Title: Novel polymorphisms in the 5′UTR of *FASN*, *GPAM*, *MC4R* and *PLIN1* ovine candidate genes: relationship with gene expression and diet

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Highlights

- We analyzed the 5´cis-regulatory regions of four genes involved in lipid metabolism in sheep.
- We identified 10 novel polymorphisms in the 5´regulatory regions in *FASN*, *GPAM*, *MC4R* and *PLIN1* genes.
- The polymorphisms fall into the core sequence of transcription factor binding sites.
- The adipogenic genes are over expressed in the intensive group (ING- GRE and IND).

Novel polymorphisms in the 5´UTR of *FASN, GPAM, MC4R* and *PLIN1* ovine candidate genes: relationship with gene expression and diet.

SNPs in the 5´UTR of *FASN, GPAM, MC4R* and *PLIN1* ovine genes

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Abstract

We have analyzed the 5´cis-regulatory regions of four genes coding for key proteins involved in lipid metabolism and energy homeostasis in Rasa Aragonesa, Assaf and Roja Mallorquina sheep breeds. We identified 10 novel polymorphisms in the 5´regulatory regions in *fatty acid synthase* (*FASN*), *glycerol-3-phosphate acyltransferase mitochondrial* (*GPAM*), *melanocortin-4 receptor* (*MC4R* and *perilipin* (*PLIN1*) genes. Due to the involvement of these genes in fat quantitative traits and the effect of all polymorphic positions on transcription factors binding sites, we tested all of them in two relevant meat reared breeds which were subjected to different feeding systems. Although no relationship was detected between the mRNA expression level of the candidate genes and the genotypes, additional studies must be conducted in older individuals, since these polymorphisms have been detected by *in silico* studies to be putatively involved in transcriptional or postranscriptional regulatory mechanisms. The expression level of *GPAM*, *MC4R* and *PLIN1* genes was analyzed and compared between feeding groups detecting over expression of adipogenic genes in the intensive groups. These results suggest that nutritional stimulation affects the expression of candidates genes involved in lipid metabolic processes, and therefore the fat quality in meat ruminant-derived food products.

Keywords: sheep, *FASN*, *GPAM*, *MC4R*, *PLIN*, polymorphisms.

1. Introduction

The amount and quality of fat in meat and dairy ruminant-derived food products are factors with a high economic and nutritional interest, but also with an impact on the human health. Quantity and quality of meat intramuscular fat (IMF) that also contributes to meat quality and consumer acceptance is influenced by the environment, age, genetics and the animal feeding system (Scerra et al., 2007; Dervishi et al., 2012).

In recent years it has detected a growing interest in the detection and characterization of markers associated with fat production traits. To date polymorphisms have been characterized in less than 5% of ovine genes, being the majority of them detected while investigating ways to increase ovine productivity (Darlay *et al.*, 2011). The high number of SNPs detected in candidate genes have increased the data interpretation complexity in association studies (Corella and Ordovas, 2005). For this reason it's recommended the identification of functional polymorphisms (i.e., those altering an amino sequence or a transcription (TFs) binding element) and avoiding the use of those nonfunctional genetic variants (Humphries *et al.*, 2004). Identifying those functional by *in silico* studies focused on prediction of transcription factor binding sites could be very interesting in nutrigenetics studies, given light in the understanding of interaction between genetic variations and diet.

In sheep, few studies have focused on genetic variation in 5´regulatoty regions of genes involved in fat synthesis and metabolism pathways, which may be good candidate genes to assess the possible use of their new variants as markers associated to fat-related traits. In this work we have studied *fatty acid synthase gene* (*FASN*), *glycerol-3-phosphate acyltransferase mitochondrial* (*GPAM*), *melanocortin-4 receptor* (*MC4R*) and *perilipin* (*PLIN1*) genes. Fatty acid synthase catalyses *de novo* fatty acid synthesis with an important role in a ruminant's production system because affects the fatty acid composition of milk sheep (Garcia-

Fernandez *et al.*, 2009). Glycerol-3-phosphate acyltransferase mitochondrial is considered a strong functional candidate gene by his function and position, as his counterpart in bovine (BTA26) contains putative QTLs related to milk and fat traits (Boichard *et al.*, 2003). Although little is known about polymorphisms in ovine *MC4R* gene, recently some SNPs in the 3´ untranslated region (UTR) have been detected in a sheep population and associated with weaning and birth weight (Song *et al.*, 2012). Finally, to our knowledge, no *PLIN1* gene polymorphisms analyses have been carried out in ovine, but many studies in humans have showed that variants in the perilipin gene are associated with obesity or metabolic traits, especially in women (Yan et al., 2004; Qi et al., 2008).

The objectives of the present study were firstly to seek for novel polymorphisms in the 5´UTR of genes related with lipid metabolism. Secondly, as it is well described that the forage type can alter the expression of genes that are associated with fat metabolism (Graugnard et al., 2009; Dervishi et al., 2010), the putative functionality of the new variants were evaluated by their relationship with gene expression in two experimental meat sheep populations subjected to different feed conditions.

2. Materials and methods

Searching for polymorphisms was performed using blood samples taken from a total of 142 individuals belonging to three sheep populations reared in Aragon (Spain): Rasa Aragonesa (n=29), Roja Mallorquina (n=50) and Assaf (n=63).

Besides, two groups of animals subjected to different feed conditions were used for testing the polymorphisms and for investigating their expression in meat intramuscular fat. The former group involved a total of 44 Rasa Aragonesa spring single-born male lambs. The suckling lambs were allocated to four dietary treatments: Grazing alfalfa (ALF; n=11); Grazing alfalfa with supplement for lambs (ALF+S; n=11); Indoor lambs with grazing ewes (IND-GRE; n=11) and Indoors (IND; n=11) (Dervishi *et al.*, 2010). The second one consisted in 48 lambs (males and females) from Churra Tensina breed whose ewes were subjected to two feed treatments: grazed pasture (GRE) or pasture hay (PH) (Dervishi *et al.*, 2012).

DNA from blood samples used for seeking polymorphisms was extracted using standard protocols and the GFX™ Genomic Blood DNA Purification Kit (GE Healthcare, UK).

DNA from individuals subjected to diet was obtained following the protocols supplied in the comercial kit NucleoSpin® Tissue, (Macherey- Nagel) and Speedtools DNA extraction kit (Biotools) (Dervishi, 2011).

Total RNA extraction and posterior cDNA syntheses was carry out from approximately 500 mg of *semitendinous* muscle (SM) from 44 Rasa Aragonesa lambs (Dervishi *et al.*, 2010) and *longissimus dorsi* (LD) from 48 Churra Tensina lambs (Dervishi *et al.*, 2012).

Searching for new polymorphisms in 5´ non-coding regions was assessed following the methodology described by Sanz et al (2013). The allele frequencies were determined by direct counting in each breed. The TRANSFAC gene tool software was used to predict the functionality of the detected polymorphisms in DNA regulatory binding sites.

Expression levels of *GPAM*, *MC4R* and *PLIN1* genes were determined by real time quantitative PCR (RT-qPCR) using the Fast SYBR Green Master Mix reagent and the StepOne Real Time System (Applied Biosystems). Primer sequences, amplicon sizes, GenBank accession number and PCR conditions are described in Supplementary Table S1. The gene expression levels were determined using the comparative Ct method and the data normalized using the housekeeping genes recommended by Dervishi *et al.* (2011; 2012) Rasa Aragonesa normalized RT-qPCR results were transformed in fold-change relative to the ALF control group (Dervishi *et al.*, 2010); therefore PCR-normalized data were represented as *n-fold* change respect to ALF. Churra Tensina normalized RT-qPCR results were also represented as *n-fold* change respect to the males for both PH and GRE feed systems, and respect to the PH feed system in all animals (males and females) (Dervishi *et al.*, 2012). The relative differences in gene expression among the different feeding systems were defined as the relative quantities after normalization. Differences between groups were calculated and defined as fold- change, setting the control means at 1. Variations in gene expression were evaluated with the Student's *t* test. Statistical significance was defined as (*$P < 0.05$).

116

117 **3. Results and discussion**

118 All the polymorphisms detected in the 5´UTR of the *FASN*, *GPAM*, *MC4R* and *PLIN1* sheep
119 genes and their allelic frequencies are showed in Table 1.

120 The novel SNP detected in the first intron of the *FASN* ovine gene (SNP g.292G>A. GenBank
121 Acc. No. JN570752.2), affects the putative binding sites for Sp1 family of transcription factors
122 which is involved in the *FASN* gene transcriptional regulation (Schweizer *et al.*, 2002). This
123 SNP could be as important as the detected in their bovine counterpart associated with milk-fat
124 content (Roy *et al.*, 2006).

125 In the *GPAM* 5´UTR screened (-800 to +100 in the 5´UTR of Glycerol-3-phosphate
126 acyltransferase mitocondrial gene: GenBank Acc. No. AY945226), five novel SNPs were
127 detected at positions g. 341 (G>C), g. 367 (C>T), g. 466 (A>G), g. 515 (A>G) and g. 705
128 (T>A), all of them containing binding sites for transcription factors. Some of these TFs regulate
129 numerous mammalian genes, either by their activating or suppressing features depending on the
130 promoter context, the cellular background, epigenetic factors, and interactions with other
131 nuclear proteins (Archer, 2011). Although not all the TFs identified seem to be closely related
132 with the physiological function of *GPAM* gene, is intelligible that these polymorphic points are
133 positioned in highly active regulatory areas in the *GPAM* gene promoter.

134 In the analyzed region of the *PLIN1* gene covering ~ -1Kb from the TSS (nt 912480:

135 NW_001494026) we detected an AT indel and 2 SNPs. In this case also were identified
136 regulatory binding elements in the three polymorphic sequences, but the most relevant was
137 Sp1 and C/EBPalpha. C/EBPalpha is an important regulator of PPARc expression, which

138 regulates the perilipin expression (Park *et al.*, 2004) implicated in lipid storage and body fat
139 mobilization in beef and dairy breeds.
140 Finally, in the examined *MC4R* 5´UTR fragment (-800 to +50) of *MC4R* gene we detected an
141 indel motif TCT (bases 34756 to 34758 according to the Genbank Ac. No. AAFC03039591).
142 Only the delected variant contains the binding site for the TFs C/EBPβ, SRBP, SRF, factor1,
143 MCM1, band I factor and DBP, some of them involved in the regulation of several lipid
144 metabolism pathways (Desvergne *et al.*, 2006). The importance of those TFs and their
145 putative relation with fat metabolism make this polymorphism a valuable candidate to be
146 studied.
147 Summarizing, in this work we report a total of 9 novel genetic variations (SNPs and indels)
148 on lipid metabolism regulatory regions of the studied four candidate genes in sheep. This
149 work provides knowledge of new polymorphisms that might affect the expression of lipid
150 metabolism related genes in ovine. Given that some SNPs have also been identified in other
151 species and associated to meat or milk production traits, our analysis may be useful to select
152 certain SNPs as genetic markers.
153 The polymorphisms detected were tested in all animals subjected to different diet systems, but
154 no differences in the least frequent allele were detected across populations or intergroup
155 differentially nourished (data not shown).
156 The putative effect of polymorphisms in the gene expression profiles was studied by means of
157 the quantitative gene expression of *GPAM*, *MC4R* and *PLIN1* genes. Data for *FASN*
158 expression was previously reported by Dervishi, et. al. (2010, 2011; 2012). No relationship
159 was detected between the genotypes, the system feeding and the mRNA expression level for
160 each studied gene. It could be possible that the age of animals could affect the transcriptional
161 activity of the studied genes. Likely in these young individuals the IMF deposition is still
162 developing and preadipocytes are still differentiating.

Relative differences in gene expression among the *semitendinous* muscle of Rasa Aragonesa lambs grouped by feeding system are shown in Figure 1. The *GPAM* expression was significantly affected by the feeding system between the ALF control and the groups ALF+S (P=0.046) and IND (P =0.043). The increase of the *GPAM* expression respect to the control group suggest its nutritional regulation (Coleman et al., 2000) and confirm the rise of lipogenic genes expression in intensive groups (Dervishi et al., 2011). A significant *PLIN1* mRNA over-expression was detected in the IND+GRE (P=0.05) group respect to the ALF control, and a tendency in the IND (P=0.06). These results shown that perilipin is over expressed in the adipose tissue under high energy intake treatments regulating the lipid storage. The mean over expression of adipogenic genes detected in the intensive group (ING-GRE and IND) respect to the grazing (ALF and ALF+S) are in agreement with Graugnard *et al* (2009) which also observed that highly energetic fed regimes stimulate adipogenesis.

Also, gene expression profiles were examined in LD muscle from Churra Tensina suckling lambs whose mothers were subjected to two feeding treatments (PH: pasture hay, and GRE: grazed pasture). The effects of lamb sex on expression of *GPAM*, *MC4R* and *PLIN1* genes in the PH and GRE groups are showed in Figure 2.A and 2.B. No statistical significant differences were detected in females respect to the males in the PH group. Otherwise in the GRE group we detected significant differences in the expression of *PLIN1* in the GRE group (P=0.02) between males and females. The most relevant result was the over expression of *PLIN* gene in female subjected to both diets. Dervishi *et al*. (2012) found in the same sample an up regulation in the expression of *PPARA* or *CEBPB* which are members of the families of TFs regulating the perilipin expression (Park *et al.*, 2004). Moreover, Muhlhausler *et al.* (2008) showed the sex effect in the expression of lipogenic genes, and also concluded the higher trend of female using the nutrients for accumulate fat during their postnatal age.

Furthermore, the effect of forage type fed to ewes on gene expression in LM in male and female suckling lambs was analyzed (Figure 2.C and 2.D). In male we found statistically significant differences in the expression of *PLIN1* gene (P=0.05) with a 0.3 fold change down-expression in the GRE group respect to the PH. On the other hand differences were detected for females in mRNA expression of *GPAM* gene (P=0.03), and a tendency in *MC4R* gene (P=0.07). Both male and female lambs, whose mother grazed mountain pastures (GRE) shown over expression for *GPAM* and *MC4R* genes, and down expression for *PLIN1* gene. This pattern suggests that the grazing pastures could influence over the expression of the mentioned genes confirming the previous results where energetic diets stimulate the adipogenesis. The down expression of *PLIN* gene regardless of the sex suggest and important hormonal regulation of *PLIN* expression.

4. Conclusion

In this work we identify novel polymorphisms in the 5´regulatory regions putatively affecting the transcription binding sites in four important ovine genes involved in fat-related traits. Relationships between mRNA abundance and the genetic variation could not be confirmed, likely by the youth of the animals analyzed. The trend of over expression among the adipogenic genes detected in the highly energetic fed groups reinforce the nutritional stimulation in the expression of candidates genes involved in lipid metabolic processes. These results are important in the understanding of interaction between the genetic variation, expression and the response to diet, with enormous interest for the treatment of human diseases related to lipid metabolism.

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CONFLICT OF INTEREST STATEMENT

213 The authors declare that there is not any conflict of interest related to the information included
214 in the Paper entitled **"Novel polymorphisms in the 5´UTR of *FASN, GPAM, MC4R* and**
215 ***PLIN1* ovine candidate genes: relationship with gene expression and diet."** by Sanz, A.,
216 Serrano C., Ranera B., Dervishi E., Zaragoza P., Calvo JH and Rodellar, C*.
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Table 1. Polymorphisms detected by breed and allele frequencies. Allele frequencies are displayed for new variants (second allele). Polymorphisms are numbered according to the bovine reference Genbank accession numbers since no ovine *GPAM*, *MC4R* and *PLIN* gene sequences have been deposited in GenBank.

Gene/Genbank Ac. No.	**Polymorphism**	**RA**	**RM**	**AS**	**RA[1]**	**ChT**
FASN JN570752.2	292G>A	0.10	0.07	0.2	0,103	
PLIN NW_001494026	Indel/AT 912993-94	0.13			0,36	0,033
	SNP A>G 912854	0.18	0.09	0.04	0,481	0,688
	SNP C>T 912498	0.09		0.43	0,093	0,081
GPAM AY945226	SNP G>C 341	0.04	0.04	0.15		
	SNP C>T 367	0.08	0.29	0.25	0,225	0,375
	SNP A>G 466	0.29	0.29	0.55	0,075	0,209
	SNP A>G 515	0.29	0.29	0.1	0,075	0,209
	SNP T>A 705	0.29	0.29	0.1	0,075	0,209
MC4R AAFC03039591	Indel TCT 34756-34758	0.59	0.5	0.82	0,357	0,389

[1]: Rasa Aragonesa breed, RM: Roja Mallorquina breed, AS: Assaf breed, RA1: Individuals from Rasa Aragonesa breed subjected to different diet, and ChT: Individuals from Churra Tensina breed subjected to different diet.

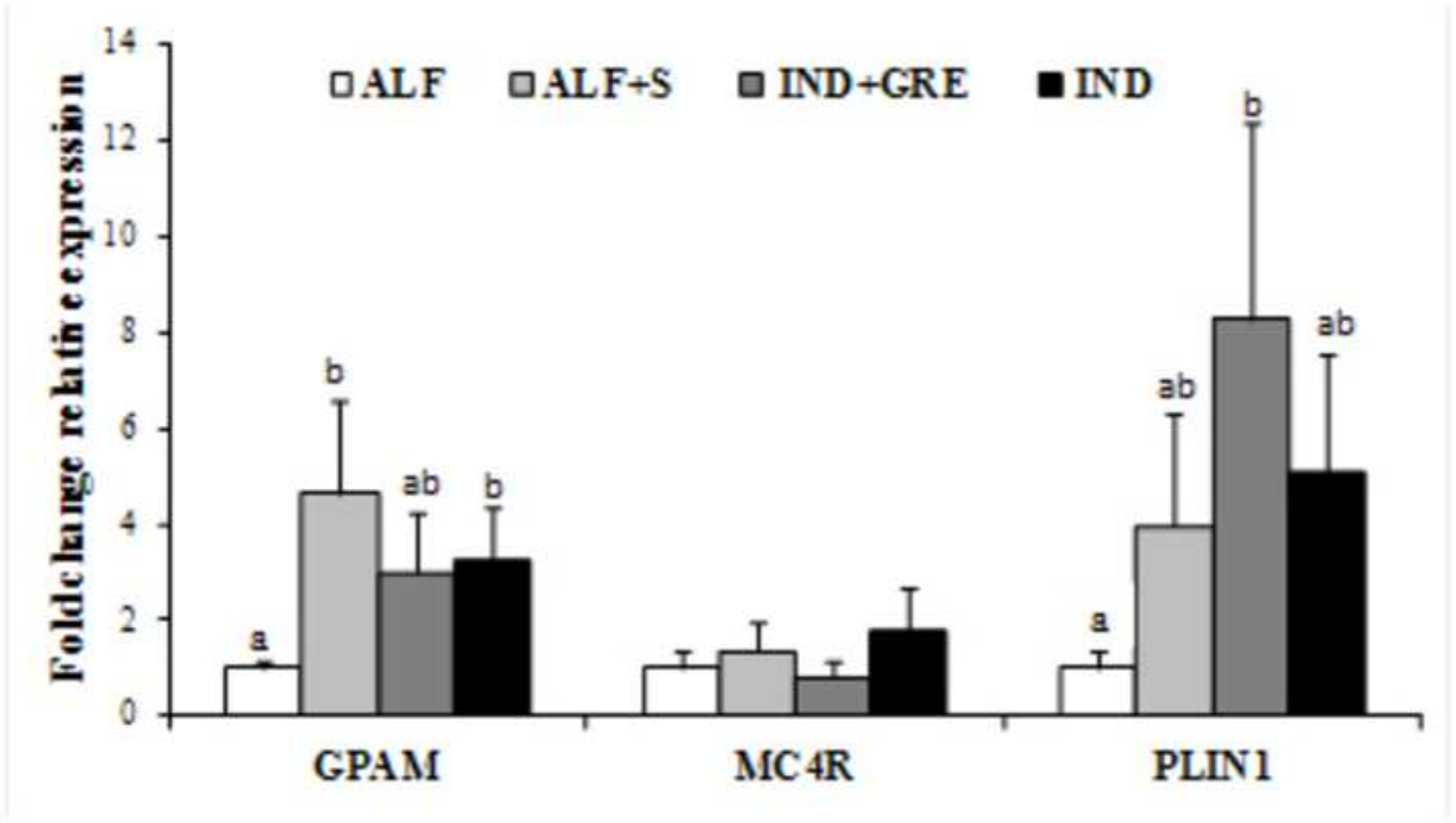
295 **Figures**

296 **Figure 1:** Effect of feeding system on mRNA expression of *GPAM*, *MC4R* and *PLIN1* genes
297 in *semitendinous* muscle of Rasa Aragonesa lambs. ALF: lambs and ewes grazing Alfalfa;
298 ALF+S: lambs and ewes grazing alfalfa with supplement for lambs; IND-GRE: Indoor lambs
299 with grazing ewes; and IND: lambs and ewes indoor. Data are shown as
300 mean ± standard errors relative to the control group ALF. Significant difference between
301 groups (*$P< 0.05$) are indicated by letters (a,b).

302 **Figure 2.** Effect of lamb sex and diet on gene expression in *L. dorsi* muscle in Churra Tensina
303 breed. A) Gene expression in male and female lambs whose mothers received the low
304 mountain hay (PH) treatment. B) Gene expression in male and female lambs whose mothers
305 grazed the low mountain vegetation (GRE) treatment. C) Gene expression in male lambs
306 depending on the feeding system (PH or GRE). D) Gene expression in female lambs
307 depending on the feeding system (PH or GRE). Data are shown as mean ± standard errors
308 relative to the males (A and B) and relative to the PH group (C and D). Significant difference
309 between groups (*$P< 0.05$) are indicated by letters (a,b).

310

Figure


ALF
ALF+S
IND+GRE
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Fold change relative expression
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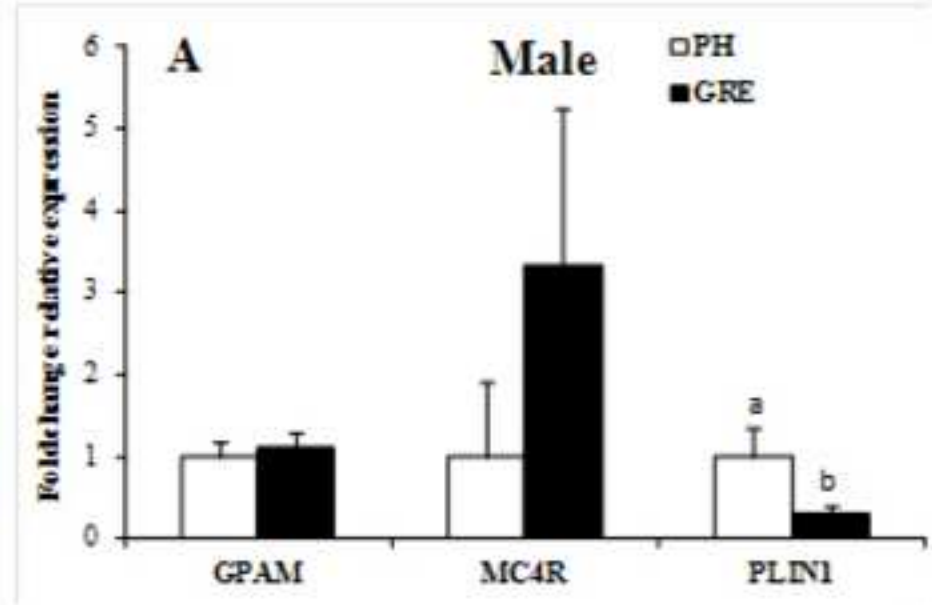
MC4R
PLIN1

Figure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Fold change relative expression
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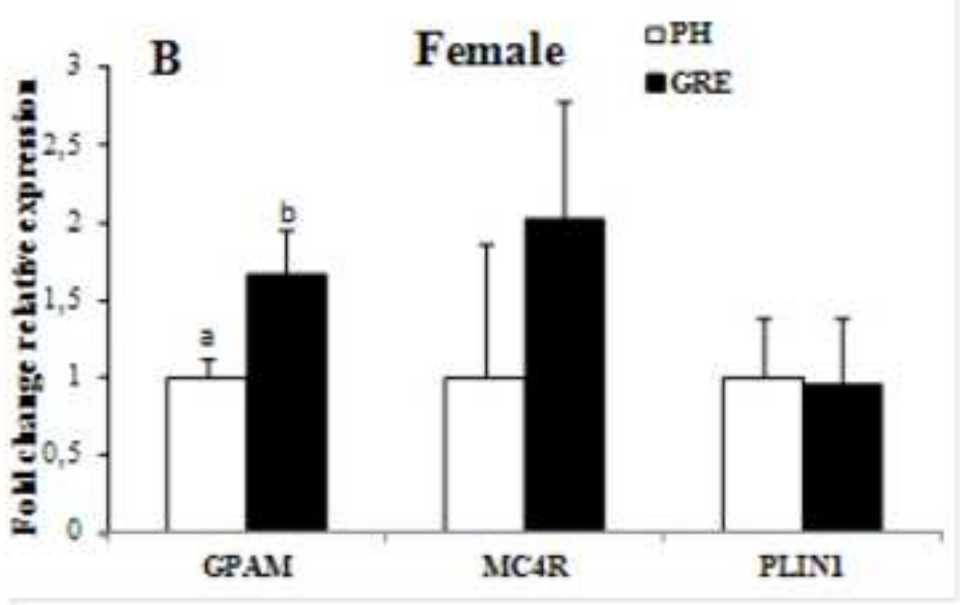
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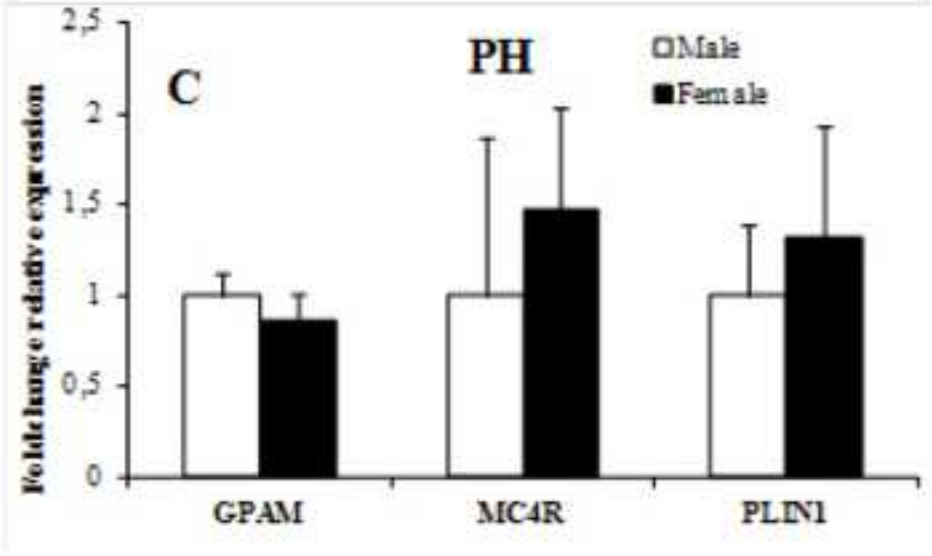
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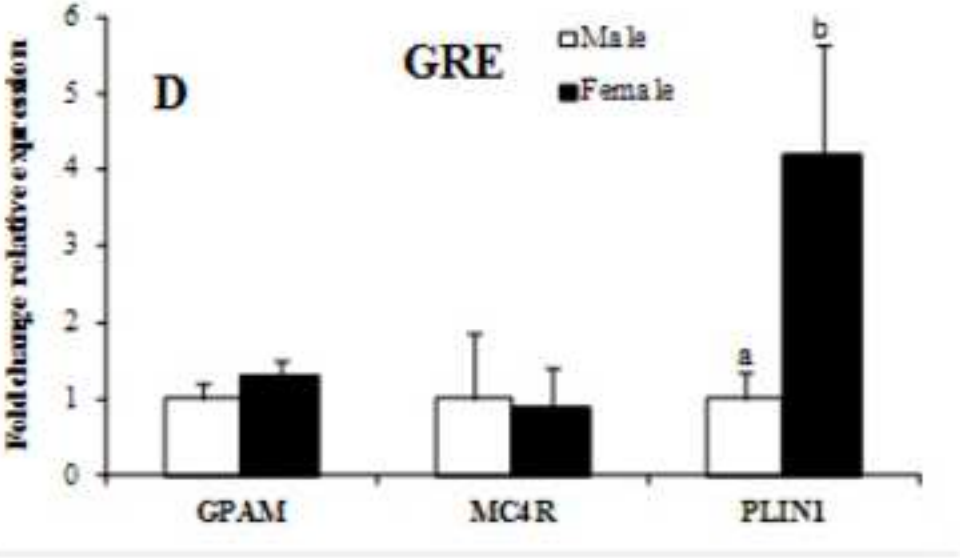
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