



UNIVERSITY OF LEEDS

This is a repository copy of *Identification of reference genes for RT-qPCR in ovine mammary tissue during late pregnancy and lactation and in response to maternal nutritional programming*.

White Rose Research Online URL for this paper:
<http://eprints.whiterose.ac.uk/97706/>

Version: Accepted Version

Article:

Paten, AM, Pain, SJ, Peterson, SW et al. (4 more authors) (2014) Identification of reference genes for RT-qPCR in ovine mammary tissue during late pregnancy and lactation and in response to maternal nutritional programming. *Physiological Genomics*, 46 (15). pp. 560-570. ISSN 1094-8341

<https://doi.org/10.1152/physiolgenomics.00030.2014>

Reuse

Unless indicated otherwise, fulltext items are protected by copyright with all rights reserved. The copyright exception in section 29 of the Copyright, Designs and Patents Act 1988 allows the making of a single copy solely for the purpose of non-commercial research or private study within the limits of fair dealing. The publisher or other rights-holder may allow further reproduction and re-use of this version - refer to the White Rose Research Online record for this item. Where records identify the publisher as the copyright holder, users can verify any specific terms of use on the publisher's website.

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.



eprints@whiterose.ac.uk
<https://eprints.whiterose.ac.uk/>

1 **Title**

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

4

5 **Authors**

6 A. M. Paten^{1,2,3}, S. J. Pain^{2,3}, S. W. Peterson^{2,3}, H. T. Blair^{2,3}, P.R. Kenyon^{2,3}, P. K. Dearden^{1,3},
7 E. J. Duncan^{1,3,*}

8

9 **Author affiliations**

10 1. Laboratory for Evolution and Development, Genetics Otago, Department of Biochemistry,
11 University of Otago, P.O. Box 56, Dunedin, Aotearoa-New Zealand.

12 2. International Sheep Research Centre, Institute of Veterinary, Animal and Biomedical
13 Sciences, Massey University, Palmerston North, Aotearoa-New Zealand.

14 3. Gravida; National Centre for Growth and Development.

15

16 *To whom correspondence should be addressed at elizabeth.duncan@otago.ac.nz

17

18 **Running head:** Ovine mammary reference genes

19

20

21 **Abstract**

22 The mammary gland is a complex tissue consisting of multiple cell types which, over
23 the lifetime of an animal, go through repeated cycles of development associated with
24 pregnancy, lactation and involution. The mammary gland is also known to be sensitive to
25 maternal programming by environmental stimuli such as nutrition. The molecular basis of
26 these adaptations is of significant interest, but requires robust methods to measure gene
27 expression. Reverse transcription quantitative PCR (RT-qPCR) is commonly used to measure
28 gene expression, and is currently the method of choice for validating genome-wide
29 expression studies. RT-qPCR requires the selection of reference genes that are stably
30 expressed over physiological states and treatments. In this study we identify suitable
31 reference genes to normalize RT-qPCR data for the ovine mammary gland in two
32 physiological states; late pregnancy and lactation. Biopsies were collected from offspring of
33 ewes that had been subjected to different nutritional paradigms during pregnancy to
34 examine effects of maternal programming on the mammary gland of the offspring. We
35 evaluated eight candidate reference genes and found that two reference genes (*PRPF3* and
36 *CUL1*) are required for normalising RT-qPCR data from pooled RNA samples, but five
37 reference genes are required for analysing gene expression in individual animals (*SEN2*,
38 *EIF6*, *MRPL39*, *ATP1A1*, *CUL1*). Using these stable reference genes, we showed that *TET1*, a
39 key regulator of DNA methylation, is responsive to maternal programming and physiological
40 state. The identification of these novel reference genes will be of utility to future studies of
41 gene expression in the ovine mammary gland.

42

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

45

46 **Introduction**

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

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

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

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

89

90 **Material and methods**

91 **Animals and sampling**

92 Ovine mammary gland tissue was sampled from a sub-set of twin-bearing, twin-born
93 ewe-offspring of a previously published maternal nutritional programming study (22, 32).
94 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
95 to either a maintenance ($M_{P50-140}$) or ad libitum ($Ad_{P50-140}$) pasture allowance during mid-to-
96 late gestation (P50-140) (Fig. 1A). The ewe offspring generated were utilised as the
97 experimental animals of the present study, and were therefore from one of six dam
98 nutritional treatment groups: SmM, SmAd, MM, MAd, AdM, and AdAd (Fig.1B, Table 1). All
99 ewe offspring (G1 offspring) were managed under the same New Zealand commercial
100 pastoral farming conditions and received the same level of nutrition (average intakes).
101 Mammary parenchymal tissue (30 - 50 mg) was sampled from 10 ewes per treatment (n=60)
102 via needle biopsy (Bard® Magnum® reusable core biopsy gun and 12G, 10cm core biopsy
103 needles, Bard Biopsy Systems) during late pregnancy (135 ± 2.4 SD days of gestation) and
104 again during lactation (15 ± 1.27 SD days post partum). Tissue samples were immediately
105

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

113 **RNA extraction and cDNA synthesis**

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

124 **Pooling of samples**

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

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

139 **Selection of potential reference genes**

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

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

170 **Primer design**

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

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

182 **Quantitative PCR reactions**

183 RT-qPCR reactions were carried out on a Bio-Rad C1000 Thermal cycler (Bio-Rad
184 CFX96 Real-Time System) using SsoFast EvaGreen Supermix (BioRad) with 10 × diluted cDNA
185 template and 300 nM of oligonucleotide primers. The following PCR program was used: 1
186 min initial incubation at 95°C followed by 40 cycles of 5 seconds at 95°C and 30 seconds at
187 60°C. On completion the reactions were held at 95°C for 10 seconds, reduced to 65°C and
188 incrementally raised by 0.5°C until reaching 95°C for a melt curve analysis. In all cases the Cq
189 measured for no template controls and –RT controls was greater than 40. Reactions were
190 carried out in duplicate for each sample to minimise effects of technical errors, duplicates
191 that differed by more than 0.5 cycles were repeated.

192 **Data analysis**

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

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

208

209 **Results**

210 ***Reference gene stability in pooled samples***

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

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

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

236

237 ***Reference gene stability in individual animal samples***

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

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

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

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

277 In both physiological states the least stable genes in this analysis were *SF1* and *PRP3*,
278 which were considered to be highly stable in the analysis of the pooled RNA samples (Fig.
279 2B). Although, when only the animals that comprised the pools were analyzed (Fig. 4), *PRP3*
280 was considered to be relatively stable in late pregnancy ($M = 0.697$), but not in lactation (M
281 $= 1.242$).

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

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

290

291 ***Sensitivity analysis of selected reference genes in RT-qPCR analysis***

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

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

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

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

324

325 **Discussion**

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

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

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

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

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

367 We observed high levels of variation in gene expression between individuals (Fig.
368 3A). This may be, at least partially, attributed to limitations in the sampling method used in
369 this study. Biopsy sites were standardised as much as practical, but the mammary gland is a
370 mixed tissue type (containing mammary epithelial cells, fibroblasts, blood vessels,
371 connective and adipose tissue) and it is likely that individual biopsy samples contained
372 different proportions of these cell types. In addition, gene expression in the mammary gland
373 is known to be patchy, with not all epithelial cells actively expressing genes for milk
374 synthesis and secretion (30). It may be possible to use cell sorting and labelling to obtain
375 more homogenous samples. Increasing sample sizes would also reduce the effect of

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

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

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

406 **Conclusions**

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

415 **Acknowledgements:**

416 The authors wish to thank Dr Anne Ridler for her advice and assistance with the mammary
417 biopsies. This work was funded by Massey University and Gravida; National Centre for
418 Growth and Development. AP was funded by Gravida; National Centre for Growth and
419 Development PhD scholarship

420

421 **Author contributions:**

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

429

430

431 **References:**

432

433

- 434 1. **Abramowitz LK, and Bartolomei MS.** Genomic imprinting: recognition and marking of imprinted loci.
435 *Current opinion in genetics & development* 22: 72-78, 2012.
- 436 2. **Aggarwal J, Sharma A, Kishore A, Mishra BP, Yadav A, Mohanty A, Sodhi M, Kataria RS, Malakar D,**
437 **and Mukesh M.** Identification of suitable housekeeping genes for normalization of quantitative real-time PCR
438 data during different physiological stages of mammary gland in riverine buffaloes (*Bubalus bubalis*). *Journal of*
439 *animal physiology and animal nutrition* 97: 1132-1141, 2013.
- 440 3. **Andersen CL, Jensen JL, and Orntoft TF.** Normalization of real-time quantitative reverse transcription-
441 PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to
442 bladder and colon cancer data sets. *Cancer Res* 64: 5245-5250, 2004.
- 443 4. **Bionaz M, and Loor JJ.** Identification of reference genes for quantitative real-time PCR in the bovine
444 mammary gland during the lactation cycle. *Physiological Genomics* 29: 312-319, 2007.
- 445 5. **Bionaz M, Periasamy K, Rodriguez-Zas SL, Everts RE, Lewin HA, Hurley WL, and Loor JJ.** Old and New
446 Stories: Revelations from Functional Analysis of the Bovine Mammary Transcriptome during the Lactation
447 Cycle. *Plos One* 7: 2012.
- 448 6. **Blair HT, Jenkinson CM, Peterson SW, Kenyon PR, van der Linden DS, Davenport LC, Mackenzie DD,**
449 **Morris ST, and Firth EC.** Dam and granddam feeding during pregnancy in sheep affects milk supply in offspring
450 and reproductive performance in grand-offspring. *J Anim Sci* 88: E40-50, 2010.
- 451 7. **Burns MJ, Nixon GJ, Foy CA, and Harris N.** Standardisation of data from real-time quantitative PCR
452 methods - evaluation of outliers and comparison of calibration curves. *BMC biotechnology* 5: 31, 2005.
- 453 8. **Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW,**
454 **Shiely GL, Vandesompele J, and Wittwer CT.** The MIQE Guidelines: Minimum Information for Publication of
455 Quantitative Real-Time PCR Experiments. *Clin Chem* 55: 611-622, 2009.

-
- 456 9. **Calcagno AM, Chewning KJ, Wu CP, and Ambudkar SV.** Plasma membrane calcium ATPase (PMCA4):
457 a housekeeper for RT-PCR relative quantification of polytopic membrane proteins. *BMC molecular biology* 7:
458 29, 2006.
- 459 10. **Cameron RC, Duncan EJ, and Dearden PK.** Stable reference genes for the measurement of transcript
460 abundance during larval caste development in the honeybee. *Apidologie* 44: 357-366, 2013.
- 461 11. **Capuco AV, and Akers RM.** Management and Environmental Influences on Mammary Gland
462 Development and Milk Production. In: *Managing the prenatal environment to enhance livestock productivity*,
463 edited by Paul L. Greenwood AWB, Philip E. Vercoe, Gerrit J. Viljoen. Dordrecht ; London: Springer,, 2010, p. 1
464 online resource (xii, 298 p.) ill. (some col.).
- 465 12. **Ferreira AM, Bislev SL, Bendixen E, and Almeida AM.** The mammary gland in domestic ruminants: A
466 systems biology perspective. *J Proteomics* 94: 110-123, 2013.
- 467 13. **Foret S, Kucharski R, Pellegrini M, Feng S, Jacobsen SE, Robinson GE, and Maleszka R.** DNA
468 methylation dynamics, metabolic fluxes, gene splicing, and alternative phenotypes in honey bees. *Proc Natl*
469 *Acad Sci U S A* 109: 4968-4973, 2012.
- 470 14. **He YF, Li BZ, Li Z, Liu P, Wang Y, Tang Q, Ding J, Jia Y, Chen Z, Li L, Sun Y, Li X, Dai Q, Song CX, Zhang**
471 **K, He C, and Xu GL.** Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA.
472 *Science* 333: 1303-1307, 2011.
- 473 15. **Hellemans J, Mortier G, De Paepe A, Speleman F, and Vandesompele J.** qBase relative quantification
474 framework and software for management and automated analysis of real-time quantitative PCR data. *Genome*
475 *Biol* 8: R19, 2007.
- 476 16. **Hilakivi-Clarke L, Clarke R, Onojafe I, Raygada M, Cho E, and Lippman M.** A maternal diet high in n - 6
477 polyunsaturated fats alters mammary gland development, puberty onset, and breast cancer risk among female
478 rat offspring. *Proc Natl Acad Sci U S A* 94: 9372-9377, 1997.
- 479 17. **Hovey RC, Trott JF, and Vonderhaar BK.** Establishing a framework for the functional mammary gland:
480 From endocrinology to morphology. *J Mammary Gland Biol* 7: 17-38, 2002.
- 481 18. **Jones PA.** Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nature reviews*
482 *Genetics* 13: 484-492, 2012.
- 483 19. **Kadegowda AK, Bionaz M, Thering B, Piperova LS, Erdman RA, and Loor JJ.** Identification of internal
484 control genes for quantitative polymerase chain reaction in mammary tissue of lactating cows receiving lipid
485 supplements. *J Dairy Sci* 92: 2007-2019, 2009.
- 486 20. **Kendzierski C, Irizarry RA, Chen KS, Haag JD, and Gould MN.** On the utility of pooling biological
487 samples in microarray experiments. *P Natl Acad Sci USA* 102: 4252-4257, 2005.
- 488 21. **Kendzierski CM, Zhang Y, Lan H, and Attie AD.** The efficiency of pooling mRNA in microarray
489 experiments. *Biostatistics* 4: 465-477, 2003.
- 490 22. **Kenyon PR, Pain SJ, Hutton PG, Jenkinson CMC, Morris ST, Peterson SW, and Blair HT.** Effects of
491 twin-bearing ewe nutritional treatments on ewe and lamb performance to weaning. *Anim Prod Sci* 51: 406-
492 415, 2011.
- 493 23. **Konczal M, Koteja P, Stuglik MT, Radwan J, and Babik W.** Accuracy of allele frequency estimation
494 using pooled RNA-Seq. *Molecular ecology resources* 14: 381-392, 2014.
- 495 24. **Leth-Larsen R, Lund R, Hansen HV, Laenkholm AV, Tarin D, Jensen ON, and Ditzel HJ.** Metastasis-
496 related plasma membrane proteins of human breast cancer cells identified by comparative quantitative mass
497 spectrometry. *Molecular & cellular proteomics : MCP* 8: 1436-1449, 2009.
- 498 25. **Lorincz MC, Dickerson DR, Schmitt M, and Groudine M.** Intragenic DNA methylation alters chromatin
499 structure and elongation efficiency in mammalian cells. *Nature structural & molecular biology* 11: 1068-1075,
500 2004.
- 501 26. **Lyko F, Foret S, Kucharski R, Wolf S, Falckenhayn C, and Maleszka R.** The honey bee epigenomes:
502 differential methylation of brain DNA in queens and workers. *PLoS biology* 8: e1000506, 2010.
- 503 27. **Maiti A, and Drohat AC.** Thymine DNA glycosylase can rapidly excise 5-formylcytosine and 5-
504 carboxylcytosine: potential implications for active demethylation of CpG sites. *J Biol Chem* 286: 35334-35338,
505 2011.
- 506 28. **Martin NP, Kenyon PR, Morel PCH, Pain SJ, Jenkinson CMC, Hutton PG, Morris ST, Peterson SW,**
507 **Firth EC, and Blair HT.** Ewe nutrition in early and mid- to late pregnancy has few effects on fetal development.
508 *Anim Prod Sci* 52: 533-539, 2012.
- 509 29. **Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D'Souza C, Fouse SD, Johnson BE, Hong C,**
510 **Nielsen C, Zhao Y, Turecki G, Delaney A, Varhol R, Thiessen N, Shchors K, Heine VM, Rowitch DH, Xing X,**
511 **Fiore C, Schillebeeckx M, Jones SJ, Haussler D, Marra MA, Hirst M, Wang T, and Costello JF.** Conserved role of
512 intragenic DNA methylation in regulating alternative promoters. *Nature* 466: 253-257, 2010.

- 513 30. **Molenaar AJ, Davis SR, and Wilkins RJ.** Expression of alpha-lactalbumin, alpha-S1-casein, and
514 lactoferrin genes is heterogeneous in sheep and cattle mammary tissue. *The journal of histochemistry and*
515 *cytochemistry : official journal of the Histochemistry Society* 40: 611-618, 1992.
- 516 31. **Neville MC, McFadden TB, and Forsyth I.** Hormonal regulation of mammary differentiation and milk
517 secretion. *J Mammary Gland Biol* 7: 49-66, 2002.
- 518 32. **Paten AM, Kenyon PR, Lopez-Villalobos N, Peterson SW, Jenkinson CMC, Pain SJ, and Blair HT.**
519 LACTATION BIOLOGY SYMPOSIUM: Maternal nutrition during early and mid-to-late pregnancy: Comparative
520 effects on milk production of twin-born ewe progeny during their first lactation. *Journal of Animal Science* 91:
521 676-684, 2013.
- 522 33. **Sati S, Tanwar VS, Kumar KA, Patowary A, Jain V, Ghosh S, Ahmad S, Singh M, Reddy SU, Chandak**
523 **GR, Raghunath M, Sivasubbu S, Chakraborty K, Scaria V, and Sengupta S.** High resolution methylome map of
524 rat indicates role of intragenic DNA methylation in identification of coding region. *Plos One* 7: e31621, 2012.
- 525 34. **Shukla S, Kavak E, Gregory M, Imashimizu M, Shutinoski B, Kashlev M, Oberdoerffer P, Sandberg R,**
526 **and Oberdoerffer S.** CTCF-promoted RNA polymerase II pausing links DNA methylation to splicing. *Nature* 479:
527 74-79, 2011.
- 528 35. **Spruijt CG, Gnerlich F, Smits AH, Pfaffeneder T, Jansen PW, Bauer C, Munzel M, Wagner M, Muller**
529 **M, Khan F, Eberl HC, Mensinga A, Brinkman AB, Lephikov K, Muller U, Walter J, Boelens R, van Ingen H,**
530 **Leonhardt H, Carell T, and Vermeulen M.** Dynamic readers for 5-(hydroxy)methylcytosine and its oxidized
531 derivatives. *Cell* 152: 1146-1159, 2013.
- 532 36. **Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR,**
533 **Aravind L, and Rao A.** Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL
534 partner TET1. *Science* 324: 930-935, 2009.
- 535 37. **Tramontana S, Bionaz M, Sharma A, Graugnard DE, Cutler EA, Ajmone-Marsan P, Hurley WL, and**
536 **Loor JJ.** Internal controls for quantitative polymerase chain reaction of swine mammary glands during
537 pregnancy and lactation. *J Dairy Sci* 91: 3057-3066, 2008.
- 538 38. **Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, and Rozen SG.** Primer3--new
539 capabilities and interfaces. *Nucleic acids research* 40: e115, 2012.
- 540 39. **Valasek MA, and Repa JJ.** The power of real-time PCR. *Adv Physiol Educ* 29: 151-159, 2005.
- 541 40. **van der Linden DS, Kenyon PR, Blair HT, Lopez-Villalobos N, Jenkinson CMC, Peterson SW, and**
542 **Mackenzie DDS.** Effects of ewe size and nutrition on fetal mammary gland development and lactational
543 performance of offspring at their first lactation. *Journal of Animal Science* 87: 3944-3954, 2009.
- 544 41. **Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, and Speleman F.** Accurate
545 normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.
546 *Genome Biol* 3: 2002.
- 547 42. **Wielscher M, Liou W, Pulverer W, Singer CF, Rappaport-Fuerhauser C, Kandioler D, Egger G, and**
548 **Weinhausel A.** Cytosine 5-Hydroxymethylation of the LZTS1 Gene Is Reduced in Breast Cancer. *Translational*
549 *oncology* 6: 715-721, 2013.
- 550 43. **Wutz A, and Gribnau J.** X inactivation Xplained. *Current opinion in genetics & development* 17: 387-
551 393, 2007.
- 552 44. **Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, and Madden TL.** Primer-BLAST: a tool to design
553 target-specific primers for polymerase chain reaction. *BMC bioinformatics* 13: 134, 2012.
- 554
555

556 **Figure Captions**

557
558 Fig. 1: Experimental design and RNA-pooling strategy used for this reference gene study. (A)
559 Maternal-feeding paradigm. Romney ewes (G0) were fed *ad libitum* until day 21 of
560 pregnancy when animals were randomly allocated to a sub-maintenance (Sm), maintenance
561 (M) or *ad libitum* (Ad) diet. At day 50 of pregnancy, ewes were randomly reallocated to
562 either a maintenance (M) or *ad libitum* (Ad) diet until day 140 of pregnancy when all ewes
563 were switched to an *ad libitum* diet. (B) The offspring (G1) exposed to maternal nutritional
564 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)

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

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

628

629 **Tables:**

630 **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	<i>Ad-libitum</i>
MM	Maintenance	Maintenance
MAd	Maintenance	<i>Ad-libitum</i>
AdM	<i>Ad-libitum</i>	Maintenance
AdAd	<i>Ad-libitum</i>	<i>Ad-libitum</i>

631

632 **Table 2:** Ranking of candidate reference genes

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

<i>PRPF3</i>	U4/U6 small nuclear ribonucleoprotein <i>PRP3</i>	14	17	14	12
<i>IPO9</i>	Importin 9	10	19	6	10
<i>MRPL39</i>	Mitochondrial ribosomal protein L39	From literature			
<i>EIF6</i>	Eukaryotic translation initiation factor 6	From literature			
<i>ATP1A1</i>	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.

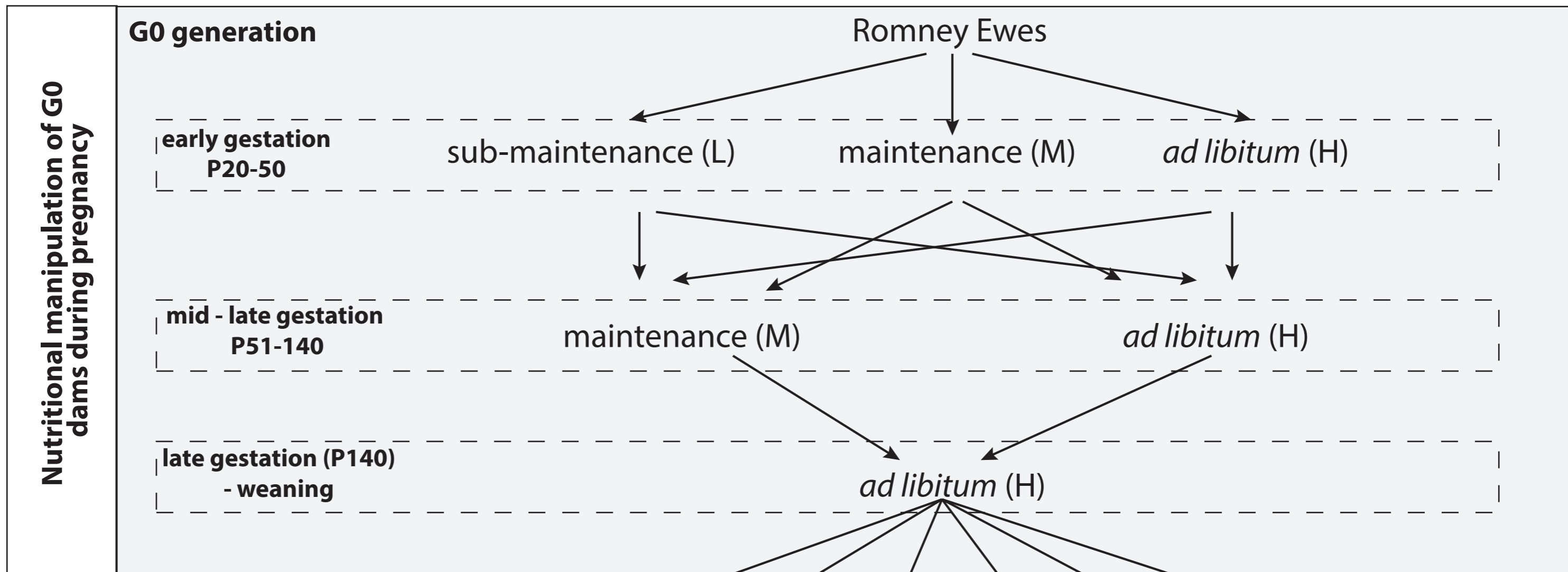
Gene	NCBI accession	Forward Primer 5' → 3'	Reverse Primer 5' → 3'	Amplicon length (bp)	PCR efficiency (%)*
<i>MRPL39</i>	XM_004002812.1	CCCTGGAAGTTGAAGCAAAA	GGTTCTGGGATGCCTTCTCT	90	98.1
<i>EIF6</i>	NM_001162563.1	AATTGAGGACCAGGATGAGC	GCACACCAGTCATTACCAC	114	103.8
<i>ATP1A1</i>	NM_001009360.1	GAGATTGTGTTCCGCCAGGAC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

637

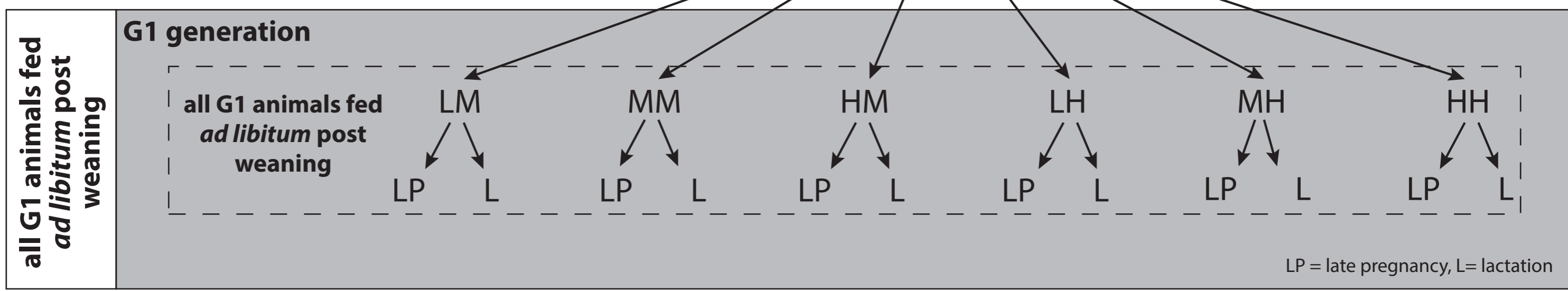
638

639

A.



B.

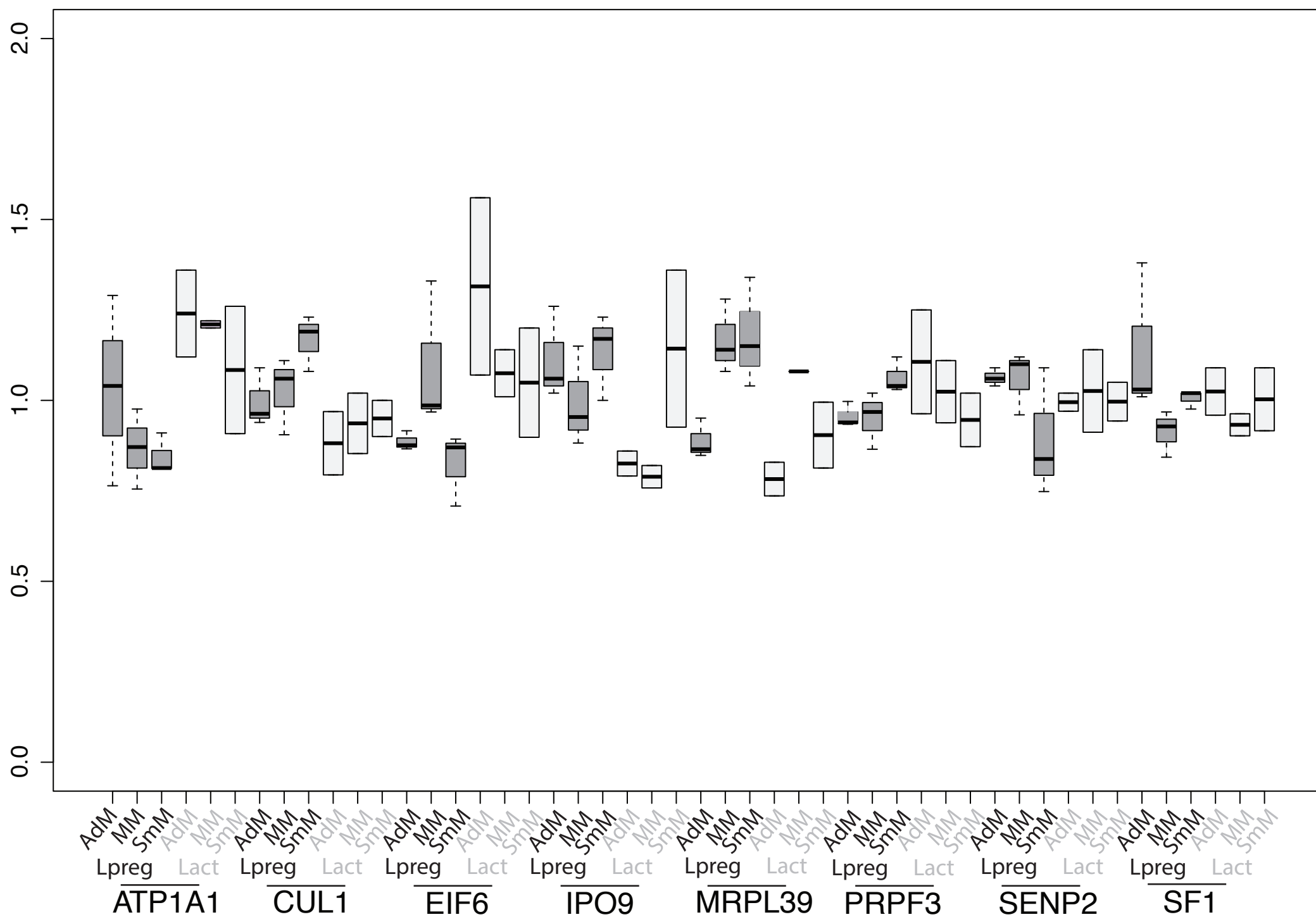


C.

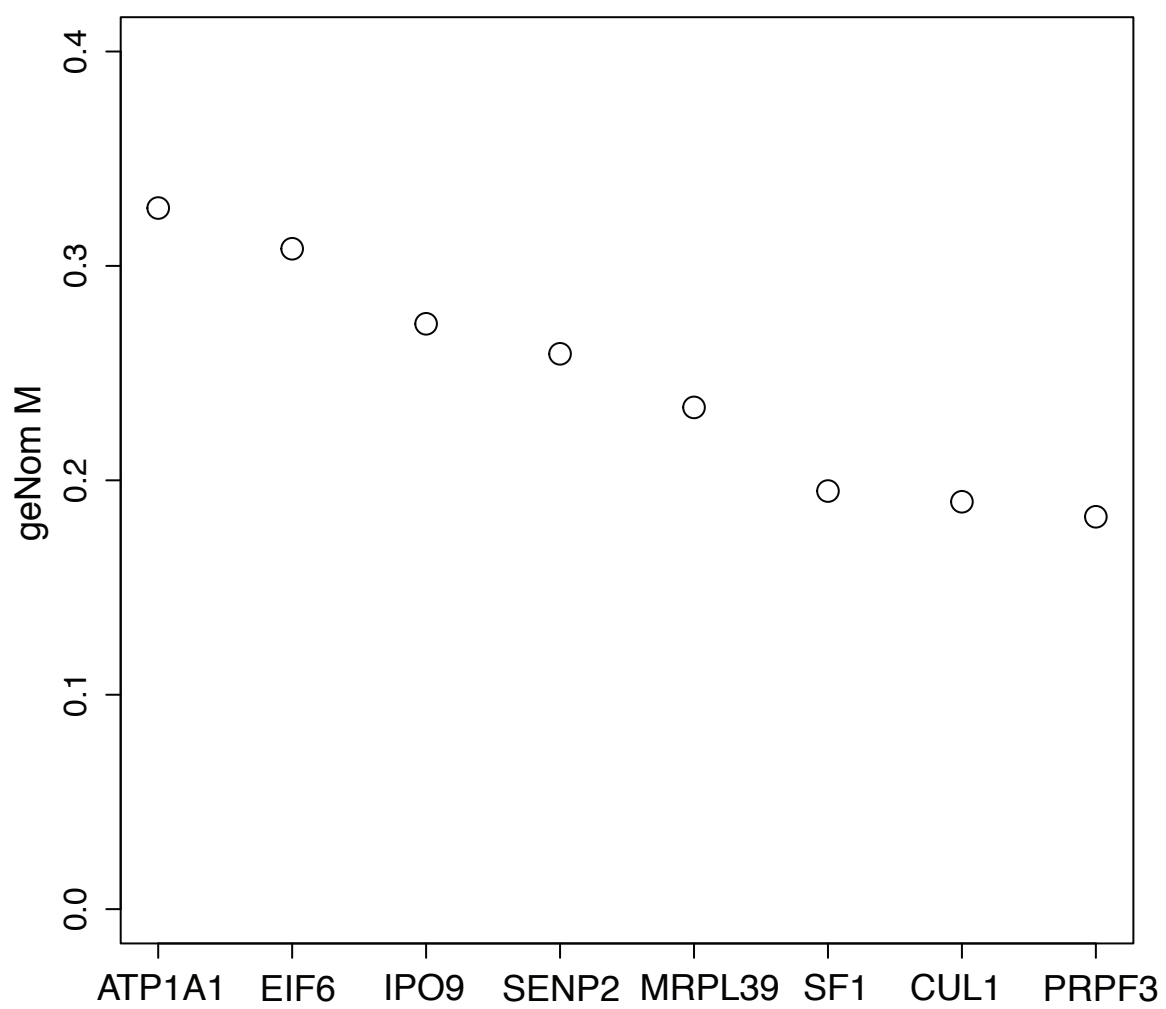
qRT-PCR samples & pooling strategy	Individual animal samples*	n=9	n=6	n=9	n=6	n=9	n=6	n=8	n=6	n=8	n=6	n=8	n=6
	Pooled animal samples	Pool 1	Pool 1	Pool 1	Pool 1	Pool 1	Pool 1	Pool 1	Pool 1	Pool 1	Pool 1	Pool 1	Pool 1
	n = 3 individual animals per pool	Pool 2	Pool 2	Pool 2	Pool 2	Pool 2	Pool 2	Pool 2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

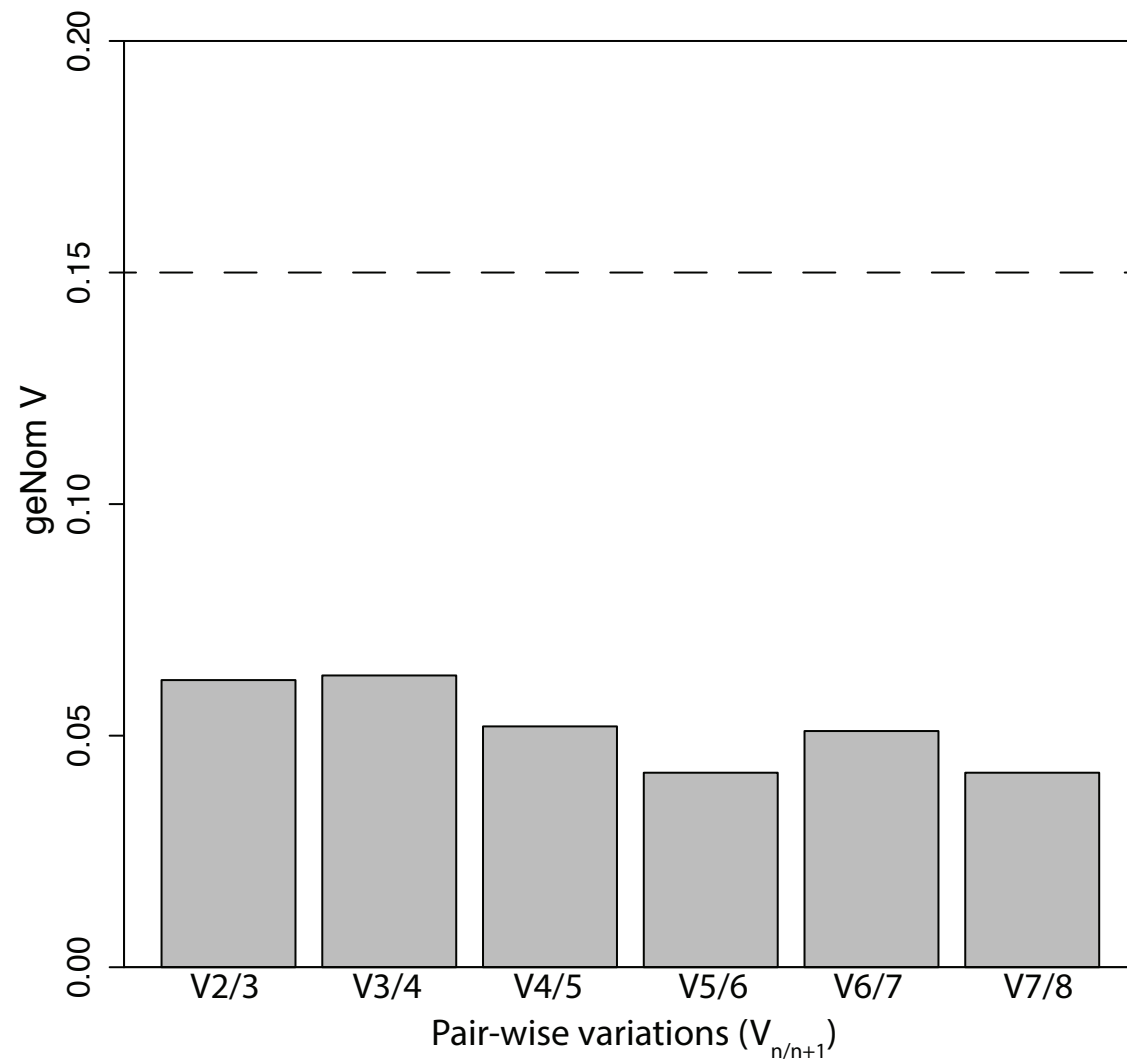
A.



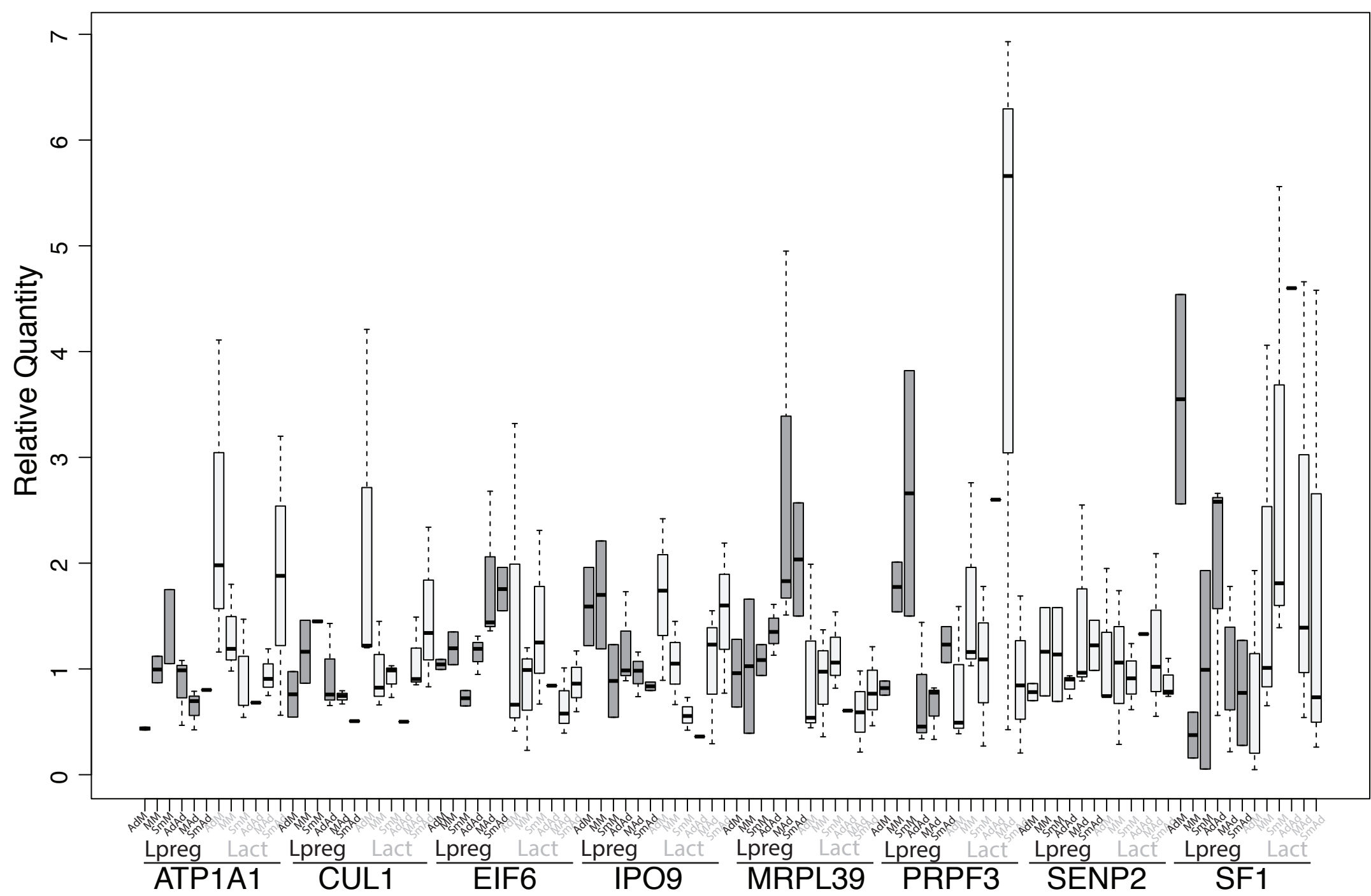
B.



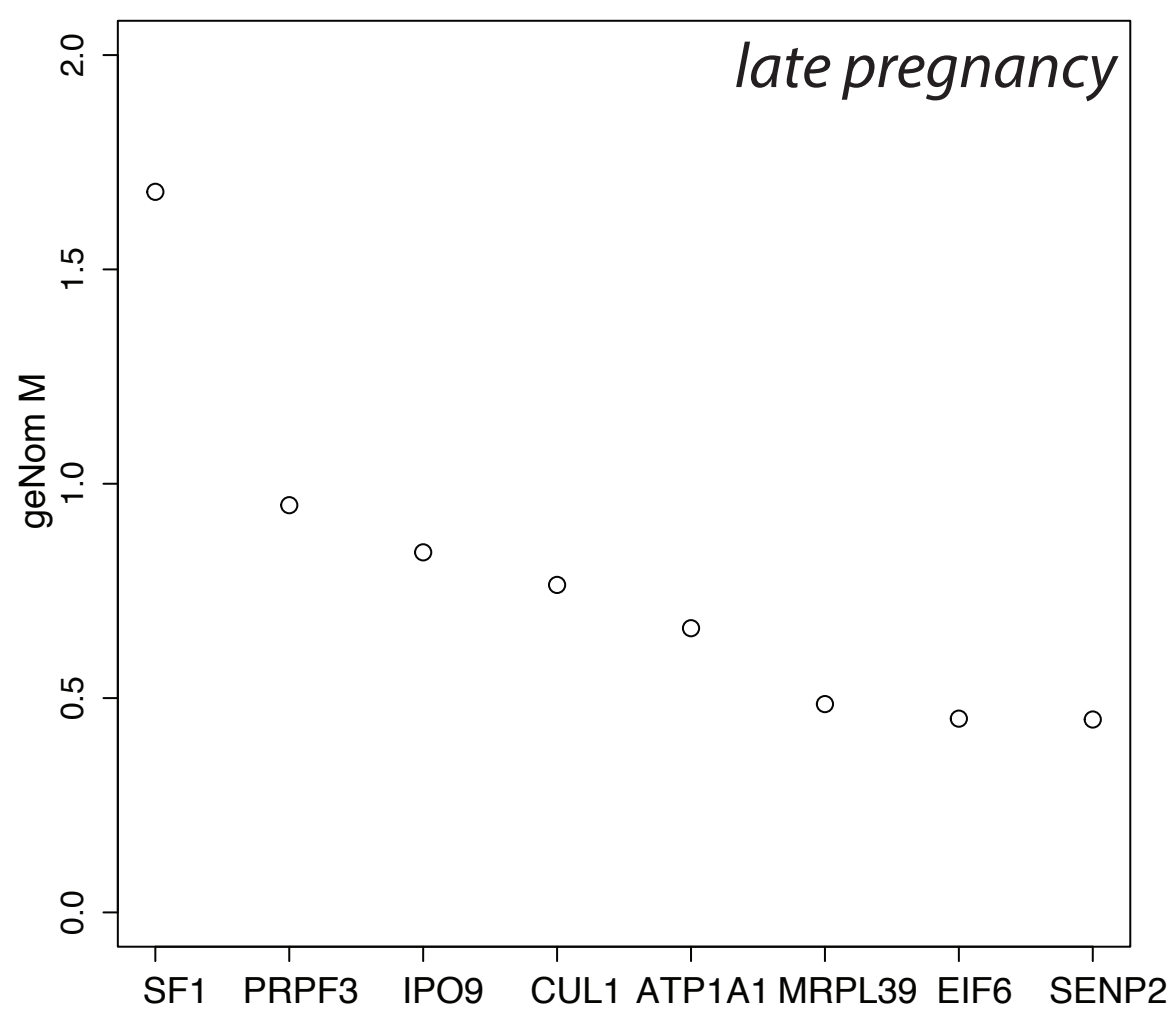
C.



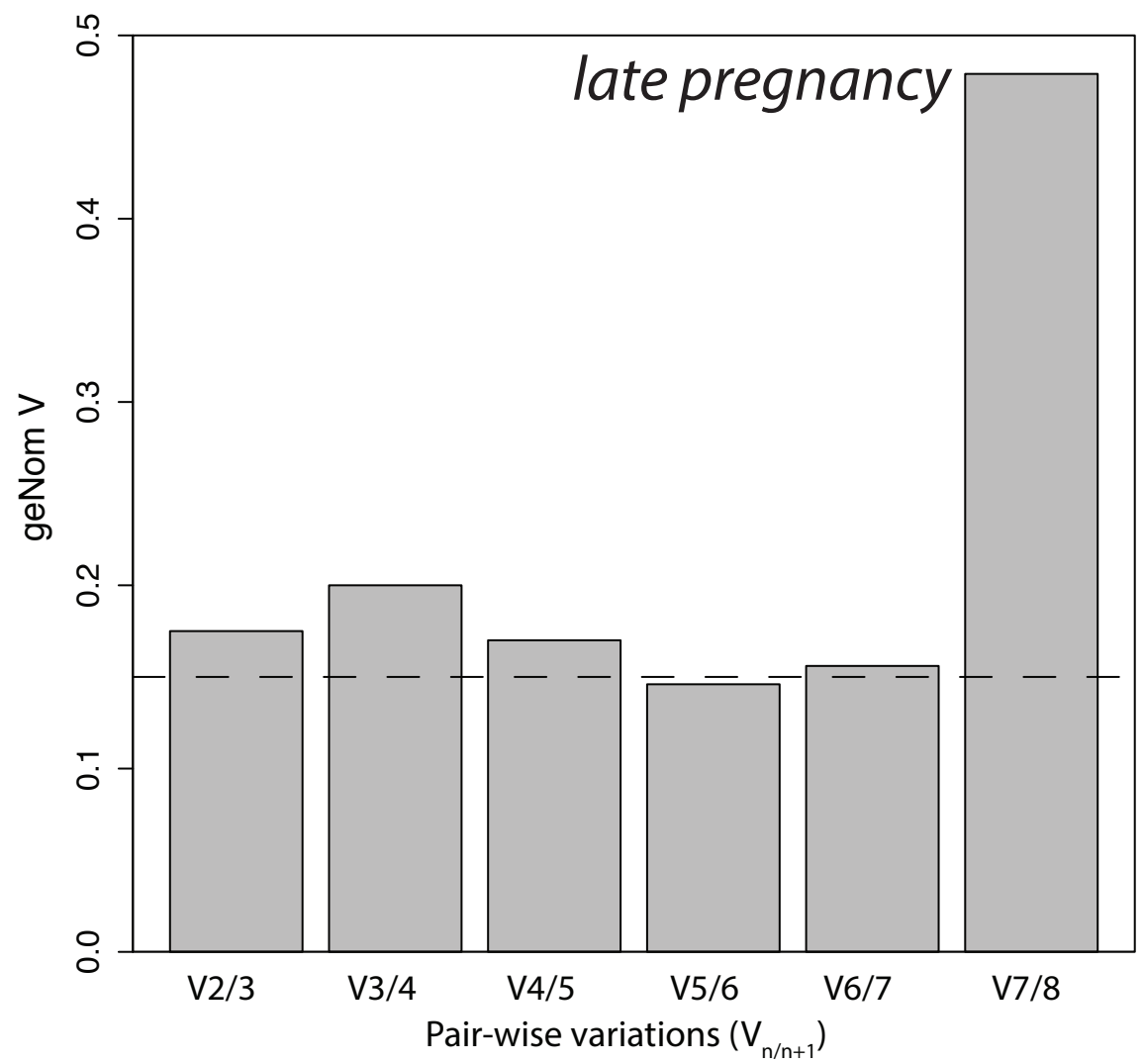
A.



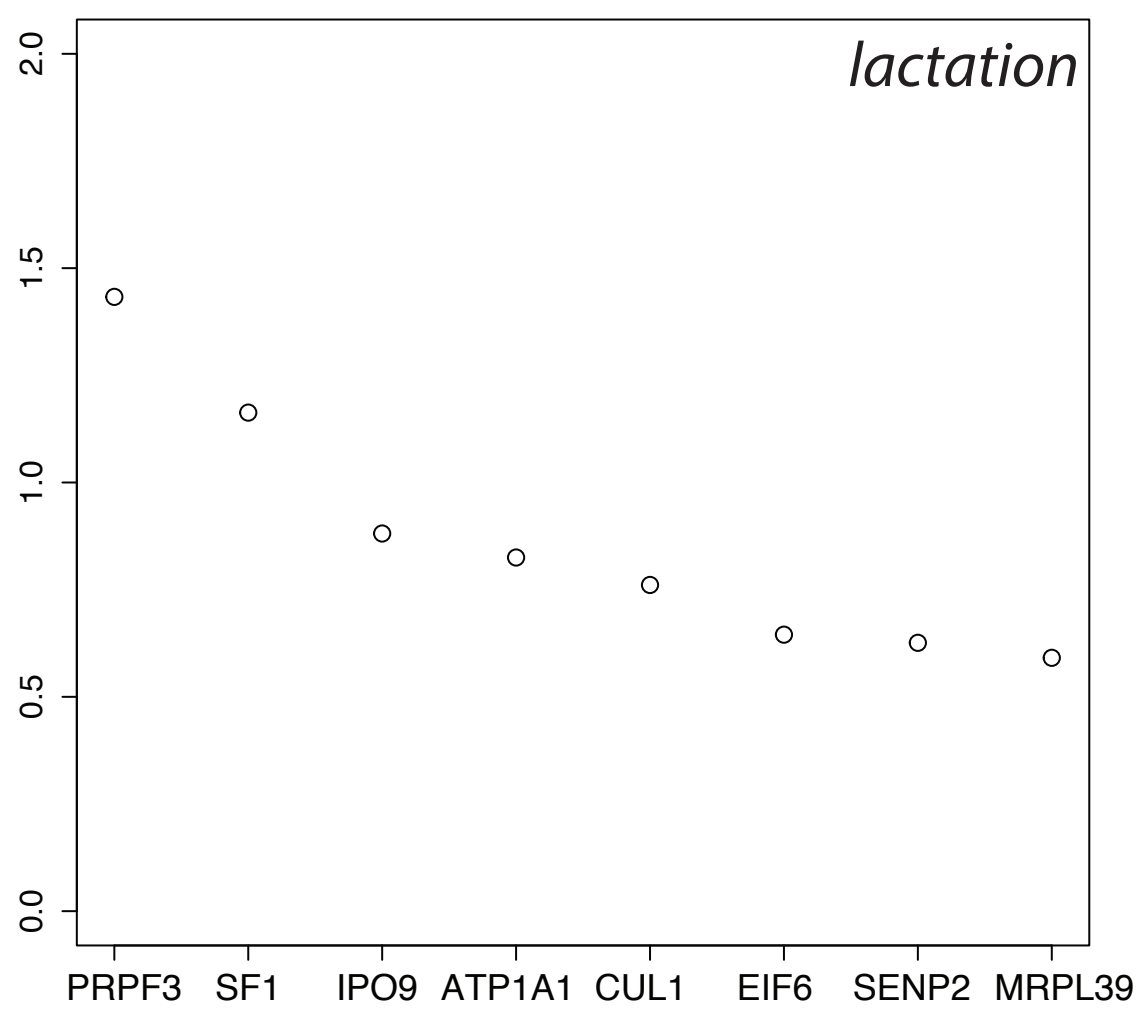
B.



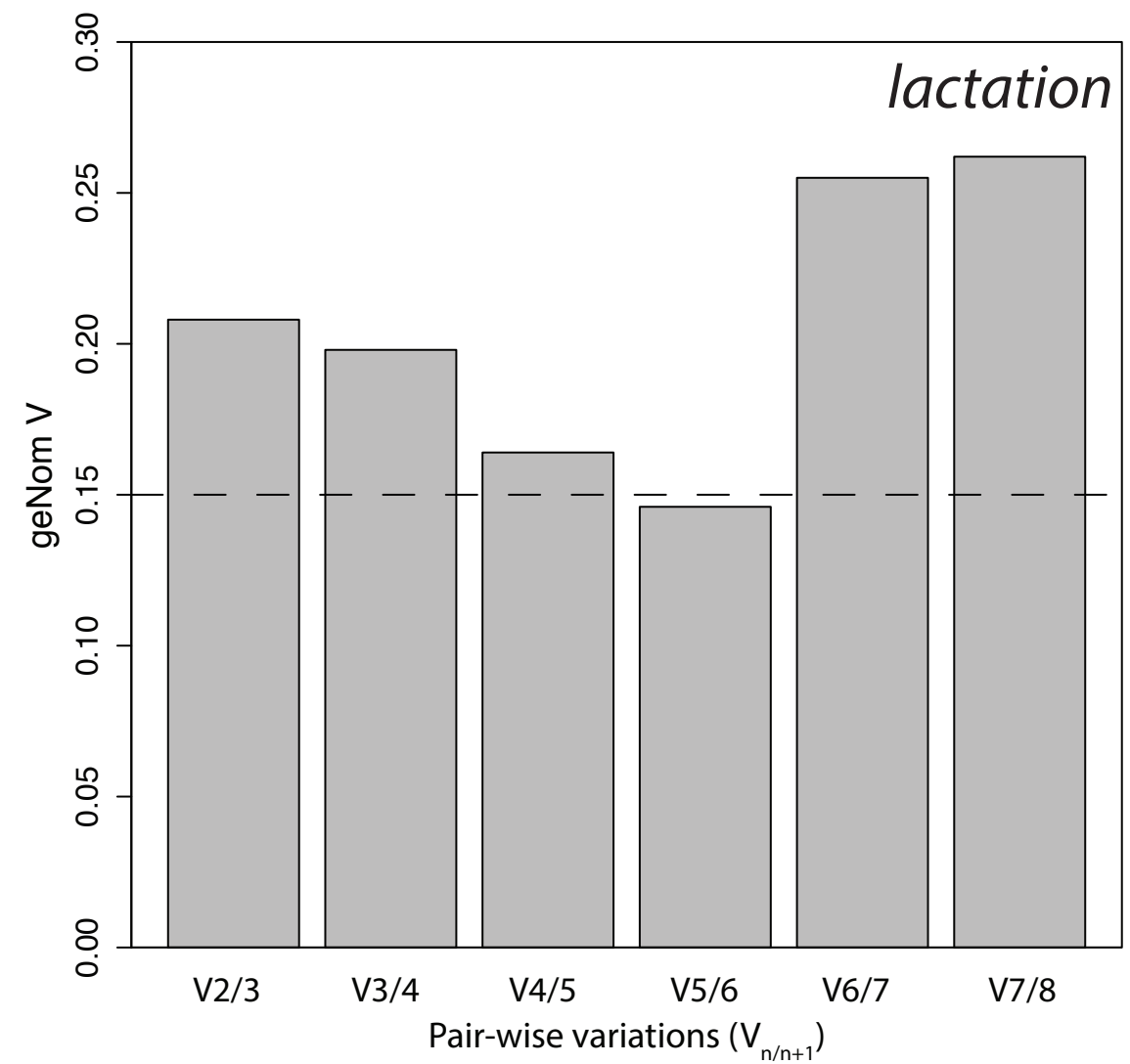
C.



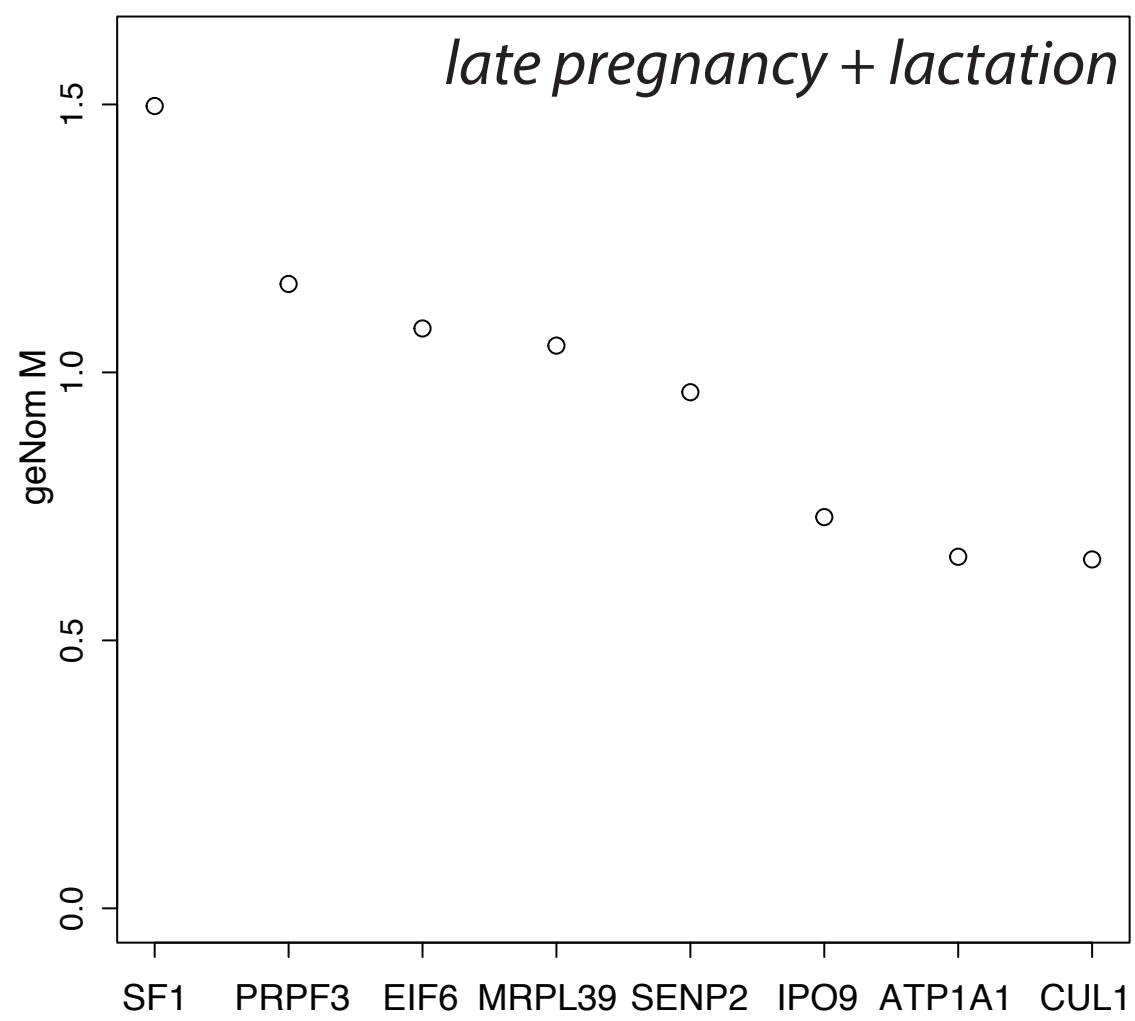
D.



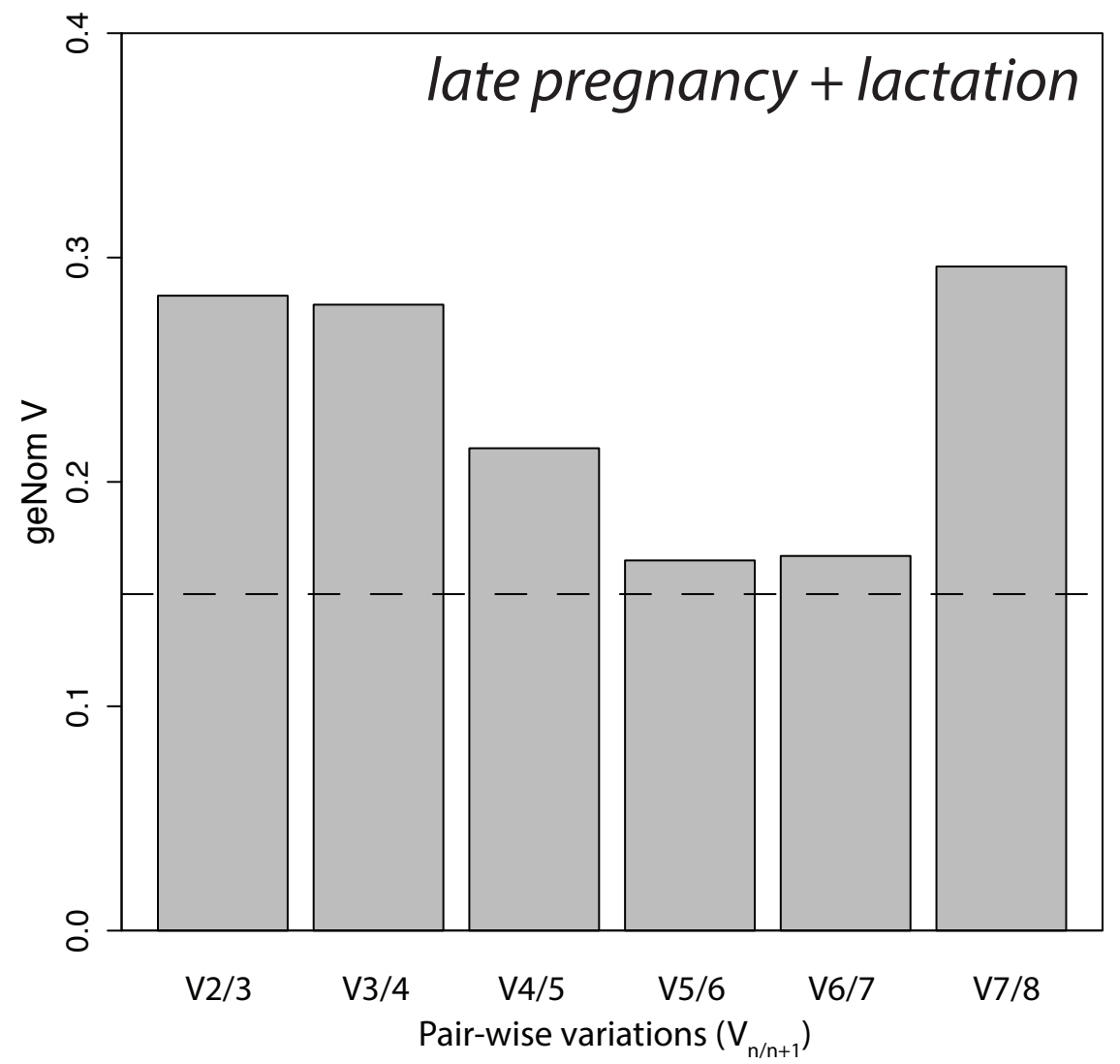
E.



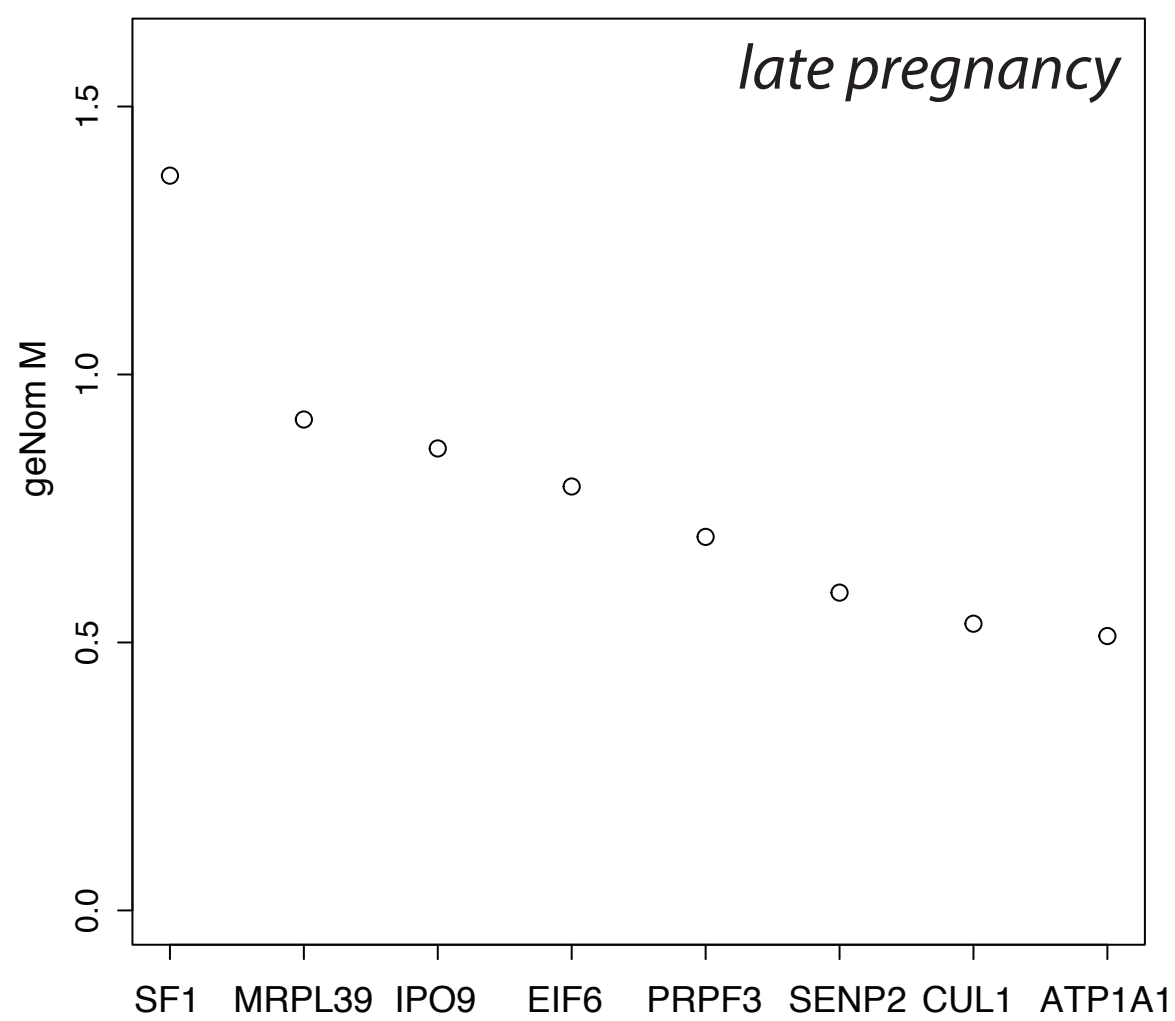
A.



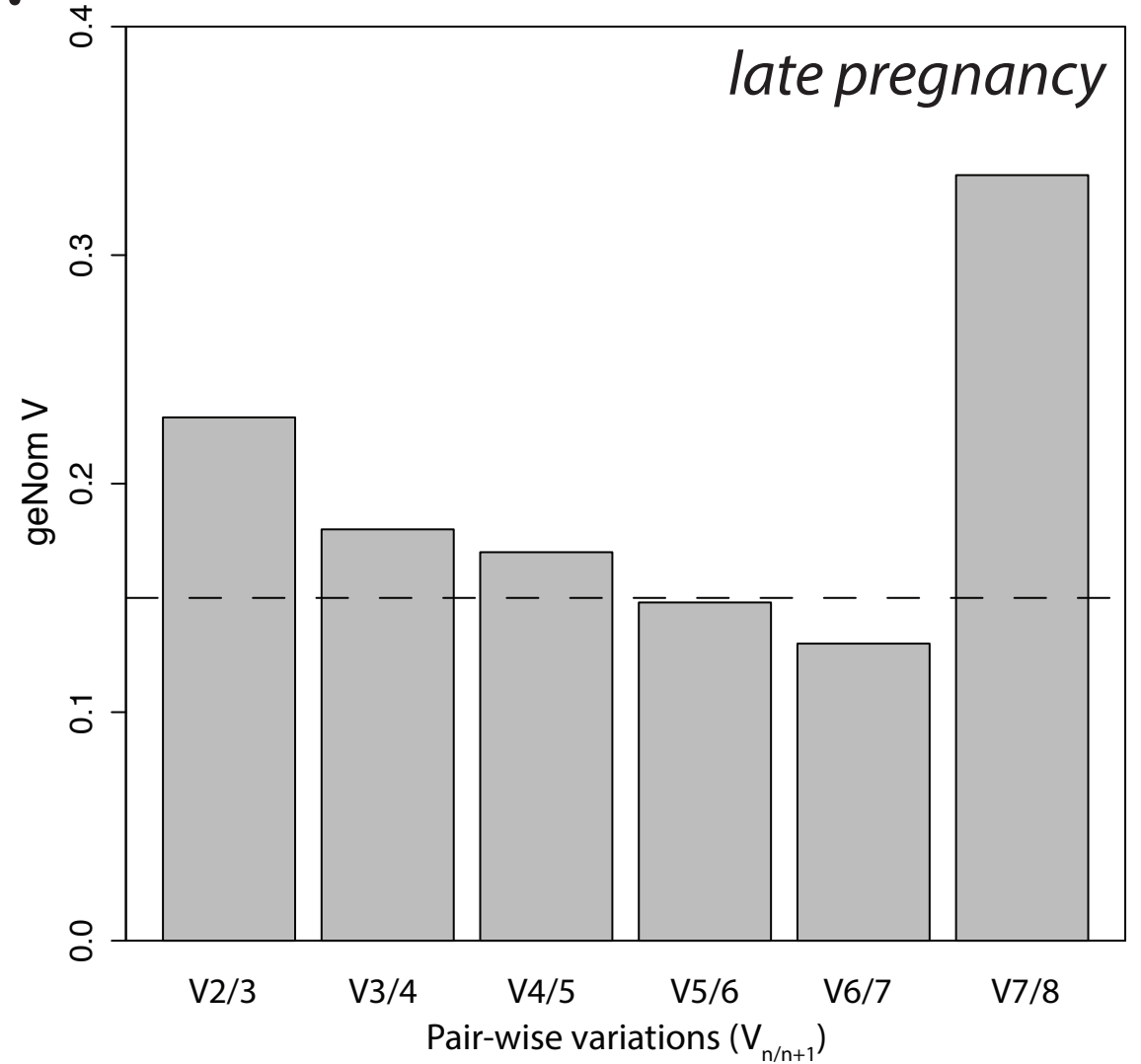
B.



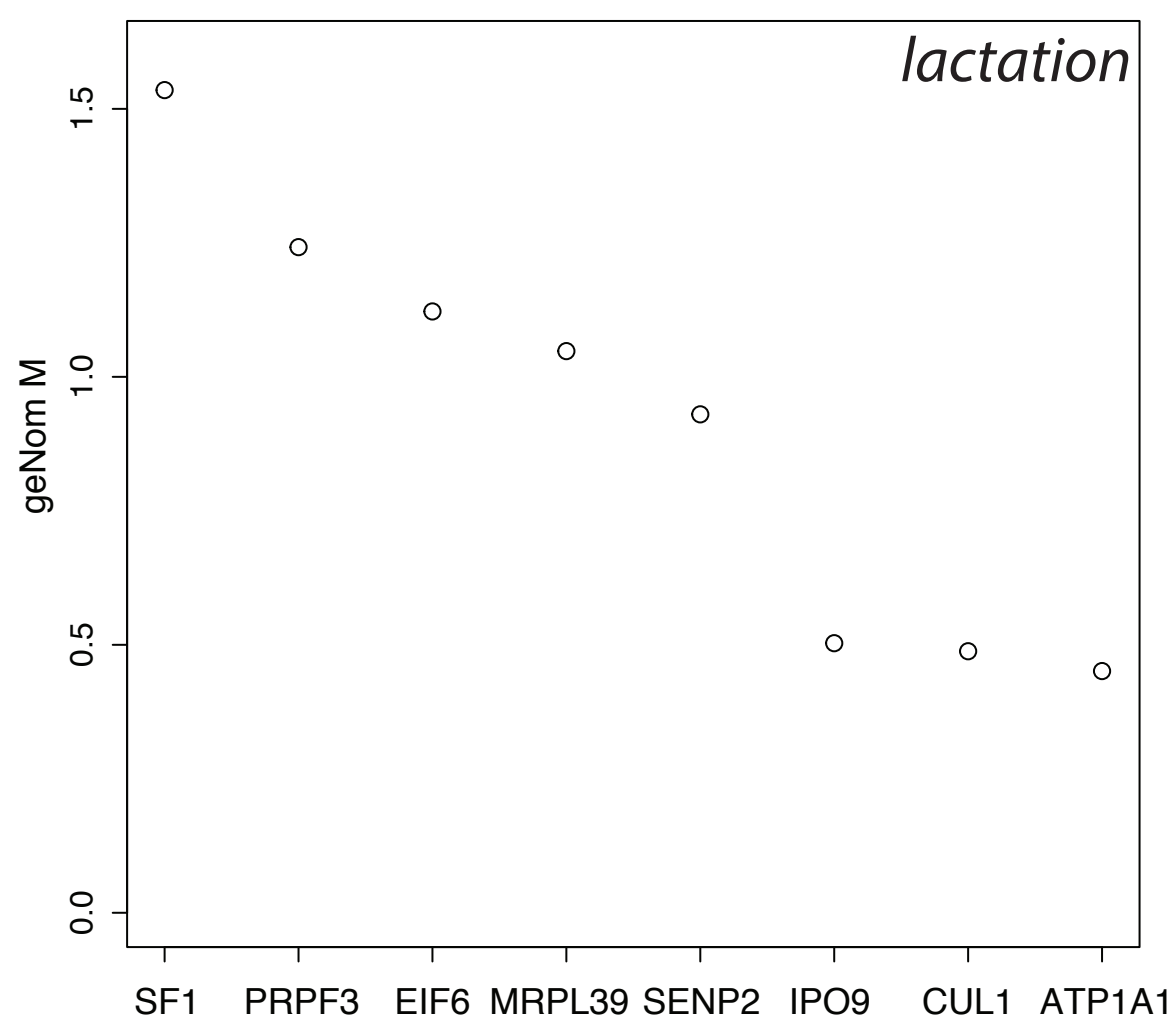
C.



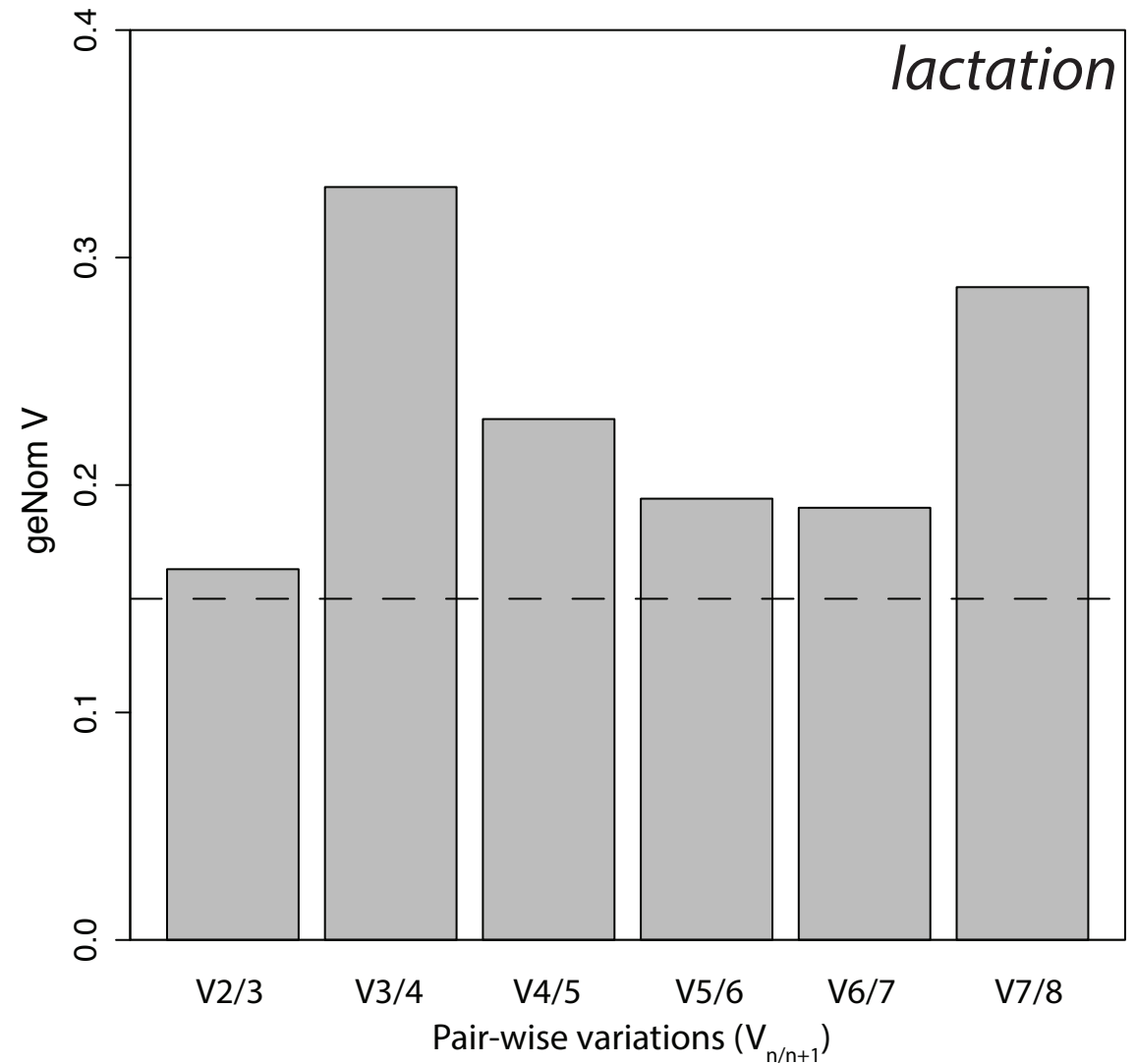
D.

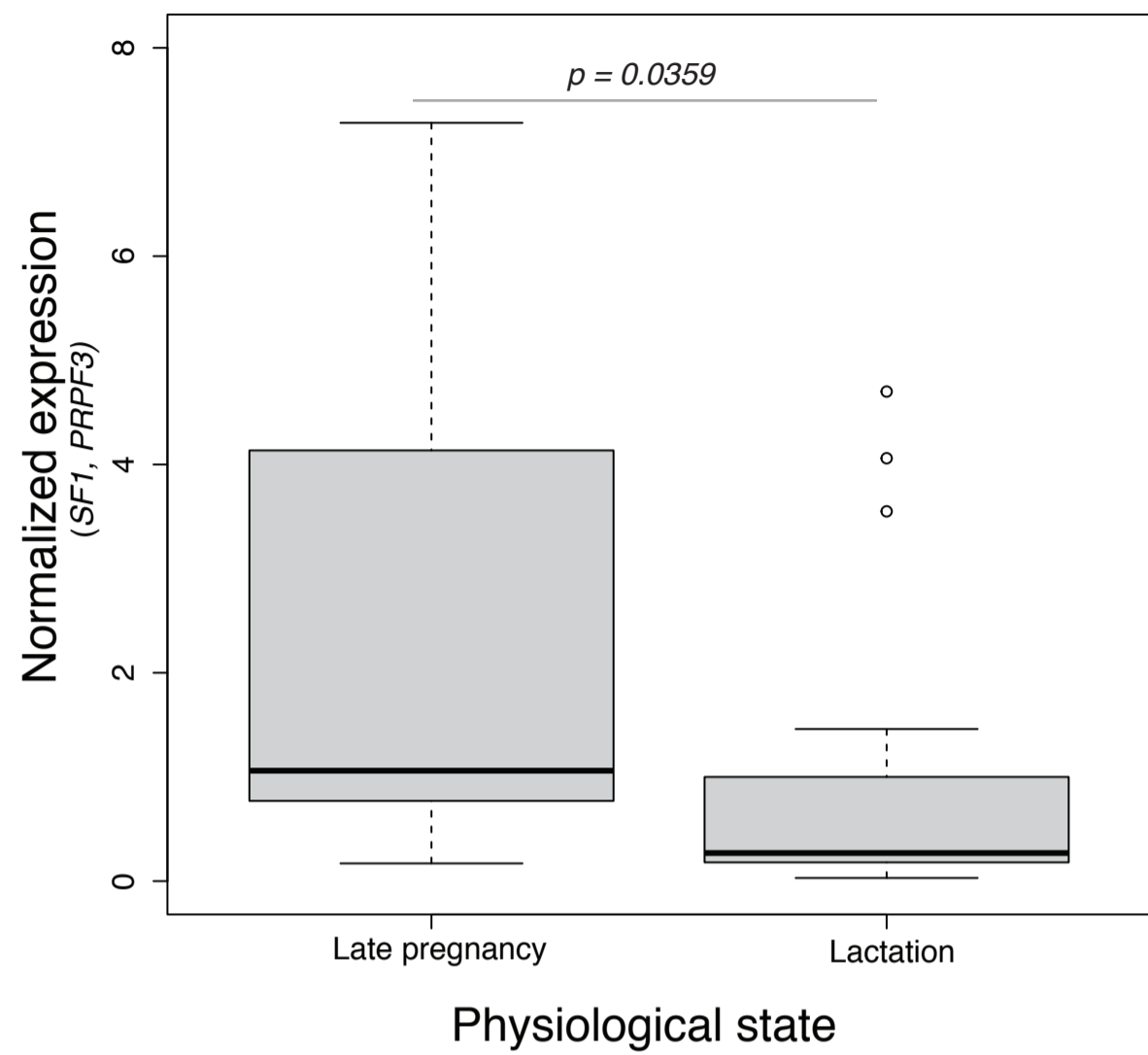
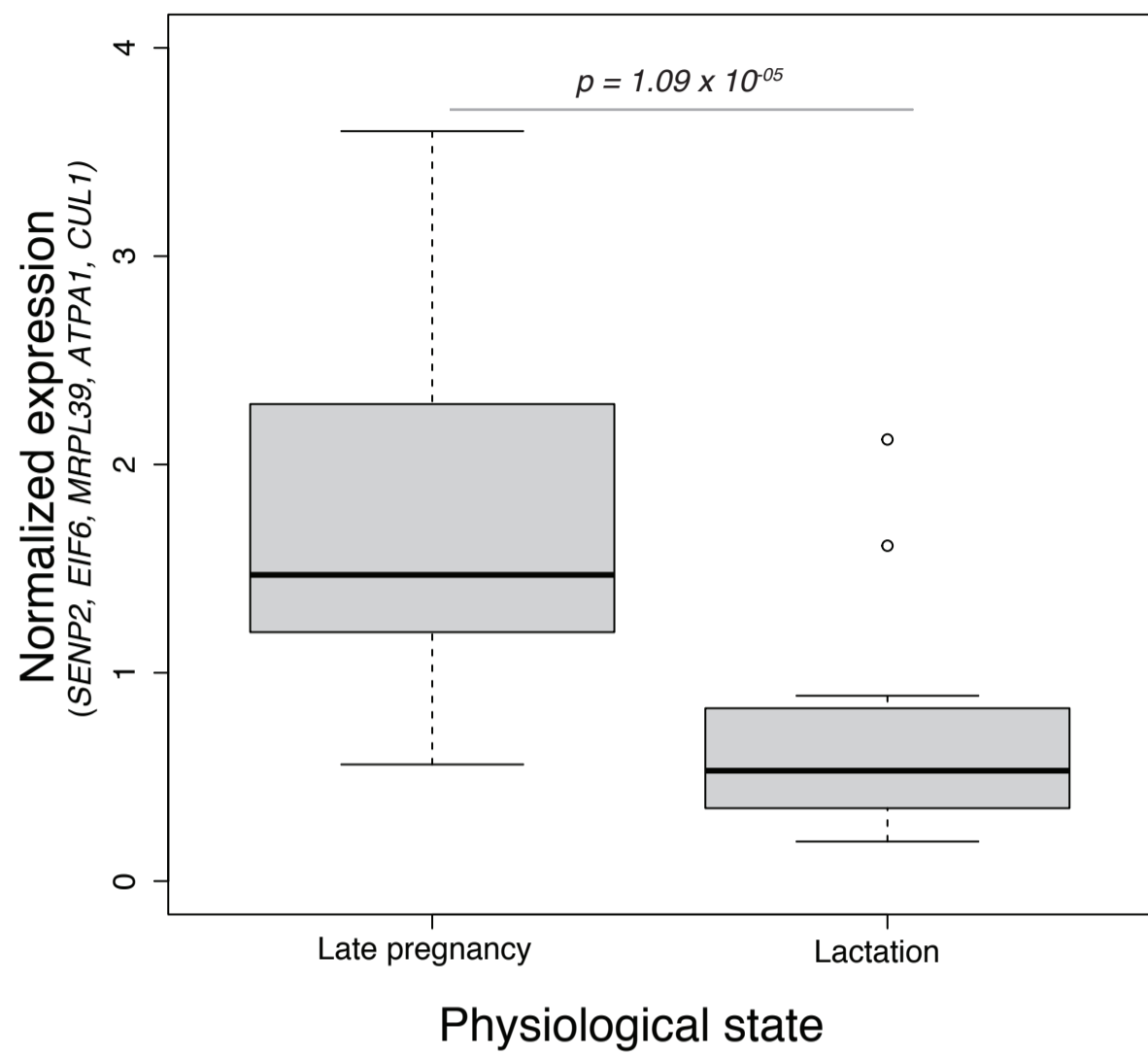


E.



F.



A.**B.**