Expression of key myogenic, fibrogenic and adipogenic genes in *Longissimus thoracis* and *Masseter* muscles in cattle

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Short title: gene expression in different muscles in cattle

Abstract

Adipogenesis, myogenesis, and fibrogenesis are related processes that can contribute to meat quality. Therefore, extending the knowledge of these processes would facilitate the identification of molecular markers that predict intramuscular fat accretion. The main purpose of this work, based on previous results, was to further study the expression of key genes related to adipogenic, myogenic, fibrogenic processes, and some cytokines in *Longissimus thoracis* (LT) and *Masseter* (***MS***) muscles of Pirenaica and Holstein young bulls. *LT* and *MS* muscles from Pirenaica (*n*=4) and Spanish Holstein (*n*=4) were sampled for

proximate analysis, determination of adipocyte size distribution and expression of key candidate genes. Fat percentage was lower in *LT* than in *MS* muscle in Pirenaica young bulls ($P = 0.023$) and was higher in *LT* muscle in Holstein than in Pirenaica young bulls ($P = 0.007$). Gene expression analysis revealed that the mRNA level of *Myogenic differentiation 1* (*MYOD)* was higher in *LT* than in *MS* muscle in both groups of animals ($P < 0.001$) and that *Myostatin* (*MSTN*) expression was also higher in *LT* than in *MS* muscle in Holstein bulls ($P = 0.001$). On the other hand, *MSTN* and *PPARG* showed higher expression in *LT* and *MS* in Pirenaica young bulls ($P = 0.026$), while the expression of *Fatty acid binding protein 4* (*FABP4*) was higher in Holstein young bulls, also in both muscles ($P < 0.001$). The results suggested that the development of intramuscular adipose depot was directly related to the expression of adipogenic genes, such as *FABP4*, but inversely related to the expression of the cytokine *MSTN* and the myogenic gene *MYOD,* genes which showed a muscle-specific expression.

Keywords: marbling, cattle, adipogenesis, myogenesis, cytokines, transcriptome.

Implications

The identification of molecular makers related to intramuscular fat (**IMF**) accretion can help to improve IMF deposition and therefore, to increase the added value of the beef. This could be of particular importance for breeds with poor tendency to accumulate IMF but which are relevant in local economies. The level of expression of the genes *MYOD* and *MSTN*, related to muscle development, and

also its possible interactions with adipogenic and other myogenic transcription factors, could be important in order to modulate IMF accretion and should be considered in future works studying factors involved in intramuscular fat deposition in cattle.

Introduction

Fat deposition in meat animals is affected by genetic and management factors and different adipose depots develop at different rates (Du et al., 2013). It has been reported that intramuscular fat (**IMF**) develops at distinct rate during growth in different breeds, leading to different IMF accumulation and carcass marbling scores. It has also been observed that IMF content could vary from 3% to 11% in different muscles within beef carcasses (Jeremiah et al., 2003) and that, within a single animal, not all intramuscular deposits are at the same stage of development (Roberts et al., 2015).

In a previous study by our group, we found that IMF percentage and adipocyte size distribution were different in four muscles. The IMF percentage was greater in *Masseter (**MS**)* and *Sternomandibularis* (***ST**)* than in *Longissimus thoracis* (***LT**)* and *Semimembranosus (**SM**)* muscles. On the other hand, *MS* and *ST* muscles had a bimodal distribution of adipocyte size while it was unimodal in *LT* and *SM*, which could indicate a more active hypertrophy process in *MS* and *ST* muscles. Nevertheless, there was not a clear muscle effect on the expression of key adipogenic genes (Martínez Del Pino et al., 2017). Although results are not yet conclusive, other authors neither found a clear relationship between the amount of fat and the expression of some adipogenic factors (Duarte et al., 2013). More

recently, studies analyzing differential gene expression of both mRNAs and miRNAs have revealed some insights on the regulation of cattle adipose tissue development, but the molecular mechanisms underlying differential IMF accretion in beef cattle still remain elusive.

During the prenatal stage, skeletal muscle development mainly involves the formation of muscle fibres (myogenesis), but a portion of mesenchymal stem cells differentiate into fibro/adipogenic progenitor cells, that diverge early during the development committing to both adipocytes (adipogenesis) and fibroblasts (fibrogenesis), processes that may be considered competitive as muscle fibers, fibroblasts and adipocytes all derive from mesenchymal stem cells (Du et al., 2013). Furthermore, because IMF is adjacent to muscle fibers, a crosstalk between myocytes and adipocytes was demonstrated (Li et al., 2017) and it was postulated that some cytokines might play a relevant role in regulating myogenesis, adipogenesis, lipogenesis and lipolysis. As reported by Li et al. (2017), myokines and adipokines secreted by muscle and adipose cells may have an important effect in maintaining a balanced ratio of skeletal muscle to fat. Intramuscular fat development might be influenced by a crosstalk between adipocytes and myocytes, probably mediated by cytokines such as *Leptin* (***LEP***) and *Myostatin* (***MSTN***) (Kokta et al., 2004). As intercellular signalling may influence IMF development, extending the knowledge of the mechanisms involved in this process would facilitate the identification of molecular markers that predict IMF accretion and could help to devise methods aiming to improve marbling scores in beef.

Then, on the basis that IMF accretion would be affected by muscle fibers, fibroblasts and cytokines, the main purpose of this work was to study the

expression of key candidate genes related to adipogenic, fibrogenic, myogenic processes, and some cytokines in *LT* and *MS* muscles of Pirenaica and Holstein. Candidate genes involved in the former processes, and according to their relevance, were selected: *Peroxisome proliferator activated receptor γ* (***PPARG***), *CCAAT/enhancer binding protein α* (***CEBPA***), *Fatty acid binding protein 4* (***FABP4***), *Zinc finger protein 423* (***ZFP423)*** and *Wingless-type MMTV integration site family member 10B* (***WNT10B***) are key genes in the regulation of adipocyte proliferation and differentiation; *Myogenic factor 5* (***MYF5***) and *Myogenic differentiation 1* (***MYOD***) have been identified as early myogenic regulatory factors while *Myogenin* (***MYOG***) is considered a terminal differentiation factor of myofibers; *Fibronectin* (***FN1****), Fibroblast growth factor receptor 1 (****FGFR1****) and Fibroblast growth factor 2 (****FGF2****)* are related to fibrogenesis and collagen synthesis. In addition, some cytokines related to the former processes were also considered: *LEP*, by its role in the regulation of energy intake and expenditure, *Adiponectin* (***ADIPOQ****)* that promotes adipocyte differentiation, *MSTN* with a well-known role in muscle development and a possible role in the regulation of adipogenesis, and *Transforming growth factor β 1 (****TGFB1****)* that induces the expression of fibrogenic genes. The two muscles were selected based on our previous results that showed differences in IMF and adipocyte size between them, and were obtained from the two types of animals that are most commonly used for beef in the area of the Pyrenees Mountains (North of Spain), in order to broaden the scope of the study: Pirenaica and Spanish Holstein. Pirenaica is one of the most important cattle breed reared in the Pyrenees area, but while it is highly appreciated for its adaptation and production system, the meat quality can

123 be compromised by its low tendency to marbling; therefore, improving this
124 attribute would increase the added value of the meat.
125
126 **Material and methods**
127 *Animals and sample collection*
128 Procedures involving animal care and handling were conducted in compliance
129 with the international guidelines (Council Directive 2010/63/EU, 2010). Animal
130 care and handling in the farm and during transportation followed European
131 guidelines (Council Directive 2008/119/EC, 2008). The slaughter was performed
132 by using stunning methods (non-penetrative captive bolt device followed
133 immediately by bleeding) following the European Union regulations that regulate
134 the protection of animals at the time of killing (Council Regulation, EC, No
135 1099/2009, 2009).
136 The animals used in this study were Pirenaica and Spanish Holstein young bulls.
137 These animals were slaughtered at the commercial endpoint for each type of
138 production, about 12 and 10 months of age respectively. Pirenaica young bulls
139 (n=4) were used in a previous work (Martínez Del Pino et al., 2017): the calves
140 were with their mothers up to five months of age and after weaning, and up to the
141 usual commercial end point (12 months), the young bulls were fed a standard
142 fattening diet based on concentrate and cereal straw, both *ad libitum*. The young
143 bulls had an average age of 11.91 ± 0.66 months and an average carcass weight
144 of 324.0 ± 17.7 kg at slaughter.
145 Holstein bulls (n=4) were weaned at 2 days of age after taking colostrum, fed with
146 artificial milk and concentrates during the suckling period, and then fed with the

147 same regimen as the Pirenaica bulls. At slaughter, Holstein bulls had an average
148 age of 9.71 ± 0.17 months and an average carcass weight of 242.7 ± 2.4 kg.
149 Immediately after the slaughter, samples of *LT* at the 10th rib of the left carcass
150 side and *MS* muscle of the left side were taken. Then, samples intended for RNA
151 analysis were snap frozen in liquid nitrogen and stored at -80°C, samples for
152 chemical characterization of the tissues were placed in ice and stored at -20°C
153 and samples for adipocyte size determination were placed in test tubes
154 containing 10 ml Tyrode´s solution (0.15 M NaCl, 6 mM KCl, 2 mM $CaCl_2$, 6 mM
155 $C_6H_{12}O_6$, 2 mM $NaHCO_3$, pH 7.62) at 39°C, and subsequently analyzed.

156 *Chemical traits*

157 IMF percentage of *LT* and *MS* muscle was determined from duplicate samples (5
158 g) by Soxhlet: samples were hydrolyzed, filtered and dried and then, the
159 extraction of chemical fat was performed for 6 h with petroleum ether
160 (International Organization for Standardization –ISO 1443: 1973). Protein
161 percentage of muscles was analyzed using the Kjeldahl method (Kjeldahl, 1883)
162 and total collagen of samples was quantified by measuring the total amount of
163 hydroxyproline according to the method described in Bergman and Loxley (1963).

164 *Adipocyte size*

165 The procedure for adipocyte size analysis was previously described in Martínez
166 Del Pino et al. (2017). Each sample was digested in a flask with 5 ml of Medium
167 199 (Gibco, Waltham, MA, USA) (pH: 7.0 to 7.4), 200 mg of bovine serum albumin
168 (Sigma-Aldrich, Madrid, Spain) and 10 mg of collagenase type II (Sigma-Aldrich,
169 Madrid, Spain) for 90 min at 39°C. Adipocyte solutions were filtered through a
170 850 µm mesh size to remove tissue debris. A preparation of the isolated
171 adipocytes was placed on the light microscope (Olympus BH.2 microscope,

Olympus Optical Co. Europe Hamburg, Germany) and the magnification was adjusted to *10x*. Four or five visual fields were registered by means of a 3CCD color video camera (model *Evolution VF,* Media Cybernetics, Inc. Silver Spring, USA) mounted on the microscope. The images were fed into a computerized image analysis system and digitized. For that purpose, each image was first calibrated and the diameter of approximately 200 adipocytes was measured (Albertí et al., 2013). Measurement of the adipocyte size was performed using a program for the acquirement and storage of the images as well as for the processing of data (Image-Pro Plus 5.1, Media Cybernetics, Inc. Silver Spring, USA). Adipocytes should meet a shape factor of 0.8–1 (shape factor of 0 indicating a straight line and 1 a perfect circle).

Adipocyte number per g of muscle tissue was approximated from the normalized histogram of adipocyte diameter distributions. First, average number and average mass of adipocytes of each histogram class was computed assuming sphericity and a fat density of 0.915 g/ml and considering the chemical fat content of the tissue determined by the Soxhlet method. Then the total number of adipocytes per g of tissue was obtained by dividing the summation, along the classes, of the number of adipocytes by the summation, along the same classes, of the mass of the adipocytes (http://www.unavarra.es/rmga/add/index_en.html).

RNA Isolation and quantitative Reverse Transcription Polymerase Chain Reaction.

Total RNA from 200 mg of muscle was isolated using TRIzol Reagent (Thermo Fisher Scientific, Carlsbad, CA, USA) and GenElute Mammalian Total RNA Miniprep kit (Merck, Darmstadt, Germany), according to the manufacturer‘s

197 instructions. Then, concentration and purity of total RNA were measured using a
198 Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA,
199 USA). A total of 750 ng of RNA was treated with DNase using RQ1 RNases-Free
200 DNase (Promega Corporation, Madison, WI, USA) and single-stranded cDNA
201 was synthesized from 500 ng of RNA using PrimeScript RT Reagent (Takara,
202 Otsu, Japan) following manufacturer's instructions.
203 The expression of candidate genes was quantified by RT-qPCR using SYBR
204 Premix Ex Taq (Takara, Otsu, Japan) in FX96 Touch Real-Time PCR Detection
205 System (Biorad, Munich, Germany). The qPCR duplicate reactions (10 µL)
206 contained 3 µL of 1:5 diluted cDNA, 5 µL SYBR Premix Ex Taq and 0.2 µL forward
207 and reverse primers (10 µM), and 1.6 µL DNase/RNase free water. Thermal
208 cycling conditions were as follows: 95°C for 30 s, followed by 40 cycles at 95°C
209 for 5 s and 3 s at 60°C, followed by amplicon melting curve. Dissociation curves
210 were examined for the presence of a single product. Sequence of primers and
211 amplicon lengths are indicated in Supplementary Table S1.
212 The stability of five reference genes (*β-actin* (***ACTB***)*, Ceroid-lipofuscinosis*
213 *neuronal 3* (***CNL3***)*, Peptidylprolyl isomerase A* (***PPIA***)*, Ribosomal protein large*
214 *PO* (***RFLPO***) and *Topoisomerase II-beta* (***TOP2B***)) was analyzed by using
215 RefFinder software (Xie et al., 2012). The most stable reference genes, *TOP2B*
216 and *ACTB,* were used for data normalization. In order to determine the primers
217 efficiency, PCR products were cloned into pGEM-T Easy vector (Promega
218 Corporation, Madison, USA) and chemically transformed into NEB 10-beta *E. coli*
219 (New England Biolabs Inc., UK). All amplified PCR products were also sequenced
220 (Macrogen Inc., Spain) to verify their identity and all the amplicons were
221 confirmed 100% homologous to their target sequences. qPCR efficiency for each

gene was estimated by standard curve method ($E = 10^{-1/slope}$) using a 10-fold dilution series of plasmid DNA (Rasmussen, 2001). The efficiencies of all primers ranged from 1.90 to 2.00 and R^2 were close to 0.99 for all genes (See Supplementary Table S2).

A nested design was considered to take into account the errors of sampling (RNA extraction), reverse transcription (**RT**), and qPCR that are introduced into measurements of gene expression. Hierarchically arranged experiments were performed with three RNA samples from each muscle, two RT reactions from each RNA sample and two qPCR replicates from each RT. In addition, RNA extraction, RT and qPCR were completely randomized in order to minimize the experimental variability (Martínez Del Pino et al., 2017).

Statistical Methods

Chemical traits, adipocyte size and number

In order to determine if there were differences in chemical traits data were analyzed by two-way analysis of the variance, and differences between muscles (*Longissimus thoracis* vs. *Masseter*) or animal groups (Pirenaica *vs*. Holstein young bulls) were tested together and for each subset of muscle and animal group.

Adipocyte size distribution was analyzed using the AdipSD software (Alfonso and Mendizabal, 2016). Unimodality *vs*. bimodality was tested considering the Hartigan's test of unimodality (Dip) (Hartigan and Hartigan, 1985). Unimodal distributions were compared for some of their distributional parameters (mean, median, mode, minimum and maximum) and for the number of adipocytes per g of tissue. Differences in these variables between muscles (*Longissimus thoracis vs. Masseter*) or animal groups (Pirenaica *vs*. Holstein young bulls) were tested

using one-way analysis of the variance. No comparison between bimodal distributions was performed as unimodality was only rejected for one of the four groups of contrast.

Gene Expression

Gene expression analysis was performed according to the method proposed by Steibel et al. (2009). Firstly, Cq data were $\log_2$ transformed using the expression $\log_2 (E^{-Cq})$. Similarly to that reported in a previous work (Martínez Del Pino et al., 2017), heterogeneous variances were observed for Animal, Sample, RT reaction and residual (qPCR) among tissues and animal groups (Supplementary Table S3). In consequence, different models were defined considering the observed heteroscedasticity, and after its comparison using Akaike's and Schwarz's Bayesian information criterion values, the model with heterogeneous variance for Sample and RT effects was chosen for the posterior analyses of differences in gene expression.

The mixed model fitted was:

$$y_{ijklmn} = \mu + T_i \times G_j + A_k + S_l(A_k) + R_m(S_l(A_k)) + e_{ijklmn}$$

where y_{ijklmn} is the individual response expressed as $\log_2 (E^{-Cq})$, μ is the mean, T_i is the fixed effect of the tissue, or the animal group, analyzed (i=2; *LT, MS,* or PIR, HOL), G_j the fixed effect of the gene studied (j=3; target gene, reference genes), A_k is the random effect of the k^{th} animal ($A \sim N(0,\sigma^2_A)$), $S_l(A_k)$ is a T_i x G_j specific random effect of the l^{th} sample taken from the k^{th} animal, ($S \sim N(0,\sigma^2_S)$), $R_m(S_l(A_k))$ *is* a T_i x G_j specific random effect of the m^{th} RT reaction of l^{th} sample from k^{th} animal ($R \sim N(0,\sigma^2_R)$), and e_{ijklmn} is the general residual random effect of each qPCR ($e \sim N(0,\sigma^2_e)$).

In order to estimate differences (*DIF*) between muscles or animal groups, different contrasts were defined using the SAS MIXED procedure (being $y_{T_iG_t}$ the $\log_2 (E^{-Cq})$ value estimated for target gene (G_t) in treatment *i* (T_i); and y_{T_iTOP2B} and y_{iACTB} the corresponding values for reference genes):

$$DIF = \left(y_{T_1G_t} - 0.5\, y_{T_1TOP2B} - 0.5\, y_{T_1ACTB}\right) - \left(y_{T_2G_t} - 0.5\, y_{T_2TOP2B} - 0.5\, y_{T_2ACTB}\right)$$

The null hypothesis that *DIF* value was equal zero was tested by the approximate t-test ($P < 0.05$) provided by MIXED procedure. Additionally, for each tissue, gene and animal group variance contribution of qPCR analysis, RT reaction, sample and animal was estimated using SAS NESTED procedure.

Finally, the individual relative gene expressions were calculated as the difference between the y_{ijklmn} values for target gene and the semi-sum values for reference genes to estimate Pearson's correlation coefficients with individual chemical traits and adipocyte size parameters estimated.

Results

Chemical traits

Chemical composition of *LT* and *MS* of Pirenaica and Holstein young bulls is shown in Table 1. In Pirenaica young bulls, the fat percentage was lower in *LT* muscle than *MS* muscle ($P = 0.023$), whereas in Holstein bulls no significant differences between muscles were observed ($P = 0.170$).

In *LT* muscle, the fat percentage was higher in Holstein young bulls ($P = 0.007$). In *MS* muscle, the fat percentage was also higher in Holstein bulls but the difference was not significantly due to the large dispersion among animals ($P = 0.171$).

295 The protein percentage, the total and soluble collagen and the moisture did not
296 show differences between muscles in both breeds ($P > 0.05$) (Table 1).

297 *Adipocyte size and number*

298 Adipocyte size distribution for Pirenaica in *LT* muscle and for Holstein young bulls
299 in *LT* and *MS* muscles and the estimated number of adipocytes are shown in
300 Table 2. The adipocyte size distribution in *LT* and *MS* muscles in Holstein young
301 bulls was unimodal (*Dip P*-value < 0.05) (Figure 1). This was also reported for *LT*
302 muscle in the Pirenaica young bulls (*Dip P*-value < 0.05), although the distribution
303 of the adipocytes in *MS* muscle in those animals was clearly bimodal (*Dip P*-value
304 > 0.05) (Martínez Del Pino et al., 2017).
305 No differences were observed between *LT* and *MS* muscles in Holstein animals
306 in the average size and number of adipocytes (Table 2), although the amount of
307 big adipocytes seemed to be greater in *MS* muscle (Figure 1). However,
308 adipocyte size distributions, showed in Figure 1, indicated differences among
309 animal groups. Holstein animals showed bigger adipocytes than Pirenaica
310 animals in *LT* muscle (Table 2) (Figure 1). Furthermore, and in contrast with the
311 results found in Holstein animals, the estimated number of adipocytes was
312 greater in *LT* than in *MS* muscle in Pirenaica animals, related to the clear
313 bimodality observed in *MS* muscle in this group (Table 2, Figure 1).

314 **Gene expression**

315 The expression of adipogenic genes *PPARG*, *CEBPA, ZFP423* and *WNT10B,*
316 were not significantly different between *LT* and *MS* muscles in both animal groups
317 (Supplementary Table S4). In Pirenaica bulls, mRNA level of *FABP4* could be

318 considered higher in *LT* than in *MS* muscle (Figure 2; $Log_2FC = 1.36$; $P = 0.020$),
319 although difference was not significant in Holstein bulls.
320 For myogenic genes, the expression of *MYOD* was significantly higher in *LT*
321 compared with *MS* muscle in both Pirenaica ($Log_2FC = 2.45$) and Holstein bulls
322 ($Log_2FC = 1.98$) ($P < 0.001$). No significant differences were found for the other
323 two myogenic genes analyzed (*MYF5* and *MYOG*). The fibrogenic genes *FN1*,
324 *FGFR1, FGF2* and *TGFB1* did not show significant differences either in their
325 expression between muscles. For the four cytokines analysed, only the
326 expression of *MSTN* in Holstein bulls was significantly higher in *LT* than in *MS*
327 muscle ($Log_2FC = 2.12$; $P = 0.001$).
328 When comparing the two animal groups, it was found that the expression of the
329 adipogenic gene *PPARG* was higher in Pirenaica than in Holstein young bulls in
330 both muscles, especially in *LT* ($Log_2FC = 1.11$; $P = 0.003$). However, the
331 expression of *FABP4* was higher in Holstein young bulls, also in both muscles
332 ($Log_2FC = -1.98$ for *LT* and -2.74 for *MS*; $P < 0.001$). The other adipogenic genes
333 analyzed did not show significant differences (Supplementary Table S5).
334 Of the myogenic and fibrogenic genes and cytokines analyzed only cytokine
335 *MSTN* showed significant differences, being higher the expression in both *LT*
336 ($Log_2FC = 2.37$; $P = 0.013$) and *MS* ($Log_2FC = 2.86$; $P < 0.001$) from Pirenaica
337 young bulls.
338 It should be highlighted that, beyond the statistical significance of differences,
339 estimated differences were especially high for *MYOD* between tissues, for *FABP4*
340 between animal groups, and for *MSTN* for both tissues and groups.
341 On the other hand, Pearson's correlation coefficients among gene expression
342 and chemical traits and adipocyte size parameters were especially high (>0.8)

343 and significant for the relationship between maximum adipocyte size and *FGFR1*
344 expression (r = 0.919; $P < 0.001$), and between minimum adipocyte size and the
345 expression of *FABP4* (r = 0.831; $P = 0.001$), *MSTN* (r = -0.829; $P = 0.001$) and
346 *PPARG* (r = -0.806; $P = 0.002$). No other very strong and significant linear
347 correlations were found (Supplementary Table S6).
348

349 **Discussion**

350 The present study arises from a previous work in which accretion of IMF in beef
351 cattle with low tendency to accumulate IMF (Pirenaica young bulls) was found to
352 be different between muscles, having *MS* more IMF than *LT* (Martínez Del Pino
353 et al., 2017). Hypertrophy of adipocytes also seemed to be a more active process
354 in *MS* and cell distribution differed between muscles, hypothesizing that these
355 differences might be related to different rates of hyperplasia and hypertrophy and
356 to specific adipocyte population characteristics. Nevertheless, there were little
357 differences in the expression of some adipogenic key genes. On the other hand,
358 and taking into account that IMF accretion could be affected by muscle fibers,
359 fibroblasts and cytokines, it was hypothesized that the observed differences in
360 intramuscular adipocyte formation between muscles could be related to the
361 expression of key genes related to fibrogenic and myogenic processes, and to
362 some cytokines, in addition to the expression of adipogenic genes. This
363 determined then the main purpose of this study.
364 The analysis of the myogenic regulatory factors (***MRF***) showed that only *MYOD*
365 expression differed between muscles, showing higher expression in *LT* than in
366 *MS* in both breeds (Figure 2; Supplementary Table S3). *MYF5* was also
367 expressed at higher level in *MS* muscle in Holstein than in Pirenaica cattle (Figure

368 3), but the difference in the expression value was low (-0.69) and the level of
369 significance was not high (*P* = 0.043). Postnatal formation of myofibers from
370 satellite cells appears to be controlled by the same set of *MRF* that control
371 embryonic myogenesis: *MYF5* and *MYOD* determine the myogenic lineage of
372 satellite cells, whereas *MYOG* and *Myogenic regulatory factor 4* drive the terminal
373 differentiation of satellite cells into myofibers (Jiang and Ge, 2014). Then, the
374 higher expression of *MYOD* in *LT* muscle might indicate a more intense
375 determination towards myofiber formation in that muscle. On the other hand, in
376 line with our results, Muroya et al. (2002) found that *MYOD* expression was
377 significantly higher in *LT* than in *MS* in Holstein cows; these authors observed as
378 well that *LT* muscle mostly expressed MyHC-2a fast isoform and also MyHC-2X
379 while *MS* exclusive expressed the MyHC-slow isoform. Also Spiller et al. (2002)
380 reported that *MYOD* was present in adult fast glycolytic fibers and is involved in
381 the maintenance of the fast IIB/IIX fiber type (Hughes et al., 1993, 1997). Then,
382 alternatively, and as *MRF*s can also mediate extrinsic signals that act as direct
383 regulators in the formation of muscle fiber type, the observed differences in
384 *MYOD* expression could be also related to the type of fiber. Similarly, higher
385 levels of *MSTN* have been found in the fast glycolytic fibers of cattle (Bass et al.,
386 1999), pig (Ji et al., 1998) and rat (Wehling et al., 2000). Moreover, they
387 suggested that *MYOD* could be regulating *MSTN* during muscle growth. In
388 relation to this, some authors suggested that *MSTN* might be regulated by
389 transcription factors related to both adipogenesis and myogenesis, as some
390 results indicate that *MSTN* could be upregulated by *MYOD* and *PPARG*, but
391 downregulated by *CEBPA* and *CEBPB* (Deng et al., 2012). In this study, similar
392 tissue-specific pattern in the level of expression of *MYOD* and *MSTN* in *LT*

393 muscle in both Pirenaica and Holstein cattle was observed, which might be
394 related to the regulatory relationship between those factors.
395 The expression of the myokine *MSTN* was higher in *LT* than in *MS* in Holstein
396 cattle and, when comparing animal groups, it was found to be overexpressed in
397 both muscles in Pirenaica young bulls. Although *MSTN* is mainly expressed in
398 muscle, where it acts as a negative regulator of skeletal muscle growth and
399 development, it can be detected as well in adipose tissue (McPherron and Lee,
400 1997) and regulate its development (Guo et al., 2009). However, its effects on
401 adipogenesis are yet controversial as it has been observed both to promote
402 adipogenesis in very early-stage mesenchymal stem cells (Deng et al., 2017) and
403 to inhibit adipogenesis in preadipocytes (Kim et al., 2001). Moreover, both
404 myoblast proliferation and differentiation can as well be negatively regulated by
405 *MSTN* via the control of the cell cycle progression and their differentiation through
406 *MYOD* activity (Langley et al., 2002).
407 The results of this work seem to suggest the involvement of *MSTN* in the
408 commitment of progenitor cells to the adipocyte lineage. Comparing genotypes,
409 *MSTN* and *PPARG* had higher expression in Pirenaica than Holstein in both
410 muscles, together with lower IMF percentage and smaller adipocytes in Pirenaica
411 in *LT* muscle. Besides, the results of the Pearson´s correlation analysis revealed
412 a significant negative correlation between the minimum adipocyte size and *MSTN*
413 ($r = -0.829$; $P = 0.001$) and *PPARG* expression ($r = -0.805$; $P = 0.002$). In line with
414 this, Gu et al. (2016) indicated that fat content decreases in *MSTN* knockout rat,
415 while there is an increase of muscle mass. On the other hand, compared to *MS*
416 muscle, *LT* muscle in Pirenaica had a higher expression of *MYOD* along with a
417 lower IMF percentage and greater number of adipocytes.

418 Genes such as *PPARG* and *ZFP423* might be also related with the commitment
419 of progenitor cells to the adipocyte lineage and adipocyte cellularity. As
420 mentioned, the lower IMF percentage in Pirenaica young bulls in *LT* muscle
421 together with higher expression of *PPARG* in Pirenaica in both muscles could
422 indicate an active differentiating population of preadipocytes, as *PPARG* peak
423 expression occurs during adipocyte differentiation (Pickworth et al., 2011), which
424 agree with the lower minimum, median and mean adipocyte size in Pirenaica
425 bulls in *LT* ($P < 0.05$).
426 The transcription factor *ZFP423* was described as a very early marker for
427 adipogenesis and was identified as a key initiator of adipogenic differentiation,
428 inducing the expression of *PPARG* (Gupta et al., 2012). This is in line with results
429 from Harris et al. (2018), who attributed the higher *ZFP423* expression and IMF
430 percentage to an increased population of adipose progenitors cells. In this work,
431 it was observed that *ZFP423* expression was slightly higher and significant in *LT*
432 than *MS* muscle in Holstein animals, which could indicate a more intense
433 commitment of progenitor cells to the adipocyte lineage. Nevertheless, the
434 relationship between gene expression levels of the former adipogenic
435 transcription factors and the amount of IMF is not always clear; besides, it should
436 be considered that in this work, the differences between Pirenaica and Holstein
437 young bulls cannot be strictly interpreted in genetic terms as both groups differed
438 in other factors, such as slaughter age and weight, and feeding during suckling
439 period.
440 On the other hand, *FABP4* expression did not show differences in *LT* and *MS* in
441 Holstein young bulls ($P = 0.265$), which could be related to the lack of differences
442 in size and number of adipocytes in Holstein, in contrast with the results for

443 Pirenaica young bulls. The expression of this gene, a marker for adipocyte
444 differentiation which is mainly expressed in white and brown differentiated
445 adipocytes, was lower in Pirenaica than Holstein cattle in both muscles. Then, it
446 could be indicative of a less intense adipocyte hypertrophy, more clearly shown
447 in *LT* muscle, as IMF content was lower in Pirenaica young bulls. At the cellular
448 level, the values of minimum, median and mean of adipocytes were significantly
449 lower in Pirenaica than in Holstein young bulls in *LT* muscle, which has a
450 unimodal distribution of adipocytes in both animal groups. Furthermore, the
451 existence of bigger adipocytes in *LT* muscle in Holstein was revealed by the fact
452 that the cell size distribution curve was displaced to the right (Figure 1).
453 Accordingly, the correlation analysis revealed that the *FABP4* expression level
454 was significantly related with the minimum adipocyte size (r = 0.831; $P = 0.001$),
455 indicating that the higher expression of *FABP4*, the larger the smaller adipocytes.
456 Although adipocyte size parameters were similar in both muscles and that
457 unimodality could not be clearly rejected in *MS* muscle, Figure 1 shows how
458 adipose cells in *MS* muscle tend to be bigger, as 70% of adipocytes are smaller
459 than 32 μm while 90% of adipocytes are below that value in *LT* muscle. In
460 addition, the number of adipocytes was lower in *MS* than *LT* in Pirenaica young
461 bulls. This could be related to the higher expression of the *ADIPOQ* gene in *MS*
462 compared with *LT* muscle though its significance was low ($P = 0.042$), which
463 might indicate more fully differentiated adipocytes in *MS* than in *LT* muscle in
464 Holstein cattle, as *ADIPOQ* is secreted by mature adipocytes and it promotes
465 adipocyte differentiation (Moisá et al., 2014). Together with *ADIPOQ*, *LEP* has
466 been reported to be positively related with IMF content or marbling in cattle

(Graugnard et al., 2009; Wang et al., 2009), although in this study no significant differences in *LEP* gene expression was observed.

Fibrogenesis leads to the synthesis and binding of collagen, related to meat tenderness, and is mediated by the *TGFB* signaling pathway (Chen et al., 2005). In this work, no differences in fibrogenic genes were observed between *LT* and *MS* muscles in both groups of animals, which would agree with the similar percentage of total and soluble collagen between muscles.

Overall, some of the genes studied, such as *CEBPA* and *WNT10B* (adipogenic genes), *FN1, FGFR1* and *FGF2* (fibrogenic genes) and the cytokines *LEP* and *TGFB1,* did not show differences between muscles or animal groups. However, other genes such as the myogenic gene *MYOD* and the cytokine *MSTN,* which showed muscle-specific expression, seem to be inversely related to IMF percentage and adipocyte size, contrary to *FABP4* expression.

Then, as a main implication, it should be highlighted that future studies, considering a higher number of animals and a screening of a broader panel of genes, should consider *MSTN* and *MYOD* and their possible interactions with other adipogenic and myogenic factors in order to better understand the role of these and other key transcription factors underlying the differences in IMF accretion in *LT* and *MS* muscles in cattle.

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Declaration of interest

The authors declare that there is no conflicts of interest.

Ethics statement

Experimental work has been carried out in compliance with European legislation on the use of animals for research.

The experimental protocol was approved by the Committee on Ethics, Animal Experimentation and Biosecurity of the Public University of Navarre (permit number PI 013/14).

Software and data repository resources

None of the data were deposited in an official repository.

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644

645 **Table 1**. *Chemical traits in Longissimus thoracis and Masseter muscles of*

646 *Pirenaica and Holstein young bulls.*

				***P*-value**					
		Group[2]		***LT vs. MS***			**PIR *vs.* HOL**		
Item	**Muscle[1]**	**PIR (*n*=4)**	**HOL (*n*=4)**	**PIR**	**HOL**	***All***	***LT***	***MS***	***All***
Fat, %	*LT*	1.30 ± 0.05[3]	3.27 ± 0.49	0.023	0.170	0.041	0.007	0.171	0.039
	MS	3.23± 0.63[3]	7.29 ± 2.54						
Protein, %	*LT*	22.16 ± 0.54[3]	20.87 ± 1.01	0.067	0.628	0.223	0.303	0.617	0.273
	MS	20.75 ± 0.32[3]	19.97 ± 1.45						
Collagen									
Total, mg/g	*LT*	4.30 ± 0.92	5.55 ± 0.45	0.652	0.272	0.305	0.265	0.200	0.071
	MS	4.90 ± 0.90	6.49 ± 0.63						
Soluble, %[4]	*LT*	17.85 ± 0.82	16.66 ± 1.17	0.901	0.506	0.574	0.437	0.763	0.892
	MS	17.68 ± 1.08	18.47 ± 2.27						
Moisture, %	*LT*	74.52 ± 0.70	72.34 ± 0.86	0.387	0.714	0.972	0.196	0.151	0.046
	MS	75.49 ± 0.58	71.28 ± 2.50						

647 Values are means ± standard error.

648 [1]*LT = Longissimus thoracis*; *MS = Masseter.*

649 [2]PIR = Pirenaica cattle*;* HOL = Holstein cattle*.*

650 [3]Values for % of fat and protein in *LT* and *MS* muscles in Pirenaica breed were presented in a

651 previous work (Martínez Del Pino et al., 2017).

652 [4]Soluble collagen is expressed as % of total collagen.

653

Table 2. *Adipocyte size parameters and estimated number of cells per gram of tissue for unimodal distributions in Pirenaica and Holstein young bulls*[1].

				P-value			
		Group[2]		*LT vs. MS*		PIR *vs.* HOL	
Item	**Muscle**[3]	**PIR**[4] **(*n*=4)**	**HOL (*n*=4)**	**PIR**	**HOL**	***LT***	***MS***
Minimum, µm	*LT*	5.68 ± 0.37	10.10 ± 0.09	--	0.361	<0.001	--
	MS[4]	--	10.33 ± 0.22				
Maximum, µm	*LT*	48.78 ± 9.68	76.97 ± 10.92	--	0.862	0.102	--
	MS	--	74.90 ± 3.19				
Median, µm	*LT*	10.11 ± 0.48	17.05 ± 1.90	--	0.221	0.012	--
	MS	--	28.29 ± 8.01				
Mean, µm	*LT*	13.25 ± 1.80	20.78 ± 1.78	--	0.169	0.025	--
	MS	--	30.15 ± 5.73				
Mode, µm	*LT*	10.35 ± 1.07	12.76 ± 0.36	--	0.141	0.077	--
	MS	--	31.88 ± 11.28				
Number, 10^6/g tissue	*LT*	7.13 ± 2.32	4.01 ± 1.07	0.047	0.664	0.269	0.337
	MS	1.26 ± 0.34	6.25 ± 4.78				

Values are means ± standard error.

[1]The adipocyte size distributions were unimodal in *Longissimus thoracis* muscle of Pirenaica and Holstein bulls and *Masseter* of Holstein bulls.

[2]PIR = Pirenaica cattle*;* HOL = Holstein cattle*.*

[3]*LT = Longissimus thoracis*; *MS = Masseter.*

661 [4]Unimodal adipocyte size distribution was rejected for *MS* in PIR ($P < 0.05$). Values for *LT* muscle

662 in Pirenaica breed were presented in a previous work (Martínez Del Pino et al., 2017).

663

664 **Figure captions**

665 **Figure 1**. Adipocyte size distribution in *Longissimus thoracis (LT)* and *Masseter*
666 *(MS)* of Pirenaica and Holstein bulls.

667 **Figure 2.** Fold change (FC) estimates, expressed as differences in normalized
668 expression values ($\log_2 (E^{-cq})$), for adipogenic, myogenic and fibrogenic genes
669 between *Longissimus thoracis* and *Masseter* in Pirenaica and Holstein bulls**.**
670 *PPARG = Peroxisome proliferator activated receptor γ*; *CEBPA =*
671 *CCAAT/enhancer binding protein α; FABP4 = Fatty acid binding protein 4*;
672 *ZFP423 = Zinc finger protein 423; WNT10B = Wingless-type MMTV integration*
673 *site family member 10B; MYF5 = Myogenic factor 5; MyoG = Myogenin; MYOD*
674 *= Myogenic differentiation 1; FN1 = Fibronectin; FGFR1 = Fibroblast growth*
675 *factor receptor 1; FGF2 = Fibroblast growth factor 2; LEP = Leptin; ADIPOQ =*
676 *Adiponectin; MSTN = Myostatin; TGFB1 = Transforming growth factor β 1.* Values
677 for *PPARG*, *CEBPA*, *FABP4* and *WNT10B* in Pirenaica breed were presented in
678 a previous work (Martínez Del Pino et al., 2017). **P* ≤ 0.05; ****P* ≤ 0.001
679 (unadjusted *P*-values for multiple testing).

680 **Figure 3.** Fold change (FC) estimates, expressed as differences in normalized
681 expression values ($\log_2 (E^{-cq})$), for adipogenic, myogenic and fibrogenic genes
682 between Pirenaica and Holstein bulls in *Longissimus thoracis* and *Masseter*
683 muscles. *PPARG = Peroxisome proliferator activated receptor γ*; *CEBPA =*
684 *CCAAT/enhancer binding protein α; FABP4 = Fatty acid binding protein 4*;
685 *ZFP423 = Zinc finger protein 423; WNT10B = Wingless-type MMTV integration*
686 *site family member 10B; MYF5 = Myogenic factor 5; MyoG = Myogenin; MYOD*
687 *= Myogenic differentiation 1; FN1 = Fibronectin; FGFR1 = Fibroblast growth*
688 *factor receptor 1; FGF2 = Fibroblast growth factor 2; LEP = Leptin; ADIPOQ =*

689 *Adiponectin; MSTN = Myostatin; TGFB1 = Transforming growth factor β 1. *P ≤*

690 0.05; ***P* ≤ 0.01: ****P* ≤ 0.001 (unadjusted *P*-values for multiple testing).

691