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**Sequencing of lipoprotein lipase gene in the Mediterranean river buffalo identified novel variants affecting gene expression**

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(Article begins on next page)

1 Novel variants at buffalo *LPL* affect gene expression

2 Gu

3 The *LPL* gene plays an essential role in the lipid metabolism being responsible for the  
4 composition of fat in adipose tissue as well as in milk. It has been poorly investigated in farm animals  
5 and little information was reported for the buffalo. Among ruminants, the buffaloes produce milk with  
6 a higher fat content; therefore, the improvement of this trait is one of the main goals for the progress  
7 of the species. We identified novel functional polymorphisms, using a combined approach based on  
8 sequencing, dual colour electrophoretic mobility shift assay and q-PCR. Our data revealed that the  
9 SNP g.-446A>G in the promoter affects the binding of a Sp1 transcription factor, resulting in the  
10 overexpression of the gene with with potential effects on milk quali-quantitative production.

11

12 **Sequencing of Lipoprotein lipase (*LPL*) gene in the Mediterranean river buffalo (*Bubalus***  
13 ***bubalis*) identified novel variants affecting gene expression**

14  
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27  
28 **NOVEL VARIANTS AT BUFFALO *LPL* AFFECT GENE EXPRESSION**

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## ABSTRACT

Lipoprotein lipase is a key enzyme for the lipid metabolism playing a fundamental role in the composition of fat in adipose tissue as well as in milk. Seldom Lipoprotein lipase (*LPL*) gene was investigated in dairy ruminants, and it has been barely studied in river buffalo so far. The aim of this work was to explore the genetic diversity of *LPL* and its promoter and to identify functional mutations using a combined approach based on sequencing, dual colour electrophoretic mobility shift assay (EMSA) and quantitative PCR (q-PCR). Thirteen consensus sequences for transcription factors were found in the promoter. A total of 11 SNPs were detected and the attention was focused on the SNPs with potential functional effects: g.-466A>G, because the presence of G created a consensus motif for the transcription factor Sp1, and g.107A>G, which was the only exonic SNP. PCR-RFLP methods for genotyping were developed for the two SNPs and the allele frequencies were calculated. A strong linkage disequilibrium ( $D' = 1$ ;  $r^2 = 0.903$ ) was found between the two SNPs. The dual colour EMSA demonstrated that only the genotype g.-446GG induced the binding of the Sp1 transcription factor, resulting in the overexpression of the gene (~2.5 fold), as confirmed by the q-PCR results. This study adds further knowledge on the structure of the *LPL* gene and its expression in river buffalo, with potential effects on milk quali-quantitative production.

Keywords: Mediterranean river buffalo, *LPL* gene, promoter, Gene expression, Sp1 transcription factor.

## INTRODUCTION

58

59 Milk fat is mostly composed of triglycerides (over 95%), whose fatty acids (FAs) can be  
60 originated by ruminal fermentation of carbohydrates (*de novo* synthesis) or come directly from the  
61 plasma by the action of lipoprotein lipase (LPL) (Barber *et al.*, 1997).

62 Lipoprotein lipase is the key enzyme involved in metabolism, transport and regulation of lipid  
63 serum levels. It acts on the surface of the capillary endothelium by hydrolysing triglycerides contained  
64 in circulating chylomicrons and very low density lipoprotein (VLDL) to provide free FAs to tissues.  
65 Regulation of Lipoprotein lipase (*LPL*) gene is very complex and responds to dietary and hormonal  
66 changes and environmental conditions being tissue-specific. The major tissues thought to control the  
67 circulating levels of plasma lipoproteins are the adipose and muscular tissues. However, *LPL* is  
68 expressed in other sites, including the mammary gland and other body compartments such as the  
69 nervous system, heart, liver, pancreatic islet cells, lungs, etc. (Merkel *et al.*, 2002).

70 The *LPL* gene has been deeply investigated in several species and single nucleotide  
71 polymorphisms have been reported in cattle (Lien *et al.*, 1995; Oh *et al.*, 2013; Tank & Pomp, 1994)  
72 and in yak (Ding *et al.*, 2012), where *LPL* has been proposed as major candidate gene for lipid  
73 deposition in the carcass and fatty acid composition. Marchitelli *et al.* (2013) reported a significant  
74 effect of the *LPL* g.25732A allele on milk fat percentage in the Piedmontese cattle breed.

75 In goats, the sequencing of the *LPL* cDNA allowed the identification of a missense mutation  
76 associated with milk fat content (Badaoui *et al.*, 2007). In the same species, Crepaldi *et al.* (2013)  
77 found an association between the transversion C50G at the exon 1 and milk yield, thus reporting for  
78 the first time an effect of *LPL* gene on this trait.

79 Among the farm animals, river buffalo is the species producing the highest milk fat, with an  
80 average content estimated in about 8.0% (<http://www.anasb.it/home.htm>). Milk yield and  
81 composition are of great economic importance for the dairy industry in Italy where this species is  
82 mainly reared for the production of *mozzarella* cheese PDO (Protected Denomination of Origin –  
83 Reg. EC 510/2006). The increasing demand of this product both on the national and international

84 market facilitated the development of the whole buffalo dairy industry, which currently involves 102  
85 cheese factories and more than 20,000 operators, with a consolidated turnover estimated in about 720  
86 million of euros (ISMEA, 2016). In this context, the discovery of genetic markers affecting milk yield  
87 and composition could contribute to accelerate the genetic improvement of the species.

88 Therefore, the aim of this study was to explore the genetic diversity of *LPL* gene in the Italian  
89 Mediterranean breed, to describe the main regulatory elements of the promoter and to investigate  
90 possible effects of the polymorphism on the gene expression.

91

## 92 MATERIAL AND METHODS

### 93 *Sample collection and nucleic acid isolation*

94 A total of 523 river buffalo cows reared in 14 farms located in Campania region (Southern  
95 Italy) were considered in this study. From all cows blood samples were collected in collaboration  
96 with the Italian National Association of Buffalo Breeders (ANASB) during the routine prophylaxis  
97 of each farm. For this reason, the Animal Care and Use Committee approval was not necessary. In  
98 addition, six individual milk samples were collected after genotyping at the SNPs g.107A>G and g.-  
99 446A>G for the isolation of RNA in order to perform the qPCR.

100 Genomic DNA was isolated using the NucleoSpin Tissue kit (Macherey-Nagel, Düren,  
101 Germany). Total RNA was isolated from milk somatic cells by Tri-Reagent<sup>®</sup> (Sigma-Aldrich, Milano,  
102 Italy) following the manufacturer's guidelines. Traces of contaminating DNA were removed using  
103 DNase I treatment (Thermo Fisher Scientific).

104 DNA and RNA concentrations and OD ratios of the samples were measured with the  
105 Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

106

### 107 *Sequencing and bioinformatics*

108 Genomic DNA of 14 individual samples randomly chosen was amplified by iCycler (BioRad)  
109 using primers designed on the buffalo genome sequence (EMBL acc. no. [AWWX01438720.1](#))  
110 (Supplementary table 1).

111 The PCR reaction mix (25 µl) comprised: 50 ng of genomic DNA, 1X PCR Buffer (Promega,  
112 Madison, WI, USA), 2.5 mM MgCl<sub>2</sub>, 5 pmol of each primer, dNTPs 200 µM each, 1 U of *Taq* DNA  
113 Polymerase (Promega). The thermal conditions were: 95°C for 4 min, 35 cycles at 95°C for 45 s,  
114 annealing for 45 s, at temperatures depending on the amplicon (Supplementary table 1), and extension  
115 at 72°C for 45 s. A final extension was carried out at 72°C for 5 min.

116 Product specificity was confirmed by electrophoresis in 1.5% agarose gel in 0.5X TBE buffer,  
117 stained with Sybr Green. PCR products were purified and sequenced on both strands at Microsynth  
118 (Vienna, Austria).

119 Homology search, comparison among sequences, and multiple alignment for SNP discovery  
120 were accomplished using DNAsis-MAX (Hitachi). The putative binding sites for transcription factor  
121 at the promoter were searched by Transfact 7.0 database under the most stringent condition of analysis  
122 (85% as minimum binding score and 100% similarity of the sequence to consensus matrix).

123

#### 124 ***Genotyping of the SNPs g.107A>G and g.-446A>G***

125 The entire panel of 523 animals was genotyped in out-sourcing (<http://kbioscience.co.uk>)  
126 using a competitive allele specific PCR system (KASPar) for the SNP g.107A>G. Also, a PCR-  
127 RFLP method was set up for the same SNP to facilitate future home made genotyping. In brief, 10µl  
128 of exon 1 amplicons from informative samples were digested with 1µl of Fastdigest *Dde* I (5'-  
129 C↓TNAG-3') endonuclease (Thermo Fisher Scientific Inc.) for 10 min at 37°C according to  
130 manufacturer's guidelines.

131 Forty samples, randomly chosen from the panel of 523 buffaloes, were genotyped also for  
132 the SNP g.-446A>G located in the promoter by digesting the amplicon with the endonuclease *Ssi* I  
133 (5'-C↓CGC-3') overnight at 37°C in the same conditions described above.

134 The digestion products were resolved on 2.5% agarose gel in 1X TBE buffer, stained with  
135 Sybr Green.

136

### 137 ***Dual colour Electrophoretic Mobility Shift Assay (EMSA)***

138 Single stranded DNA probes (table 1) corresponding to the nucleotides -458 to -434 of the  
139 *LPL* promoter were co-denatured at 95°C for 5 min in 0.2X SSC buffer and annealed at room  
140 temperature for 2 hours with their complementary sequence to obtain double strand probes  
141 (homozygous AA and GG). Concentrations and OD ratios were measured with the Nanodrop ND-  
142 1000 spectrophotometer as reported previously and dilutions were prepared to a final concentration  
143 of 80ng/μl.

144 Electrophoretic Mobility Shift Assay reactions were carried out according to [Gu et al. \(2019\)](#).  
145 Briefly, binding reactions were set-up by incubating 0.5 BFU of Sp1 transcription factor (Enzo Life  
146 Science Inc., Farmingdale, NY, USA) in 1X Enzo buffer (EB) with 40 ng DNA probe. After  
147 incubation at 37°C for 20 min, the samples were loaded in 5% native polyacrylamide gel and run at  
148 150V for 90 min in pre-chilled TBE 0.5X according to [Jing et al., 2003](#). Staining was performed as  
149 sequential steps in dual colour, Sybr™ Green for the DNA and Sypro™ Ruby for the protein,  
150 according to the manufacturer's guidelines of EMSA kit (ThermoFisher Scientific). The image was  
151 acquired using the Gel Doc™ EZ System (Bio-Rad) and the digital images pseudocolored and  
152 overlaid.

153

### 154 ***Quantitative real-time PCR analysis (qPCR)***

155 Comparative *LPL* gene expression was examined in 6 Mediterranean river buffaloes chosen  
156 according to the genotypes at the SNPs g.-466A>G and g.107A>G (2 samples double homozygous



157 AA, 2 samples double heterozygous AG and 2 samples double homozygous GG). The animals  
158 belonged to the same farm, and were at the 3<sup>rd</sup> lactation, at  $210 \pm 11$  days in milk, free from mastitis.

159 Reverse transcription was accomplished by Verso cDNA synthesis kit (ThermoFisher  
160 Scientific) starting from 100 ng of total RNA in a final reaction volume of 20 $\mu$ l and using a mixture  
161 of oligo dT<sub>18</sub> and Random Hexamer as primers, according to the protocol suggested by the company.

162 Quantitative PCR was performed using 2X QuantiTect SYBR Green PCR Master Mix in  
163 presence of ROX passive reference dye (Qiagen). Analysis was carried out with a Mini Opticon Real-  
164 time PCR System (Bio-Rad) for 40 cycles at 95°C for 15 s, 55°C for 15 s and 72°C for 20 s.  
165 Amplification specificity was checked using melting curve following the manufacturer's instructions.  
166 Primers used for *LPL*,  *$\beta$ -actin* and 18S rRNA expression are listed in table 1. Two biological replicates  
167 per sample were analysed and each replicate was amplified in triplicate (technical replicates). Relative  
168 gene expression of *LPL* was determined using  *$\beta$ -actin* and 18S rRNA as endogenous controls using  
169 the geometric mean values for normalization. Results are reported as fold-change relative to the mean.

170

### 171 *Statistical analysis*

172 Allelic frequencies were calculated by Popgene software (University of Alberta, Edmonton,  
173 Alberta, Canada). Haploview 4.2 was used to estimate the rate of linkage disequilibrium ( $D'$  and  $r^2$ )  
174 between the SNPs g.107A>G and g.-446A>G. Unpaired Students's t-test was applied to evaluate the  
175 differences in the gene expression.

176

177

## RESULTS AND DISCUSSION

178 The DNA regions covering the promoter, the 5'UTR and all coding regions until the stop  
179 codon of the river buffalo *LPL* gene were amplified and sequenced, for a total of 5510 bp.

180 The analysis of the first 750 bp of the river buffalo *LPL* promoter greatly contributed to  
181 evaluating the factors involved in the regulation of the gene expression. In this respect, *LPL* was

182 scarcely investigated in ruminants, with information limited to a short report in sheep (Volpe *et al.*,  
183 1994).

184 The bioinformatics analysis revealed thirteen consensus sequences for transcription factors  
185 (figure 1). Twelve of them were equally represented: three TATA-boxes (-301/-306; -606/-611; -648/-  
186 653), three Oct-1 (-66/-75; -155/ -164; -559/-608), three Sp1 (-3/+7; -6/-15; -444/-449) and three  
187 C/EBP $\alpha$  (-57/-64; -89/-95; -545/-551). In addition, a motif AP2 was found in position +34/+41.

188 Part of these regulatory elements have been described also in human where transfection  
189 experiments in adipocytes identified proximal positive (-368 to -35) and distal negative (-724 to -565)  
190 *cis*-acting motifs (Previato *et al.*, 1991). In particular, the proximal regulatory region was shown to  
191 contain multiple conserved binding sites including C/EBP and Oct-1.

192 C/EBPs are known to be crucial components of terminal adipocyte development and play,  
193 together with PPAR $\gamma$ , a master role in the regulation of adipogenesis in the network of transcription  
194 factors which interact at different stages of the process (Farmer, 2006). *LPL* is one of the adipocyte-  
195 specific genes that are trans-activated by C/EBP $\alpha$  (Olofsson *et al.*, 2008). In river buffalo, C/EBP $\alpha$   
196 motifs have been amply found also in the promoter region of *SCD*, another gene involved in lipid  
197 metabolism, and associated with milk fat quality (Pauciullo *et al.*, 2012b).

198 Oct-1 belongs to octamer-binding proteins (OCTs), a group of highly conserved transcription  
199 factors that specifically bind to the octamer motif (ATGCAAAT) and to closely related sequences  
200 found in promoters and enhancers (Zhao, 2013). Oct-1 regulates the expression of a variety of genes,  
201 including  $\kappa$ - (Pauciullo *et al.*, 2013) and  $\beta$ -casein genes (Pauciullo *et al.*, 2014), oxytocin (Cosenza  
202 *et al.*, 2017a) and immunoglobulin genes (Dreyfus *et al.*, 1987). Furthermore, Oct-1 it is not a strong  
203 transcriptional activator by itself, but in conjunction with other co-activators as TATA-binding  
204 proteins (Zwilling *et al.*, 1994).

205 In human, Oct-1 plays a major role in the modulation of *LPL* gene expression with three  
206 elements involved, one distal and two proximal (Previato *et al.*, 1991). In fact, the deletion of the  
207 region containing the distal octamer sequence resulted in a 75-100% increase of activity. Conversely,

208 the deletion of the region with the two proximal octamers resulted in the decrease of transcription  
209 activity of approximately 65% (Previato *et al.*, 1991). Human octamer motifs are conserved in mouse  
210 and chicken *LPL* promoters (Yang *et al.*, 1995) and homology analysis with buffalo promoter  
211 confirmed a similarity of 96%. Therefore, it is reasonable to suggest that the presence of conserved  
212 Oct-1 motifs leads to similar regulation mechanisms of *LPL* gene expression mediated by this  
213 transcription factor.

214 Two cis-acting regions of the human *LPL* promoter, known as LP- $\alpha$   
215 (GCGACTATCTTCTTTCACTTATCATAACTCAATACGG) and LP- $\beta$   
216 (GGACGCAATGTGTGTCCCTCTATCCCTACATTGACTTTGC), were also considered important  
217 for transcriptional activation of the gene during the adipocyte differentiation (Enerbäck *et al.*, 1992).  
218 The comparative analysis of the LP- $\alpha$  and LP- $\beta$  consensus sequences with the reference representative  
219 genomes available and annotated for the *LPL* showed that the human LP- $\alpha$  sequence was found across  
220 many species (with different level of homology), including river buffalo with 85% of similarity  
221 (Figure 1). Conversely, no significant similarity was found in buffalo for the LP- $\beta$ , which is present  
222 only in primates, in the sea otter, and in the Hawaiian monk seal (similarity ranging from 85 to 100%).  
223 This result suggests a major impact of LP- $\alpha$  on the *LPL* regulation among different species. Further  
224 studies will be necessary to evaluate the real influence of this element on the transcriptional regulation  
225 of *LPL* in river buffalo.

226 The sequence analysis revealed 11 SNPs in total (Table 2). Although the sequenced region  
227 covers only 20.3% of the *LPL* (taking as reference the bovine gene sequence [NC\\_037335.1](#) and the  
228 buffalo genome [AWWX01438720.1](#)), the variability observed in river buffalo is high compared to  
229 that reported for other ruminants (Badaoui *et al.*, 2007; Crepaldi *et al.*, 2013; Oh *et al.*, 2013). This  
230 finding is probably the result of the lower selective pressure applied on buffalo compared to other  
231 ruminants. Therefore, the natural genetic diversity is more preserved in this species, as happens also  
232 in humans, where about 100 polymorphisms have been identified in the *LPL* gene, mostly associated

233 with the occurrence of familial hyper-lipidemia and atherosclerosis due to a reduction or loss of LPL  
234 function (Merkel *et al.*, 2002).

235 Six polymorphic sites were detected in the intron 3. None of them affected key sites of the  
236 spliceosome machinery (acceptor sites, branch points, poly-pyrimidine tracts, donor sites). Therefore,  
237 these mutations are not expected to influence the gene expression.

238 The other five SNPs were observed in the promoter (four SNPs) and in exon 1 (one SNP). Of  
239 these, two are of special interest for their potential functional effects. The mutation g.-446A>G falls  
240 within the consensus sequence of a Sp1 transcription factor, which also regulates the expression of  
241 other lipogenic genes in river buffalo as, for instance, the stearyl CoA desaturase gene (*SCD*), as  
242 recently demonstrated by Gu *et al.* (2019). Conversely, the exonic mutation (g.107G>A) falls in the  
243 5' un-translated region (UTR), which is a non-coding region, but plays an important role in the  
244 translational regulation. In fact, in addition to consensus sequences for transcription factors, it  
245 contains Kozak consensus sequences and cis-acting regulatory elements known as upstream Open  
246 Reading Frames (uORFs) (Wethmar *et al.*, 2010).

247 The PCR-RFLP protocols set up for the SNP genotyping proved to be effective for the allele  
248 discrimination: for g.107G>A the digestion of the PCR product (200 bp) with the *DdeI* endonuclease  
249 produced two fragments (195 and 5 bp) for the G allele, and three fragments (103, 92, 5 bp) for the  
250 A allele (Figure 2A). For the SNP g.-446A>G, using the endonuclease *SsI*, the allele A remains  
251 undigested (151 bp), whereas the allele G is restricted into two fragments (84 and 67 bp) (Figure 2B).

252 Frequencies of 0.63 and 0.68 were calculated for the alleles g.107G and g.-446G respectively.  
253 At both SNPs the genotype frequencies were in agreement with the Hardy-Weinberg equilibrium.

254 A strong linkage disequilibrium ( $D'=1$ ) was found between these two SNPs and the high level  
255 of  $r^2$  (0.903) allows the imputation of an allele at one SNP, known the allele at the other SNP.

256 In order to investigate the effects of the SNP g.-446A>G on the *LPL* expression, we tested *in*  
257 *vitro* whether a genotype-dependent binding reaction exists between the transcription factor Sp1 and  
258 this genetic marker, using the electrophoretic mobility shift assay (EMSA). This technique is a

259 powerful tool to assess the selective binding of a protein to a double stranded DNA. The specific  
260 protein-DNA interaction results in the reduced migration rate of the complex compared with the free  
261 DNA (Garner and Revzin, 1981). Therefore, dual colour EMSA experiments were performed using  
262 specific double stranded DNA constructs (green colour) corresponding to homozygous genotypes (g.-  
263 446AA and g.-446GG), each treated with a recombinant Sp1 transcription factor (red colour). The  
264 presence of the dual colour band (yellow) and the shift in the mobility of the DNA construct (Figure  
265 3) demonstrated that the Sp1 interacted only with the genotype g.-446GG, generating a dual colour  
266 band (yellow) and a shift in the molecular weight of the DNA construct (Figure 3) confirming, *de*  
267 *facto*, the bioinformatics analysis (Figure 1). The transcription factors Sp1 are considered enhancer  
268 motifs for the basal expression of many genes, including *LPL*, whose transcription in human is  
269 stimulated by this transcription factor itself or by a synergistic interaction between Sp1 and SREBP-  
270 1 (Yang and Deeb, 1998).

271 The exclusive interaction of the transcription factor Sp1 with the promoter having genotype  
272 g.-446GG led us to investigate also the effect of the SNP on the *LPL* transcription level. The q-PCR  
273 showed a significant overexpression of the genotype GG (~2.5 fold higher) compared with both AA  
274 (P=0.0019) and AG (P=0.0017) genotypes (Figure 4), in agreement with the results of the EMSA  
275 experiments. In particular, the genotype GG induced the Sp1 binding, which consequently up-  
276 regulated the expression of the *LPL* gene.

277 These results could have practical implications for the improvement of milk quality. In fact,  
278 the up-regulation of the *LPL* in mammary gland is mediated by the anterior pituitary through the  
279 release of prolactin (Thomson, 1992), in agreement with hormonal regulation of the mammary gland  
280 during the lactation period and nutrient transport supporting the milk production (Bionaz and Looor,  
281 2008). In addition, the change of *LPL* gene expression in milk somatic cells may affect the natural  
282 function of LPL enzyme, i.e. the hydrolysis of fatty acids from triglycerides and phospholipids in the  
283 milk.

284 LPL regulates the hydrolysis of circulating triglycerides carried by chylomicrons and VLDL  
285 (Very Low Density Lipoprotein), and the uptake of fatty acids by most tissues, including mammary  
286 gland and adipose tissues. In particular, lipoprotein lipase is critical for the uptake and secretion of  
287 the long-chain fatty acids in milk (Iverson *et al.*, 1995). In the lactating females, LPL levels are almost  
288 completely depressed in adipose tissue, while those in the mammary gland are high. As a  
289 consequence, circulating fatty acids are directed to the mammary gland for milk fat production  
290 (Iverson *et al.*, 1995). Therefore, LPL may affect the quali-quantitative characteristics of milk fat  
291 through this mechanism. The effect of the *LPL* polymorphism on milk production in river buffalo is  
292 under investigation.

293

294

## CONCLUSIONS

295 The genetic progress of Italian river buffalo focuses on the improvement of the milk  
296 production traits; therefore, the genes regulating fat metabolism are important targets of study. In the  
297 present investigation, we provided the first description of *LPL* promoter and reported on the genetic  
298 diversity found in that region and at exon level. The SNP g.-446A>G in the promoter region created  
299 a consensus sequence for the transcription factor Sp1, whose binding activity was demonstrated to be  
300 genotype-dependent. The specific bond to the GG genotype induced a significant increase in the *LPL*  
301 gene expression, which could be associated with the milk yield and composition.

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415

1 **Table 1.** Oligonucleotide primers sequences, positions and applications.

| Application     | Gene           | Region         |                  | Primer sequence                          | EMBL ID               | Size (bp) | Endonuclease |
|-----------------|----------------|----------------|------------------|--|-----------------------|-----------|--------------|
| Genotyping      | <i>LPL</i>     | Promoter       | <i>Forward</i>   | 5'-GCAAGTTCAAGCTGCAGAAA-3'               | <u>AWWX01438720.1</u> | 151       | <i>Ssi</i> I |
|                 |                |                | <i>Reverse</i>   | 5'-ATTTGGGCAATACCCAGCTT-3'               |                       |           |              |
|                 | <i>LPL</i>     | Exon 1         | <i>Forward</i>   | 5'-GGAAACCTGCCGCTTCTA-3'                 | <u>AWWX01438720.1</u> | 200       | <i>Dde</i> I |
|                 |                |                | <i>Reverse</i>   | 5'-GCTCAGAGCCAGCAGAA-3'                  |                       |           |              |
| Realtime-PCR    | <i>LPL</i>     | Exon 1         | <i>Forward</i>   | 5'-GGAAACCTGCCGCTTCTA-3'                 | <u>AWWX01438720.1</u> | 200       | -            |
|                 | <i>β-actin</i> | Exon 1         | <i>Forward</i>   | TCCTCCCTGGAGAAGAGCTA                     | <u>NM_001290932</u>   | 101       | -            |
|                 |                |                | <i>Reverse</i>   | AGGAAGGAAGGCTGGAAGAG                     |                       |           |              |
| <i>18S rRNA</i> | Exon 1         | <i>Forward</i> | CGTTCTTAGTTGGTGG | <u>NR_036642</u>                         | 76                    | -         |              |
|                 |                |                | <i>Reverse</i>   | GTAAGTAGTTAGCATGC                        |                       |           |              |
|                 |                |                | <b>Genotype</b>  | <b>Probes (5' to 3')</b>                 |                       |           |              |
| EMSA            | <i>LPL</i>     | promoter       | AA               | 5'-AGGAGAATTGGC <u>A</u> GGGGAGGGGGGG-3' | <u>AWWX01438720.1</u> | 25        | -            |
|                 |                |                | GG               | 5'-AGGAGAATTGGC <u>G</u> GGGGAGGGGGGG-3' |                       |           |              |

2  
3 Primers were designed by means of DNAsis software (Hitachi Software Engineering Co., San Bruno, CA).  
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5

1 **Table 2.**  
 2 Polymorphisms found at river buffalo *LPL*. European Variation Archive (EVA) BioProject Acc. No.  
 3 [PRJEB28835](#)  
 4

| Location | Position (1) | Position (2) | Polymorphism |
|----------|--------------|--------------|--------------|
| Promoter | -517         | 13606        | A/T          |
|          | -446         | 13677        | A/G          |
|          | -433         | 13690        | A/G          |
|          | -358         | 13765        | C/T          |
| Exon 1   | +107         | 14229        | A/G          |
| Intron 3 | +33          | 27206        | A/G          |
|          | +38          | 27211        | A/G          |
|          | +43          | 27216        | T/G          |
|          | +64          | 27237        | A/G          |
|          | +70          | 27243        | T/A          |
|          | +149         | 27322        | A/G          |

5 (1) Numbering refers to each location and re-starts from +1 (exon 1 and intron 3) or -1 (promoter)  
 6 each time.

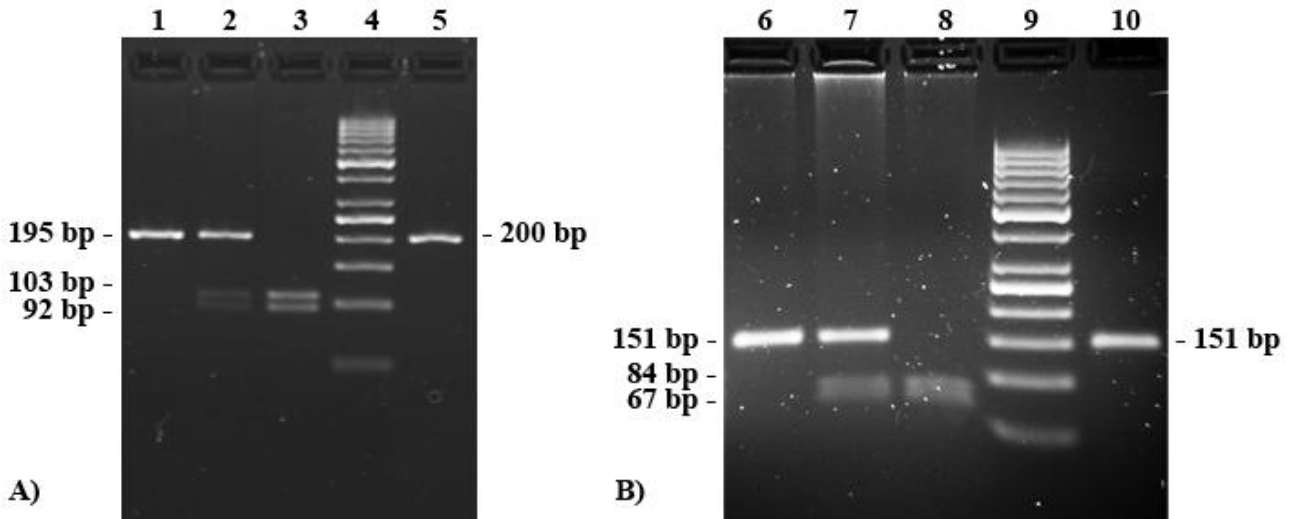
7 (2) Numbering refers to the buffalo genome sequence (EMBL ID: [AWWX01438720.1](#))  
 8

1 LP- $\alpha$   
2 tatgcaaaggaataataggatgtgcatcttctgtgactggccttctttcacttagcataa -699  
3  
4 TATA  
5 caatacactttcaggctgtctggcctcaacattctgcctctgaggataaaaatggttacttt -639  
6  
7 TATA Oct-1  
8 gcatttcttgatgagtttgaggattgagtataattttgcataaggcaaaaattagaaacta -579  
9  
10 C/EBP $\alpha$   
11 gagcaagagggccttttctcaactacaagccaatccatattaccaaagttaaacaccaga -519  
12  
13 wgtgcaagttcaagctgcagaaaaatgtgagtcctgagggtccttagattgacattgtcc -459  
14  
15 Sp1  
16 aggagaattggcrgggaggaggggggracaaaaaacaaaacagacactgcaagttacaac -399  
17  
18 aggagccaccaaagaagctgggtattgcccaaatgtagtytaacaacttaacttcctt -339  
19  
20 TATA  
21 tcttagaaaacagatgattacagagtatttcaataaaagccataaaaacctttgtttggt -279  
22  
23 gtttggttaggggaccaggagagccagacctcttaggaaaagcaaatgcccctcctaaa -219  
24  
25 Oct-1  
26 gaaagtaaaatcttctgccccttccccttcttctccctggcagagttgaatctca -159  
27  
28 ttacagtttgctcaaatatttgaaagtgaatttagtatcctcccccaacttaagatttt -99  
29  
30 C/EBP $\alpha$  Oct-1 C/EBP $\alpha$   
31 atagccaatagatgatgaggttcatttgcataattccaatcacataagcagccgtggagt -39  
32  
33 Sp1 Sp1 5' UTR  
34 ggaaacagtgctcggactcattttctcctccccctcctcctcctcctccgaggaaacctgc 22  
35  
36 AP2 Exon 1  
37 cgcttctagctcccaccctcccctttaaagggtgacttgctctgtgccagaccgctgct 82  
38  
39 ccagcctgctgccgccttgggctcrgcgggtctactgttctgtccgcgctcgcgctggt 142  
40  
41 M E S K A L L L L  
42 gccctgcatctcctacagaggacatccccgagATGGAGAGCAAGGCCCTACTTCTGCT 202  
43  
44 A L S  
45 GGCTCTGAGC...  
46

47 **Figure 1.**

48 Key regulatory motifs found in the 5' promoter region of the river buffalo *LPL* gene. Numbering is  
49 relative to the first nucleotide of the first exon (+1). The 5' UTR (Un-Translated Region) of the exon 1  
50 is reported in italics, whereas the coding region is in upper case. Genetic diversity is indicated in bold  
51 (w = a/t; r = a/g; y = c/t).  
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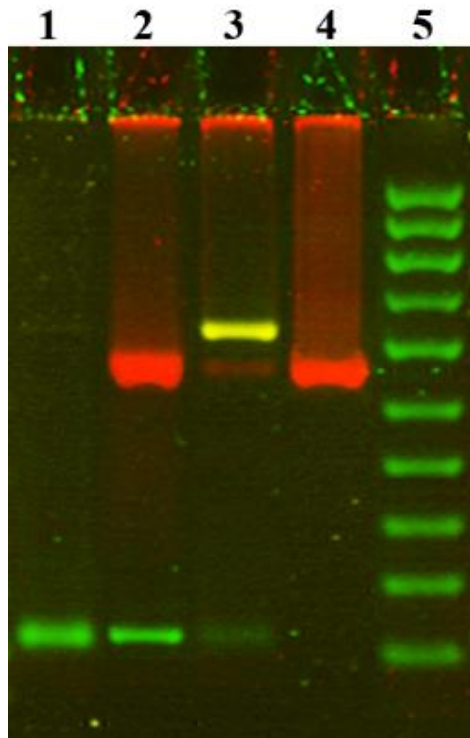
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**Figure 2.**

5 **A)** Genotyping of the SNP g.107A>G in the exon 1 of river buffalo *LPL* by *Dde* I PCR-RFLP. Line  
6 1, GG homozygous sample; line 3, AA homozygous sample; line 2, heterozygous sample. Line 4,  
7 GeneRuler 50 bp DNA Ladder (Thermo Scientific); line 5, undigested PCR product. The band 5bp  
8 long was not visible on the gel. **B)** Genotyping of the SNP g.-446A>G in the *LPL* promoter by *Ssi* I  
9 PCR-RFLP. Line 6, AA homozygous sample; line 8, GG homozygous sample; line 7, heterozygous  
10 sample. Line 9, GeneRuler 50 bp DNA Ladder (Thermo Scientific); line 10, undigested PCR product.

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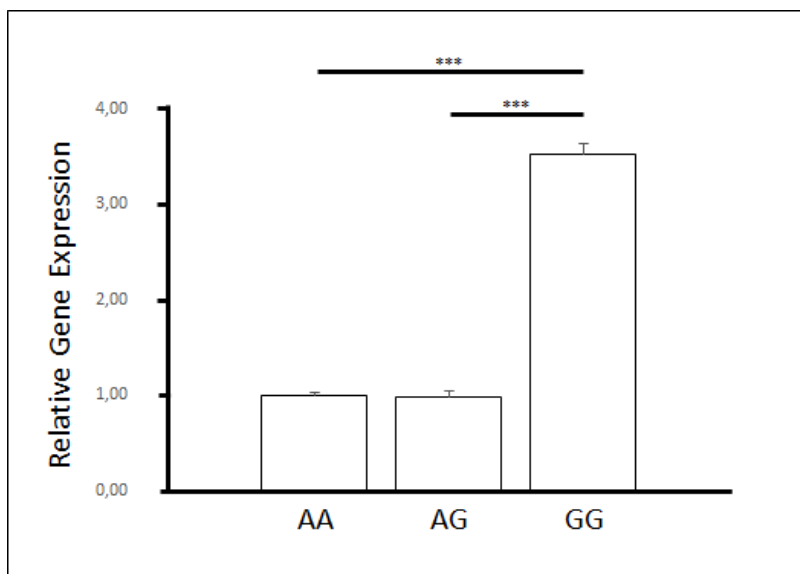


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**Figure 3**

Dual colour electromobility shift assay (EMSA) testing the interaction between the *LPL* promoter and the transcription factor Sp1. Line 1, free DNA sample (Green); line 2, DNA probe with genotype AA (no binding); line 3, DNA probe with genotype GG (binding and shift in yellow); line 4, free Sp1 protein (Red); line 5, 20 bp DNA Ladder (Jena Bioscience).





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**Figure 4**

Relative expression values (mean ± SEM) of LPL in 6 buffaloes (2 animals for each genotype at g.107A>G and g.-446A>G). Unpaired Student's *t*-test was used to assess the differences among the genotypes (\*\**P* < 0.01).

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**Supplementary table 1.**

Primer sequences, position according to the genome sequence [AWWX01438720.1](#), annealing temperature and amplicon size used for the sequencing of river buffalo *LPL* gene.

| Region covered       | Position      | Primers        | Sequence                    | Ta °C | Size (bp) |
|----------------------|---------------|----------------|-----------------------------|-------|-----------|
| Promoter/Exon 1      | 13321-13340   | <i>Forward</i> | 5'-TAATCTACTCTCTGTCCCTA-3'  | 62.0  | 1014      |
|                      | c 14334-14318 | <i>Reverse</i> | 5'-GCTCAGAGCCAGCAGAA-3'     |       |           |
| Int.1/Exon 2/Int.2   | 20301-20320   | <i>Forward</i> | 5'-AACCGTTCTCTTGCAATCCA-3'  | 61.5  | 449       |
|                      | c 20749-20731 | <i>Reverse</i> | 5'-ACCTGAGCCAGAACTGTTT-3'   |       |           |
| Exon 3/Exon 4        | 26997-27017   | <i>Forward</i> | 5'-ACAGGAATGTATGAGAGTTGG-3' | 61.5  | 1880      |
|                      | c 28876-28857 | <i>Reverse</i> | 5'-CAGACTTCCTGCAATACCAG-3'  |       |           |
| Int. 4/Exon 5/Int. 5 | 29772-29790   | <i>Forward</i> | 5'-CCAAAGGAAAGTTCCCACG-3'   | 61.4  | 494       |
|                      | c 30273-30254 | <i>Reverse</i> | 5'-GATGACCATGCTAAGAGGTG-3'  |       |           |
| Int. 5/Exon 6/Int. 6 | 31294-31314   | <i>Forward</i> | 5'-ACACCACATCTCAGCTATATT-3' | 60.1  | 481       |
|                      | c 31774-31756 | <i>Reverse</i> | 5'-ACAAATGAGGGCTCAGAAC-3'   |       |           |
| Int. 6/Exon 7/Int. 7 | 32734-32753   | <i>Forward</i> | 5'-GCTTCCTGTTGATCACTAGT-3'  | 60.5  | 347       |
|                      | c 33085-33067 | <i>Reverse</i> | 5'-ACAAAAGGTCAGGGACTG-3'    |       |           |
| Int. 7/Exon 8/Int. 8 | 33761-33780   | <i>Forward</i> | 5'-ACTCCCCAAACAATAAAGCT-3'  | 59.0  | 464       |
|                      | c 34224-34205 | <i>Reverse</i> | 5'-GGCTCAGAAGGTAAAGAATC-3'  |       |           |
| Int. 8/Exon 9/Int. 9 | 35403-35422   | <i>Forward</i> | 5'-GAATGGCCTGAGTATAGATA-3'  | 58.8  | 381       |
|                      | c 35783-35763 | <i>Reverse</i> | 5'-CCTGAAGGTTCTTATCACAAG-3' |       |           |

6 c = complementary to

