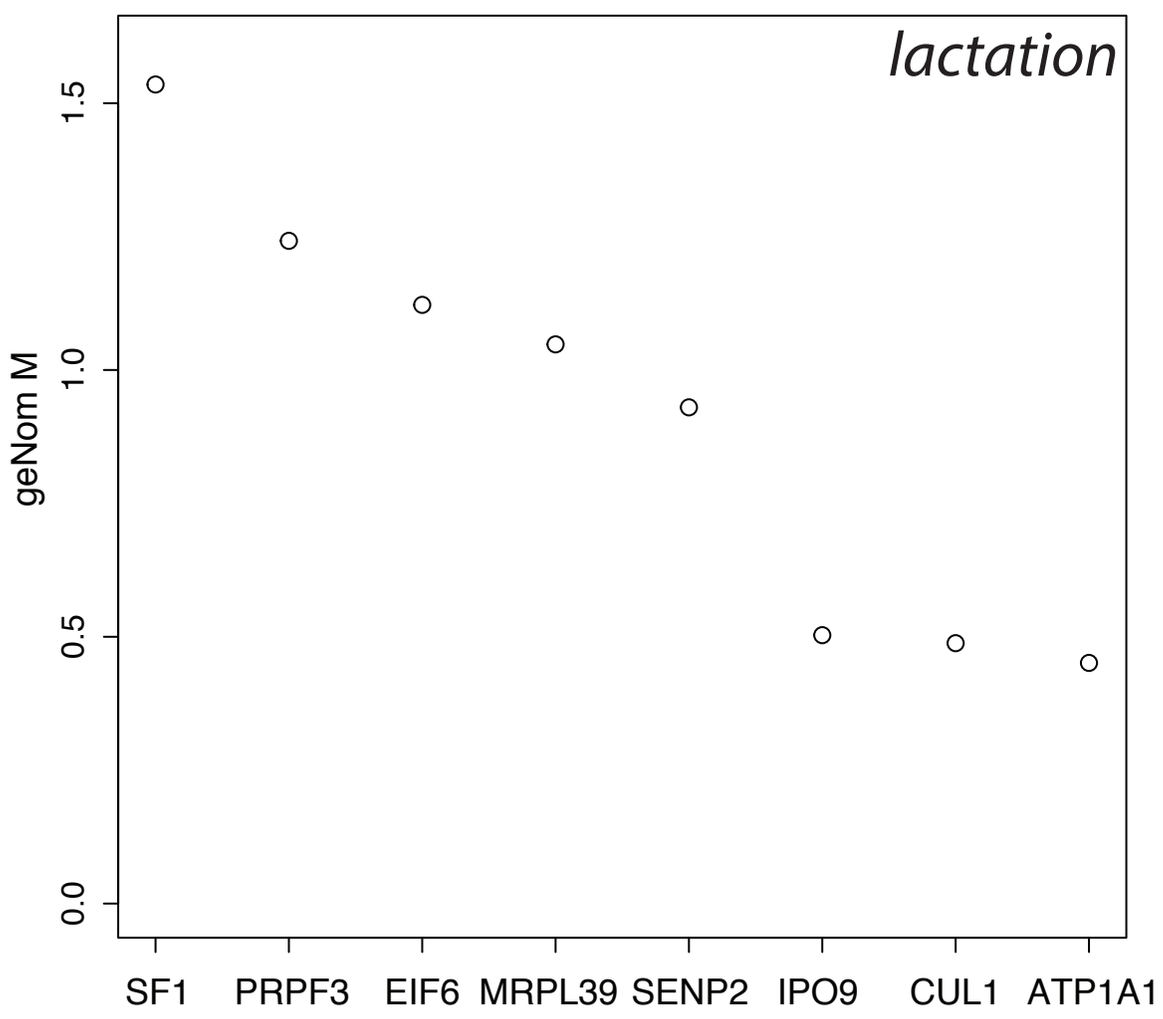
0.1
0.2
0.3
0.4
V2/3
V3/4
V4/5
V5/6
V6/7
V7/8
Pair-wise variations ($V_{n/n+1}$)

E.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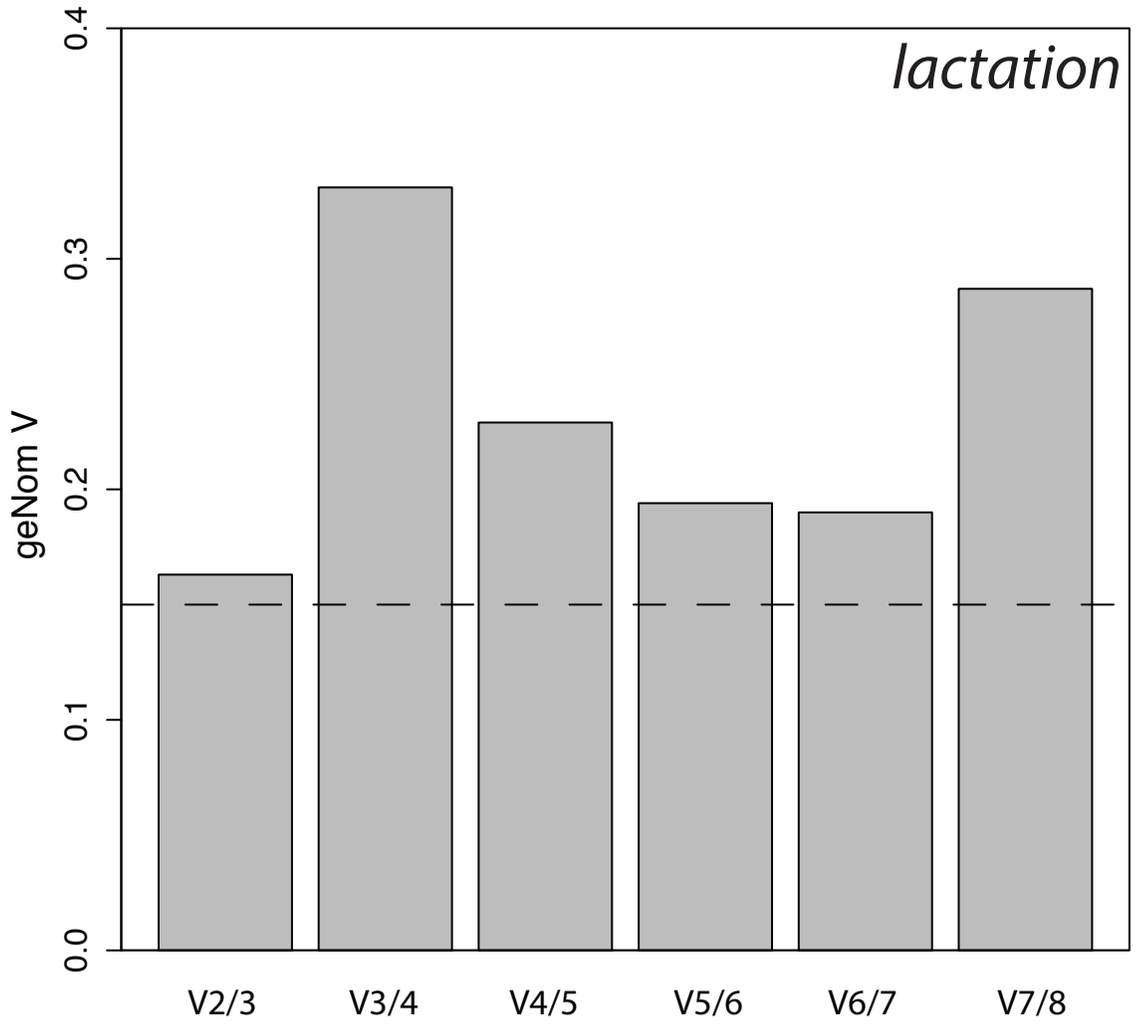
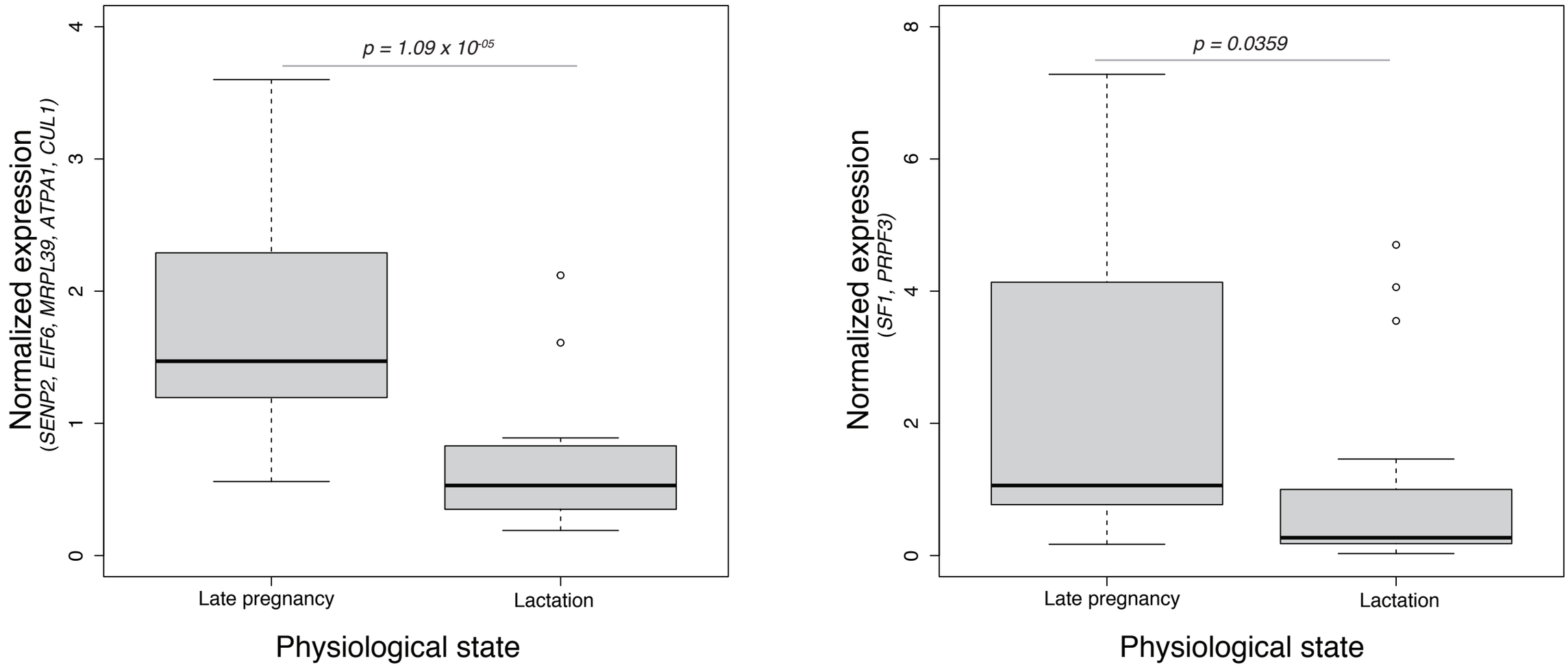
SF1
PRPF3
EIF6
MRPL39
SENP2
IPO9
CUL1
ATP1A1

F.

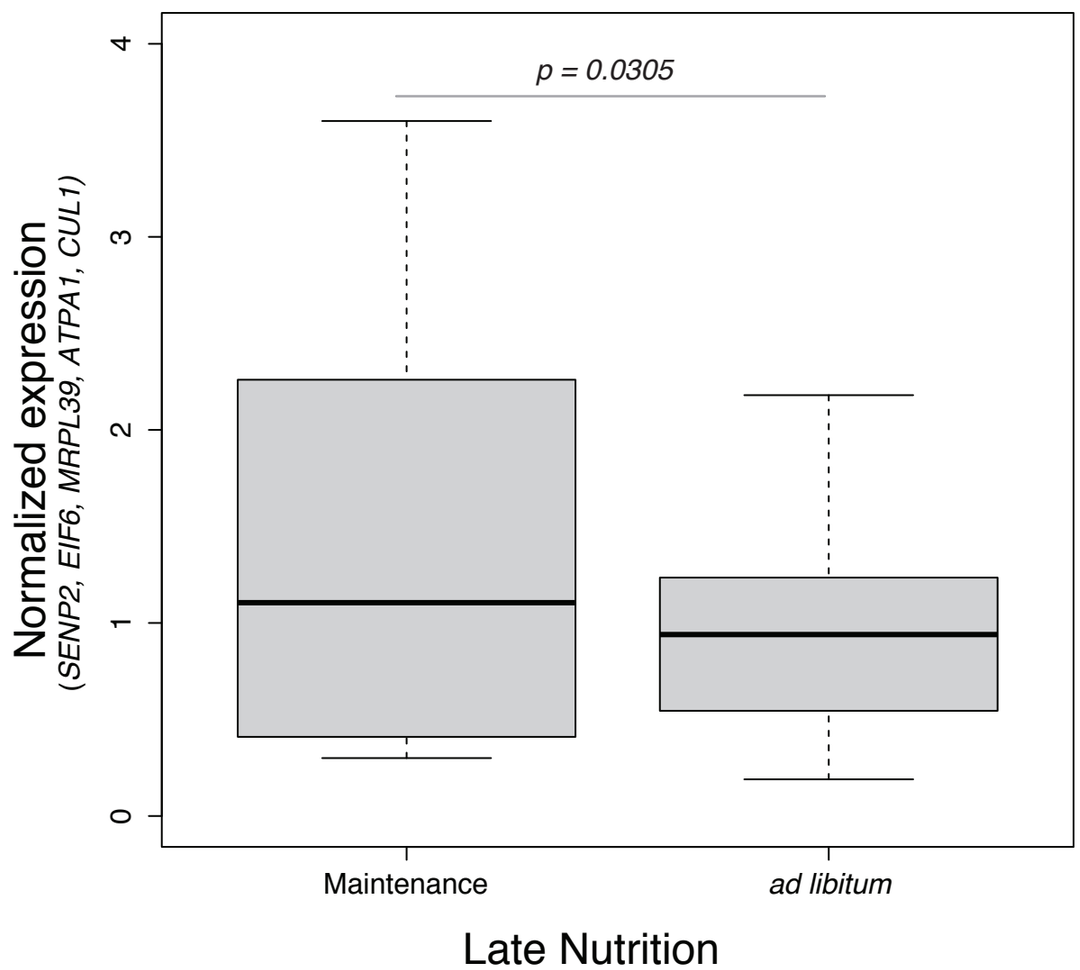

lactation
geNom V
0.0
0.1
0.2
0.3
0.4
V2/3
V3/4
V4/5
V5/6
V6/7
V7/8
Pair-wise variations ($V_{n/n+1}$)

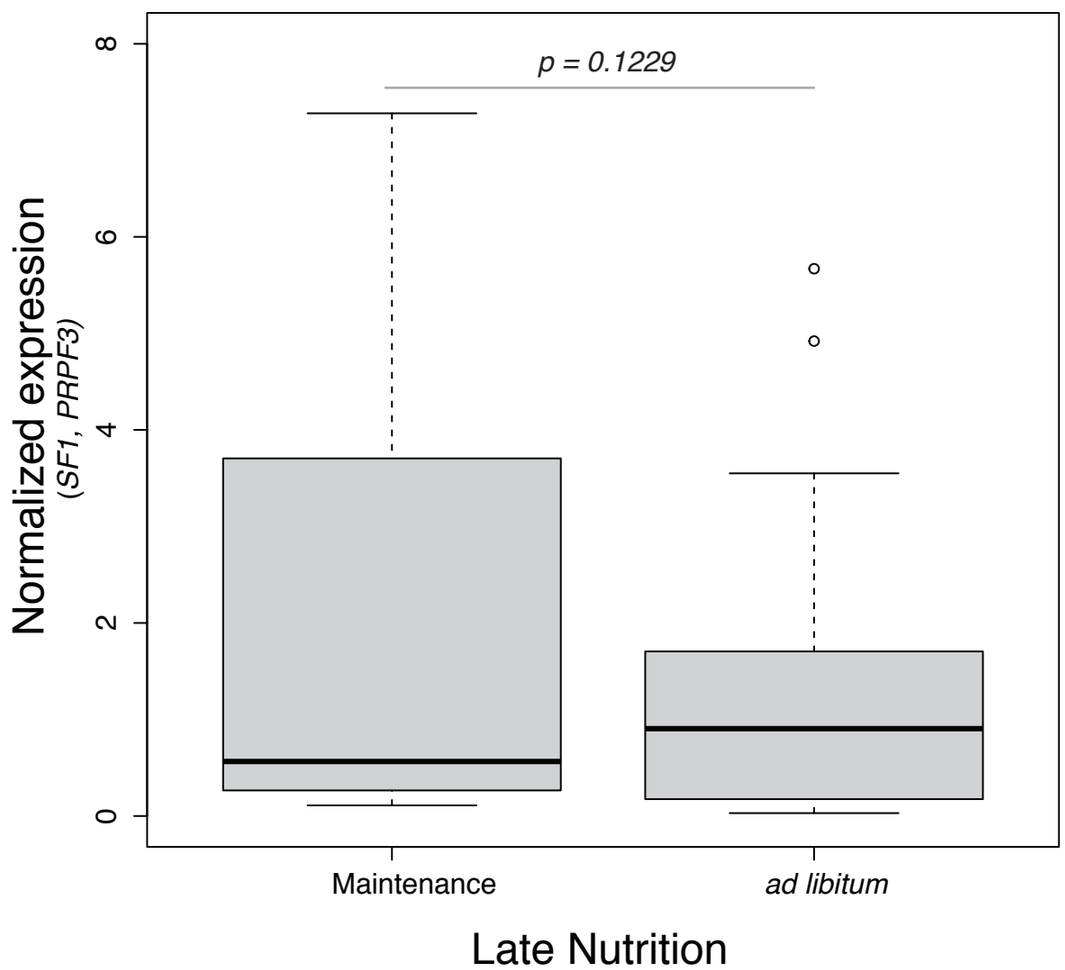
A.


p = 1.09 x 10-05
Normalized expression
(SENP2, EIF6, MRPL39, ATPA1, CUL1)
0
1
2
3
4
Late pregnancy
Lactation
Physiological state

p = 0.0359
Normalized expression
(SF1, PRPF3)
0
2
4
6
8
Late pregnancy
Lactation
Physiological state

B.


p = 0.0305
Normalized expression
(SENP2, EIF6, MRPL39, ATPA1, CUL1)
0
1
2
3
4
Maintenance
ad libitum
Late Nutrition


p = 0.1229
Normalized expression
(SF1, PRPF3)
0
2
4
6
8
Maintenance
ad libitum
Late Nutrition