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Genome-wide analysis reveals that the *cytochrome P450 family 7 subfamily B member 1* gene is implicated in growth traits in Rasa Aragonesa ewes

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ABSTRACT

Sheep are very well adapted to changing environments and are able to produce and reproduce with low inputs in feed and water better than other domestic ruminants. Indeed, the ewe body condition score (**BCS**) and live weight (**LW**) play a significant role in productive and reproductive performance. This work conducts a genome-wide association study (**GWAS**) to detect genetic variants associated with growth traits in 225 adult ewes of the Rasa Aragonesa breed by using the genotypes from 50 k and HD Illumina Ovine BeadChip. These ewes were measured for LW, BCS and growth rate (**GR**) for 2 years, from January to September. Corrected phenotypes for BCS, LW and GR were estimated and used as input for the GWAS. Only one single nucleotide polymorphism (**SNP**) rs425509273 in chromosome 9 (**OAR9**), associated with the GR, overcame the genome-wise significance level. One, three and nine SNPs were associated at the chromosome-wise level (FDR 10%) for traits BCS, LW and GR, respectively. The *cytochrome P450 family 7 subfamily B member 1 (**CYP7B1**)* candidate gene, located 83 kb upstream from SNP rs425509273 in OAR9, was partially isolated and Sanger-sequenced. Fifteen polymorphisms comprising 12 SNPs, two indels and one polyC, were detected in promoter, exon 1, 3, 5, and intron 1–3 region. The SNP association analysis of the polymorphisms located close to the transcription start site (**TSS**) showed that a 22 bp insertion located at −58 nucleotides from the TSS (indel (−58)), a polyC (−25), and two A/G SNPs (SNP3 (−114) and SNP5 (−63)) were associated with the GR trait, whereas only the indel (−58) was associated with the BCS trait. The haplotype analysis confirmed these results. The functional characterisation of the polymorphisms at *CYP7B1* gene in liver by real-time quantitative PCR analysis confirmed that the mutations in the promoter region affected *CYP7B1* gene expression. Our results demonstrated the involvement of the *CYP7B1* gene promoter on GR and BCS traits in Rasa Aragonesa. These findings suggest that variations in ovine *CYP7B1* may serve as potential genetic markers to be used in breeding programmes to improve growth characteristics that could influence reproductive traits.
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Implications

Climate change had devasting effects on sheep productivity and reproductive efficiency through the exposure of animals to heat stress, water scarcity and lower grazing lands. Finding genetic markers associated with resilience and environmental stress resistance may help sheep breeding programs to achieve animals with better adaptive ability to manage their body reserves to cope with environmental stressors. Genome-wide association study revealed *cytochrome P450 family 7 subfamily B member 1* as a candidate gene related to growth rate in Rasa Aragonesa ewes. Our results demonstrated that *cytochrome P450 family 7 subfamily B member 1* promoter polymorphisms influence the gene expression of the gene.

Introduction

The effect of climate change is expected to directly and indirectly affect livestock physiology and health by increasing exposure to environmental stressors like heat (Rojas-Downing et al., 2017) and water stress (Chedid et al., 2014). Therefore, sheep farming systems should look at working with more robust animals that

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combine productivity and adaptive ability, and a more efficient use of natural resources. Body reserves in adult animals can be considered an important trait, and both, life weight (**LW**) and body condition score (**BCS**), are related traits to this. In this context, Macé et al., (2019) showed different profiles in BCS and LW dynamics in a flock suggesting the existence of proven intra-flock variability in ewes' adaptive capacity to manage their body reserves. Furthermore, ewes' BCS plays an important role in both fertility and growth rate (**GR**) of its progeny. Many studies have investigated the sheep BCS in relation to productive and reproductive performances (Kenyon et al., 2014, 2004; Macé et al., 2019) to define the suitable BCS profile throughout a ewe's breeding cycle. In Masham ewe breed, the number of lambs weaned per ewe exposed to the ram was positively correlated with the BCS upon breeding and/or at mid-pregnancy (Newton et al., 1980 as cited in Kenyon et al., 2014). Likewise, ewes' LW upon mating has been shown to influence subsequent litter size and ewes' productivity (Paganoni et al., 2022). In the Romane sheep breed reared under extensive conditions, a study confirmed that LW and BCS levels are influenced by the age at first lambing, parity or litter size (Macé et al., 2019), and breeding season is also influenced by the BCS and LW. The literature suggests an association between a poorer BCS and a shorter breeding season, which implies that the BCS can influence ewes' response to seasonal signals (Kenyon et al., 2014). Ewes with a low BCS are expected to display less reproductive performance compared to those with a higher BCS and would, thus, affect farmers' profitability (Young et al., 2010).

Studies underlying genetic mechanisms of LW and BCS are scarce. Kizilaslan et al., (2022) reported heritability estimates of growth traits ranged from 0.29 (pre-weaning average daily gain) to 0.52 (180 days weight). Recently, Tao et al., (2020) identified 39 genes associated with birth weight and body conformation traits. These genes were mainly involved in the cell cycle and body development.

Rasa Aragonesa is a meat local Mediterranean sheep breed from northeast Spain, reared in extensive or semi-extensive farming systems. This breed shows a marked reproductive seasonality where maximal breeding activity is associated with short days from August to March. Forcada et al., (1992) have reported that Rasa Aragonesa ewes with a higher BCS have a longer breeding season, which is predominately due to later seasonal anoestrous onset and a shorter whole anoestrous period (Rondon et al, 1996). Therefore, the objective of this study was to detect genetic variants associated with growth traits in ewes by using the genotypes from 50 k and HD Illumina Ovine BeadChip and investigated the association between polymorphisms in the *cytochrome P450 family 7 subfamily B member 1* (***CYP7B1***) gene and growth traits.

Material and methods

Animals and phenotypes used for genome-wide association study

The experiment was conducted on an experimental farm ("Pardina de Ayés") owned by Oviaragón S.C.L. (NE Spain, 42°29′N 0°23′W, 848 m.a.s.l.). The annual average rainfall is bimodally distributed with peaks in spring and autumn, dry summers and some snow in winter. Two hundred and twenty-five Rasa Aragonesa ewes were farmed according to the local traditional system for this breed. Briefly, ewes were kept indoors and fed a commercial concentrate from October to March. From March to the end of the experimental period (August), ewes were grazed on mountain pastures and received the same commercial concentrate *ad libitum*. All the ewes were handled in a single lot. The experimental period lasted from January to August in 2011 and 2012. Every 3 weeks, the individual LW and BCS on a scale from 1 to 5 (Russel et al., 1969) were measured. The pooled overall means and SDs for LW and the BCS were 45 ± 9.5 kg and 2.9 ± 0.3, respectively, in 2011, and 52.4 ± 8.4 kg and 3 ± 0.2 in 2012, with an age of 3.98 ± 1.85 years at the beginning of the experiment. Distribution of records by year and age of BCS and LW are shown in Table 1. Corrected phenotypes for growth traits were estimated as described later in sub-section "Corrected phenotype values for growth traits" for the 225 ewes.

Animals used for functional validation studies

Liver samples were obtained from a previous study (Ripoll et al., 2013) in which 48 Rasa Aragonesa breed lambs were fed *ad libitum* a commercial concentrate from weaning to slaughter, supplemented with 500 mg of dl-α-tocopheryl acetate kg^{-1} (n = 36; ranging from 4 to 28 days) and without this supplementation (n = 12). Ripoll et al. (2013) study was performed to evaluate the effects of finishing period length with vitamin E (dl-α-tocopheryl acetate) in the meat colour and lipid oxidation of light lambs. Briefly, the experiment began upon weaning (48.7 ± 0.21 days old) with an average LW of 18.5 ± 0.16 kg and finished at a slaughter age and weight of 75.20 ± 1.84 days old and 23 ± 0.24 kg, respectively, according to the Ternasco de Aragón Protected Geographical Indication specifications (Regulation (EC) No. 1107/96). Lambs were slaughtered in a commercial abattoir in accordance with EU laws. Immediately after slaughter, a piece of liver was cut, frozen in liquid nitrogen and stored at −80 °C until RNA isolation.

Genome-wide association study genotyping

Genomic DNA from blood samples was obtained using the SpeedTools DNA Extraction kit (Biotools, Madrid, Spain). The 225 ewes were genotyped using the OvineSNP50 Infinium Beadchip (n = 110; Illumina Inc., San Diego, CA, USA), and the 680 k IlluminaAgResearchSheep HD chip (n = 115). Genotyping services were conducted at the "Centro Nacional de Genotipado (CEGEN-ISCIII)" (https://www.usc.es/cegen/) and the "Xenetica Fontao" company (https://www.xeneticafontao.com/).

Validation of the genome-wide association study results

Structural characterisation of the CYP7B1 gene

The gene annotation based on the genome-wide association study (**GWAS**) results showed that the *CYP7B1* gene could be involved in growth traits. The *CYP7B1* gene is located in chromosome 9 (**OAR9**), covering approximately 173.7 kb with six exons (GenBank acc. Number NC_056062). The primers designed from sheep sequences NC_056062 and XM_004011703 were used to amplify total 5′UTR and coding region (exons 1–6), and also partial promoter and 3′UTR regions (Supplementary Table S1). The primers for PCR were designed with the Primer3 software (https://primer3.ut.ee/).

Genomic DNA (50 ng) from 13 ewes with different genotypes for single nucleotide polymorphism (**SNP**) rs425509273 (5 CC, 5 CT, and 3 TT) was amplified in a final PCR volume of 25 μl, which contained 5 pmol of each primer, 200 nM dNTPs, 2.25 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100 and 1 U Taq polymerase (Biotools, Madrid, Spain) for fragments 3–7. DNA AmpliTools Master Mix (Biotools, Madrid, Spain) was used for fragments 1 and 2. The PCR cycling conditions were set according to the protocol provided in the manufacturer's recommended procedures: an initial denaturation cycle at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s (57 °C for fragments 1 and 2), and 72 °C for 30 s, with a final elongation step at 72 °C for 5 min. PCR products were sequenced in both directions using Sanger technology in an ABI 3730XL sequencer (STAB VIDA. Caparica, Portugal).

Table 1
Distribution of records by year and age of ewe body condition score (BCS) and live weight (LW).

Year	Trait	Number of Records	Min	Max	Age Group		
					Mature	Young	Lamb
2011							
	BCS	1 491	2	3.50	2.83 ± 0.28	–	2.76 ± 0.23
	LW	1 491	24	63	47.57 ± 6.65	–	30.92 ± 2.98
2012							
	BCS	2 681	2	3.75	2.94 ± 0.34	2.69 ± 0.28	2.98 ± 0.26
	LW	2 681	27.50	72.50	57.84 ± 6.17	44.43 ± 3.87	39.01 ± 4.43

Previously, PCR clean-up was performed using the FavorPrep Gel/PCR purification mini kit (Favorgen, Ibian, Zaragoza, Spain). BLAST (https://www.ncbi.nlm.nih.gov/BLAST/) and CLUSTAL Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) tools were used for searching regions of similarity between sequences and multiple sequence alignment, respectively. The SNP position in the genome was determined based on the ovine Rambouillet v1.0 genome (GCA_002742125.1). The software Alibaba 2.1 (https://gene-regulation.com/pub/programs/alibaba2/) was used to predict transcription factor-binding sites within the surrounding sequence where the SNP was located (plus and minus 30 nucleotides).

Genotyping of the polymorphism in the CYP7B1 gene

The polymorphisms detected in the 5′ region of the gene were genotyped by Sanger sequencing in the 225 and 48 animals of the GWAS and the functional validation studies, respectively. For this purpose, a pair of primers (forward: 5′-GCTCATGTCTTCCGCT GTC-3′; reverse: 5′-AAATCTCAGCCCCTTCCCC-3′) was designed by amplifying a 360 bp fragment. The PCR conditions were the same as those for fragments 3–7 (Supplementary Table S1), but at the annealing temperature of 57 °C. PCR was purified and Sanger-sequenced as described above.

Functional characterisation of the polymorphism in the CYP7B1 gene by a real-time quantitative PCR analysis

Total RNA was extracted from approximately 500 mg of liver using the RNeasy Tissue mini kits (QIAGEN, Madrid, Spain) according to the recommended manufacturer's protocol. The RNA concentration was determined by a Qubit® fluorometer (Fisher, Madrid, Spain). Integrity of the RNA was assessed by visualisation of the 28S and 18S ribosomal RNA bands after agarose gel electrophoresis, being both bands clearly visible without observed degradation. To completely remove genomic DNA, 1 µg RNA was treated with deoxyribonuclease (Invitrogen, Carlsbad, CA, USA), and first-stranded cDNA was performed using the SuperScript III Reverse Transcriptase kit (Invitrogen) following the manufacturer's instructions. Gene expression levels were determined by real-time quantitative PCR analysis (**RT-qPCR**). To normalise the results of the *CYP7B1* gene, three candidate housekeeping genes (*GUSB, RPL37, RPL19*) were used. Gene expression stability was determined using NormFinder (Andersen et al., 2004). Specific exon-spanning primers for genes for RT-qPCR were designed with the Primer3 software (https://primer3.ut.ee/). RT-qPCR was performed in a 10 µl PCR total reaction mixture containing the SYBR Green Master Mix: SYBR Premix Ex Taq II (Tli RNase H Plus, Takara, Demlab, Zaragoza, Spain) on an ABI Prism 7500 platform (Applied Biosystem, Madrid, Spain). All qPCR reactions were run in triplicate following the manufacturer's instructions. Post-amplification melting-curve analyses were carried out for each gene to ensure primer specificity and to confirm the presence of a unique PCR product. The PCR amplification efficiency were assessed by generating standard curves for each target gene using a 10-fold serial dilution of pooled cDNA that were included on each plate. The correlation coefficient and slope were calculated. The formula: amplification efficiency = $10^{(-1/\text{slope})}$ was used. The annealing temperature, primer concentration, primer sequences, efficiencies and correlation coefficient for these genes are described in Supplementary Table S2.

Statistical analysis

Corrected phenotype values for growth traits

The phenotypes used in the GWAS were obtained from the BCS and LW data collected over the 2-year experiment. In all, 4 172 BCS and LW data belonging to the 225 ewes were recorded. The corrected phenotypes were estimated by running a mixed linear regression analysis. To estimate the BCS-corrected phenotype, LW and ewes age were taken as covariates. For the LW-corrected phenotype, the BCS and age were considered to be covariates. The fitted model included the collection date of growth traits as a fixed effect (21 levels) and ewes' permanent environmental effect as random (225 levels). The individual BCS and LW estimates, adjusted by the factors included in the model, were obtained and used as inputs in the GWAS. BLUPF90 (Misztal et al., 2018) was used to run the mixed linear regression analyses.

The third phenotype was estimated by a linear regression analysis with the lm command in the R v3.5.1 software by taking ewes' LW as the dependent variable and their age as the independent variable. The obtained individual slope estimates were the GR-estimated phenotype (kg/day) utilised for the GWAS.

Quality control and genome-wide association study analysis

The quality control criteria applied to the raw genotypes using PLINK 1.9 software (Chang et al., 2015) were based on excluding the individuals with a low genotype call rate (<0.90), the SNPs with a genotype call rate <0.97, SNPs with a minor allele frequency (**MAF**) < 0.01, and the SNPs with a failure of Hardy-Weinberg equilibrium (*P*-value < 0.001). The datasets from the two genotyping platforms were merged with PLINK 1.9 (Chang et al., 2015). Imputation of 50 k to HD genotypes was conducted with Beagle v5.4 software (Browning et al., 2018). Genotype imputation approach use genotype data in a panel of reference samples to infer ungenotyped markers in target samples (Marchini and Howie, 2010). The Genome-wide Complex Trait Analysis software (Yang et al., 2011) was run for the GWAS analysis using the mixed linear model association approach. The genomic relationship matrix was constructed and included in the mixed model analysis to account for population structure. Bonferroni (*P* < 0.05) and the false discovery rate (FDR < 10%) multitest correction tests were applied to adjust the threshold of the genome and chromosome-wide significant values. The genomic inflation factor was calculated in the R v3.5.1 software for each trait as the observed median χ^2 over the expected median χ^2. The Rambouillet v1.0 genome assembly based on Ensembl release 101 was employed to identify any candidate genes within a window of 500 kb centred on the significant SNP.

CYP7B1 gene association analysis

Statistical analyses were carried out as a regression of the corrected phenotypes for the three growth traits on the polymorphism genotypes by fitting a linear model with the Rcmdr package of the R software (Fox et al., 2020). The model included the genotype of SNPs and ewes' clusters to which ewes belonged as a fixed effect. These clusters were obtained with PLINK 1.9 (Chang et al., 2015) using genotypes utilised for the GWAS analysis. Briefly, the SNPs that passed the quality control were subsequently pruned for linkage disequilibrium (**LD**) in PLINK, using a 50 SNP window size, a 10 SNP window shift, and $r^2 < 0.2$. The SNPs that remained after pruning were used to calculate the genome-wide identity-by state pairwise distances. Clustering analysis was performed by applying the pairwise population concordance test constraint (−ppc option). Multidimensional scaling analyses were performed on identity-by state pairwise distances to examine for population structure. Differences between genotypes were estimated by the least square means for each genotype pairwise comparison. Bonferroni correction was applied, and all the polymorphisms were independently analysed with the same statistical model.

A haplotype association analysis was performed between haplotypes and the corrected phenotypes for the growth traits using the Rcmdr package by fitting a similar linear model to that employed for the polymorphism association studies but including the number of copies of each haplotype instead of the genotype of SNP (this was recoded as 0, 1 or 2 copies). The same statistical model was applied for analysing independently each haplotype. The LD blocks were evaluated with the Haploview software v4.2 using the 4-gamete rule (Barrett et al., 2005). Polymorphisms were phased with PLINK1.9 (Chang et al., 2015) using the expectation–maximisation algorithm to assign individual haplotypes, considering only those haplotypes with a frequency ≥1%. The diplotypes with a posterior probability below 0.7 were discarded. Least square means were applied to estimate the differences between the number of copies of each haplotype. To adjust for multiple testing, Bonferroni correction was applied.

Analysis of expression results

Differences in the ovine *CYP7B1* gene expression rates of the alternative genotypes/haplotypes located at the gene promoter were estimated by the approach described by Steibel et al., (2009). The Haploview software v4.2 was used to construct haplotype blocks, and the LD analysis was done as previously mentioned. The quantification cycle values were rescaled with the equation proposed by Steibel et al., (2009) because amplification efficiencies of all the genes differ from 2. A mixed model using the SAS statistical package v. 9.3 (SAS Institute, Cary NC, USA) was employed:

$$y_{gomr} = \mu + MG_{og} + b_1(SA)_m + b_2(SW)_m + b_3(VE)_m + A_m + e_{gomr}$$

where y_{gomr} is the quantification cycle for the gth gene (*CYP7B1* and the three housekeeping genes) from the rth well, corresponding to the mth animal; MG_{og} is the fixed interaction between the oth genotype (AA, AB and BB) or the haplotype (0, 1 and 2 copies) and the gth gene (M is the effect of the oth genotype or haplotype of the *CYP7B1* gene, and G is the effect of the gth gene). The SA (slaughter age), SW (slaughter weight) and VE (number of days of concentrate enriched with dl-α-tocopheryl acetate intake) effects were included as a covariate; A_m ($A_m \sim N(0, \sigma_a^2)$) and e_{gomr} were the random effects of the animal and residual, respectively. Gene-specific residual variance (heterogeneous residual) was also included in the model ($e_{gomr} \sim N(0, \sigma_{eg}^2)$).

The *CYP7B1* expression was normalise using the three housekeeping genes as suggested by Steibel et al., (2009). The expression rate differences ($diff_{CYP7B1}$) between genotypes/haplotypes and the fold change values were estimated from MG fixed interaction differences. The *t* statistic was determined to calculate the significance of the $diff_{CYP7B1}$ estimates. The *P*-values were adjusted for multiple testing according to Bonferroni correction. The SE for each fold change value of $diff_{CYP7B1}$ was used to calculate asymmetric 95% confidence intervals (upper and lower).

Results

Genome-wide association study results

The 225 ewes genotyped for 582 880 SNPs passed the quality control criteria and were included in the GWAS analysis. For the multidimensional scaling analysis, 184 076 autosomal SNPs were used to calculate the pairwise identity-by state distance after SNP pruning. The multidimensional scaling analysis revealed a substructure in the total dataset and identified four principal clusters in the population (Supplementary Fig. S1). The genomic relationship matrix was included in the mixed model analysis to correct the putative effect of the population substructure. This was checked by calculating the genomic inflation factors for each trait, which were ≤1 (LW: 0.95; BCS: 0.96; GR: 1). The GWAS results obtained by the mixed linear model association analysis for the significant SNPs at the chromosome and genome-wise levels for the studied growth traits are reported in Table 2. Only one SNP (rs425509273), located in OAR9 and associated with GR, overcame the genome-wise significance level after Bonferroni correction. At the chromosome-wise level of significance (FDR 10%), 1, 3 and 9 SNPs were identified for the BCS, LW and the GR respectively. The nine SNPs associated with GR variability were located in eight chromosomes. In most cases, the less frequent allele (**A1**) of the associated SNP showed a negative effect on the trait with allele substitution values (b) ranging between −0.008 and −0.04, and frequencies from 0.01 to 0.13. In general, the A1 alleles showed a negative effect on the LW trait, with b values from −2.01 to −2.85 and frequencies ranging between 0.13 and 0.38. For the BCS, the associated SNP (rs424629620) was located in chromosome 6 and had an effect of 0.09 and an MAF of 0.38. Several annotated genes were found near the significant SNPs for GR and LW, whereas only one gene was found for BCS (Table 2).

Validation studies

Structural characterisation of the CYP7B1 gene

To validate the GWAS results, the SNP that overcame the genome-wise significance level was chosen. This SNP was 83 kb downstream from the *CYP7B1* gene. To search for the polymorphisms that could be involved in the studied phenotypes, the total 5′UTR and coding region (exons 1–6), partial promotor and 3′UTR regions of the *CYP7B1* gene were sequenced. Fifteen polymorphisms were found: 12 SNPs, two indels and one polyC (Table 3). None of these polymorphisms produced amino acid changes. Eight of the total polymorphisms, not previously described, were located in the promoter region. It is noteworthy that one of the polymorphisms was an insertion of 22 bp at position −58 in relation to the transcription start site (**TSS**) (Table 3). This insertion was a repeat of the 22 nucleotides located between OAR9: g.45801535 and OAR9: g.45801556 according to the ovine genomic map Oar_rambouillet_v1.0. *In-silico* analysis of the transcription factor-binding motifs identified several overlapping motifs containing polymorphisms indel (−621), SNP3 (−114), SNP4 (−97), SNP5 (−63), indel (−58) and polyC (−25). For SNP3 G > A (−114), the CACCC-bi and RAP1 binding consensus sites were pre-

Table 2
Significant Single Nucleotide Polymorphisms (SNPs) at the chromosome-wise level associated with each growth traits in Rasa Aragonesa breed. Minor allele frequency (MAF) is also indicated. Putative causal genes located in the 250 kb region on both sides of the significant SNPs are indicated. Position of markers are based on Oar_rambouillet_v1.0 genome assembly in bp.

Traits[1]	Chr[2]	SNP	dbSNP[3]	Position	MAF	b[4]	*P*-value	p-Bonf[5]	Fdr_thr[6]	Genes within 250 kb on either Side
GR	7	oar3_OAR7_82708460	rs405607259	89 550 547	0.01	−0.040 ± 0.008	5.90E−07	0.34	4.40E−06	*LIN52-VSX2-ABCD4-VRTN-SYNDIG1L-ISCA2-NPC2-LTBP2-AREL1-FCF1-YLPM1*
	9	oar3_OAR9_42035112	rs425509273	45 546 608	0.06	−0.014 ± 0.003	4.63E−08	0.03	9.50E−06	*BHLHE22-**CYP7B1***
	9	oar3_OAR9_88434565	rs427506082	97 921 953	0.03	−0.017 ± 0.004	1.60E−06	0.93	9.50E−06	*CNBD1-CNGB3*
	15	oar3_OAR15_47073330	rs430194623	51 439 713	0.01	−0.037 ± 0.008	7.43E−06	1	7.50E−06	*ZNF667-TRIM5 like (LOC105614607)-TRIM5 Like (LOC114118421)-OR51I2-TRIM34-TRIM6-LOC101102669-OR52B6-OR52B4 like-OR52H1 (LOC101119670)-OR52H1 (LOC105602420)-UBQLNL-UBQLN3-LOC101120692-OR52D1-LOC101120949-OR51I2 like-LOC101121967-LOC101103421-LOC101122215-LOC101122462*
	20	oar3_OAR20_22070146	rs417043327	23 839 961	0.06	−0.012 ± 0.003	2.07E−06	1	8.40E−06	*MEP1A like-MMUT-CENPQ-GLYATL3- C20H6orf141-RHAG-CRISP2-CRISP3-PGK2*
	23	oar3_OAR23_51434165	rs427416766	56 897 122	0.06	−0.014 ± 0.003	2.23E−06	1	7.20E−06	-
	25	oar3_OAR25_6873226	rs425329469	6 487 542	0.05	−0.013 ± 0.003	1.13E−05	1	1.20E−05	*SLC35F3*
	26	oar3_OAR26_3943857	rs398535453	4 490 102	0.13	−0.008 ± 0.002	8.35E−06	1	1.00E−05	*CSMD1*
	27	oar3_OARX_6627610	rs422252289	7 456 631	0.08	−0.011 ± 0.002	1.64E−06	0.96	3.80E−06	*MAGEB2 like-MAGEB1 like*
BCS	6	oar3_OAR6_99760786	rs424629620	110 458 659	0.38	0.092 ± 0.02	2.29E−06	1	3.90E−06	*ARHGAP24*
LW	12	oar3_OAR12_22059564	rs430155342	25 998 332	0.13	−2.850 ± 0.608	2.77E−06	1	5.50E−06	*RAB3GAP2-MARK1-C12H1orf115*
	13	oar3_OAR13_33247804	rs422199592	35 228 082	0.14	−2.671 ± 0.592	6.35E−06	1	6.40E−06	*ZEB1-ZNF438*
	24	oar3_OAR24_28384805	rs408865776	29 194 526	0.38	−2.013 ± 0.448	6.87E−06	1	1.00E−05	*RABGEF1-TMEM248-LOC101111335- SBDS-CALN1*

[1] GR: growth rate; BCS: Body condition score; LW: Live weight.
[2] Chr: chromosome.
[3] dbSNP: Single Nucleotide Polymorphism database.
[4] b: effect of the allele substitution values.
[5] p_Bonf: *P*-value after Bonferroni correction.
[6] Fdr_thr: False Discovery rate threshold = 10%.

Table 3
The *CYP7B1* gene polymorphisms. The position in the ovine genomic map Oar_rambouillet_v1.0., its identifier in the Single Nucleotide Polymorphism variant database (dbSNPs), and the polymorphism are indicated. The distance in bp from the transcription start site (TSS) to the polymorphism in the SNPs located in the promoter is indicated in parentheses.

Name	dbSNP	Region	Position	Polymorphism[1]
Indel (−621)	–	promoter	g.45802097	insC
SNP1 (−558)	–	promoter	g.45802034	A/G
SNP2 (−199)	–	promoter	g.45801675	A/G
SNP3 (−114)	–	promoter	g.45801590	G/A
SNP4 (−97)	–	promoter	g.45801573	C/A
SNP5 (−63)	–	promoter	g.45801539	A/G
indel (−58)	–	promoter	g.45801534	insACCCACACGCACCGCCCCGCTC
PolyC (−25)	–	promoter	g.45801510	polyC
SNP6	–	exon 1	g.45801489	C/A
SNP7	rs417515909	intron 1	g.45660532	C/T
SNP8	rs404047939	intron 2	g.45660360	A/T
SNP9	rs421906566	intron 3	g.45652963	G/T
SNP10	rs408370224	intron 3	g.45652933	T/C
SNP11	–	exon 3	g.45652588	T/C
SNP12	rs160653444	exon 5	g.45642126	T/C

[1] ins = insertion.

dicted with the A allele, while these motifs were replaced with an AP-2alpha motif for the G allele. For SNP5 A > G (−63), the substitution of the G by the A allele resulted in the loss of an SP1 and the Adf-1 binding consensus site. However, the strongest effect was found for the 22 bp insertion (indel (−58)), which led to several TF-binding motifs appearing: SP1, REB1 and AP-2alph. PolyC and the other polymorphisms were not predicted to produce any major changes in the binding consensus sites.

CYP7B1 single nucleotide polymorphism association studies

Polymorphisms SNP3 G > A (−114), SNP4 C > A (−97), SNP5 A > G (−63), indel (−58), and polyC (−25) located at the promoter

Table 4
CYP7B1 gene polymorphisms effects on growth traits in the Rasa Aragonesa breed. Different letters indicate significant differences after Bonferroni correction: a,b: <0.10; c,d: <0.05; e,f:< 0.01; g,h:<0.001.

Polymorphism[1]	Trait[2]	P-value Polymorphism	Lsmeans Genotype[3]					
			A/A	A/G	G/G			
SNP3 (−114)	GR	2.00E−03	0.004 ± 0.004e	0.012 ± 0.002	0.016 ± 0.001f			
	BCS	1.60E−01	0.044 ± 0.048	0.050 ± 0.027	−0.004 ± 0.014			
	LW	9.16E−01	0.115 ± 1.27	−0.412 ± 0.71	−0.137 ± 0.36			
			A/A	A/C	C/C			
SNP4 (−97)	GR	3.54E−01	0.008 ± 0.005	0.014 ± 0.004	0.015 ± 0.001			
	BCS	9.16E−01	0.031 ± 0.07	−0.005 ± 0.05	0.010 ± 0.01			
	LW	6.12E−01	−0.692 ± 1.80	1.017 ± 1.28	−0.236 ± 0.32			
			A/A	A/G	G/G			
SNP5 (−63)	GR	1.89E−04	0.009 ± 0.002e,g	0.016 ± 0.001f	0.018 ± 0.001 h			
	BCS	2.70E−01	−0.017 ± 0.023	0.009 ± 0.017	0.036 ± 0.022			
	LW	9.74E−01	−0.053 ± 0.61	−0.228 ± 0.45	−0.193 ± 0.59			
			N/N	Ins22/N	Ins22/Ins22			
Indel (−58)	GR	5.17E−03	0.017 ± 0.015e	0.015 ± 0.012a	0.010 ± 0.006f,b			
	BCS	4.25E−02	0.043 ± 0.019c	−0.001 ± 0.019	−0.036 ± 0.026d			
	LW	5.76E−01	0.120 ± 0.67	−0.594 ± 0.51	0.057 ± 0.49			
			C7/C7	C7/C8	C7/C9	C8/C8	C8/C9	C9/C9
PolyC (−25)	GR	2.24E−04	0.008 ± 0.019e,g	0.016 ± 0.001f	−0.008 ± 0.012	0.018 ± 0.001 h	0.011 ± 0.006	0.008 ± 0.007
	BCS	1.26E−01	−0.032 ± 0.026	0.002 ± 0.020	−0.271 ± 0.017	0.041 ± 0.019	−0.001 ± 0.084	0.029 ± 0.10
	LW	5.11E−01	0.451 ± 0.67	−0.897 ± 0.52	−0.328 ± 4.37	0.087 ± 0.49	1.737 ± 2.19	−1.865 ± 2.52

[1] SNP: Single Nucleotide Polymorphism; Ins22 indicates the allele with the insertion of 22 bp, otherwise N as normal allele; C7, C8 and C9 indicate alleles with 7, 8, and 9 cytosine repetitions, respectively.
[2] GR: growth rate; BCS: Body condition score; LW: Live weight.
[3] Lsmeans = Least square means.

region were genotyped in the GWAS population. Table 4 provides the association results for each polymorphism after Bonferroni correction. SNP3 (−114), SNP5 (−63), the indel (−58), and polyC (−25) were associated with the GR trait. Lower GR was observed for the indel (−58) in the homozygous animals for the 22 bp insertion (0.010 ± 0.006) with respect to the homozygous animals for the alternative allele (0.017 ± 0.015). Likewise, the GR of the ewes carrying the GG genotype for SNP5 (−63) (0.018 ± 0.001) and SNP3 (−114) (0.016 ± 0.001) was higher than in the AA animals for the two SNPs (0.009 ± 0.002 and 0.004 ± 0.004 for SNP5 (−63) and SNP3 (−114), respectively). For SNP5 (−63), the heterozygous ewes (0.016 ± 0.001) obtained a higher GR than the AA ewes. Finally, a lower GR was observed in the homozygous animals with seven repetitions (0.008 ± 0.019) compared to the homozygous ones with eight repetitions (0.018 ± 0.001) or to the heterozygous (0.016 ± 0.001) ewes for these two alleles for polyC (−25). The indel (−58) was also associated with the BCS trait insofar as the homozygous animals for the 22 bp insertion had an estimated lower BCS value (−0.036 ± 0.026) than the homozygous animals for the alternative allele (0.043 ± 0.019).

CYP7B1 haplotype association studies

Two haplotypic blocks in the promoter region of the *CYP7B1* gene were detected with the Haploview software (Supplementary Fig. S2). The first one comprised polyC (−25), the indel (−58) and SNP5 (−63), while the second one included SNP3 (−114) and SNP4 (−97). Considering that one block included all the SNPs, 22 haplotypes were defined (Supplementary Table S3).

The haplotype association analysis confirmed the SNP association results. Only haplotypes H2 (G-C-A-Ins22-C7 for SNP3 (−114) – SNP4 (−97) – SNP5 (−63) – indel (−58) – polyC (−25) polymorphisms) and H12 (G-C-G-N-C8) presented a frequency above 5%. Ins22 indicated the allele with the insertion of 22 bp, and otherwise N as a normal allele. C7 and C8 indicated alleles with seven and eight cytosine repetitions, respectively. These haplotypes were significantly associated with the GR trait in such a way that having two copies of the H2 haplotype led to a lower GR than for the animals with one or no copies ($P < 0.05$) (Table 5). Similarly, this haplotype was also significant for the BCS, with significant differences between having two and no copies. The H2 haplotype had the A, Ins22, and C7 alleles for the SNP5 (−63), indel (−58) and polyC (−25) polymorphisms, which were significantly associated with a lower GR in the SNP association studies. The H12 haplotype, which was associated with a higher GR, had alleles G, G, N and C8 for the SNP3 (−114), SNP5 (−63), indel (−58) and polyC (−25) polymorphisms, which were also significantly associated with an increased GR. In this case, having the H12/H12 diplotype implied a higher GR than the diplotypes with one or no copies. These results confirmed the involvement of the *CYP7B1* promoter in both the GR and BCS in Rasa Aragonesa.

Functional characterisation of the polymorphism in CYP7B1

The genotype frequencies for the SNPs located in the 5′ region of gene *CYP7B1* in the population used for the expression studies are shown in Supplementary Table S4. Housekeeping genes *GUSB*, *RPL37* and *RPL19* were employed to normalise gene expression because they were all stabler than the *CYP7B1* gene (Supplementary Fig. S3). The *CYP7B1* expression was affected by the number of days of concentrate enriched with dl-α-tocopheryl acetate intake ($P < 0.05$), SNP5 (−63) ($P < 0.0001$) and polyC (−25) ($P < 0.0001$). The insertion of 22 nucleotides (indel (−58)) was not significant. SNP5 (−63) and polyC (−25) were completely linked ($r^2 = 1$). The *CYP7B1* expression in the AA lambs for SNP5 (−63) (n = 13) (homozygous animals for the C7 allele at the polyC (−25) polymorphism) was 2.9-fold higher than that in the AG lambs (n = 17; $P < 0.0001$) (heterozygous for seven and eight cytosine repetitions at the polyC (−25) polymorphism). Furthermore, *CYP7B1* expression in the GG lambs for SNP5 (n = 17) (homozygous animals for eight cytosines at the polyC (−25) polymorphism) was 3.21-fold higher than that in the AG lambs (n = 17; $P < 0.0001$) (heterozygous for seven and eight cytosine repetitions at the polyC (−25) polymorphism) (Table 6). Whether the *CYP7B1* expression was affected by the haplotypes defined with PLINK 1.9 was also tested (Chang et al., 2015). Haplotypes were built using the five polymorphisms genotyped in the 5′ region of the gene. In Supplementary Table S5, both haplotypes and their frequency in the population for the functional studies are shown. The *CYP7B1* expression was also affected by the number of days of concentrate

Table 5
Haplotype effects for the single nucleotide polymorphism (SNP) located at the promoter region of the *CYP7B1* gene (SNP3 (−114)-SNP4 (−97)- SNP5 (−63)- ins22 (−58)- polyC (−25)) on growth traits in the Rasa Aragonesa breed. The least square means (Lsmeans) indicate 0 copies of the haplotype (0), 1 copy of the haplotype (1), or 2 copies of the haplotype (2). Different letters indicate significant differences after Bonferroni correction: c,d: <0.05; g,h:<0.001.

Haplotype[1]	Trait[2]	*P*-value	Lsmeans		
			0	1	2
H2	GR	1.26E−02	0.016 ± 0.002c	0.016 ± 0.002c	0.008 ± 0.001d
	BCS	1.39E−02	0.035 ± 0.02c	−0.004 ± 0.02	−0.068 ± 0.03d
	LW	3.95E−01	−0.115 ± 0.413	−0.663 ± 0.556	0.694 ± 0.85
H12	GR	8.84E−05	0.009 ± 0.002 g	0.016 ± 0.001 h	0.019 ± 0.002 h
	BCS	1.22E−01	−0.014 ± 0.02	0.006 ± 0.02	0.053 ± 0.03
	LW	8.12E−01	0.0498 ± 0.551	−0.175 ± 0.451	−0.509 ± 0.668

[1] H2: G-C-A-Ins22-C7; H12: G-C-G-N-C8.
[2] GR: growth rate; BCS: Body condition score; LW: Live weight.

Table 6
Differences in the *CYP7B1* expression rate between the different genotypes in lambs for the single nucleotide polymorphism (SNP5 (−63). Estimates, SE, *P*-values, fold change (FC) and the 95% FC confidence interval (FCup-FClow) for pairwise genotype contrasts are included.

Genotype contrast	Estimate	SE	*P*-value	FC	FCup	FClow
AA vs AG	−1.5351	0.3815	<0.0001	2.90	4.87	1.73
GG vs AA	−0.1458	0.397	0.7136	1.11	1.90	0.65
GG vs AG	−1.6808	0.3391	<0.0001	3.21	5.08	2.02

Table 7
Differences in the *CYP7B1* expression rate in lambs between the different numbers of haplotype 3 (H3) copies. Haplotype 3: G-C-A-Ins22-C7 alleles for single nucleotide polymorphism (SNP)3 (−114) – SNP4 (−97) – SNP5 (−63) – indel (−58) – polyC (−25) polymorphisms. Estimates, SE, *P*-values, fold change (FC) and the 95% FC confidence interval (FCup-FClow) between 0 copies of the haplotype (0), 1 copy of the haplotype (1), or 2 copies of the haplotype (2) contrasts are included.

Haplotype contrast	Estimate	SE	*P*-value	FC	FCup	FClow
0 vs 1	−0.8129	0.3157	0.0103	1.76	2.70	1.14
2 vs 0	−1.7835	0.5979	0.003	3.44	7.76	1.53
2 vs 1	−2.5964	0.6152	<0.0001	6.05	13.95	2.62

enriched with dl-α-tocopheryl acetate intake (P < 0.05) and haplotype H3 (G-C-**A**-Ins22-C7 for the SNP3 (−114) – SNP4 (−97) – SNP5 (−63) – indel (−58) – polyC (−25) polymorphisms) (P < 0.0001). The animals with two copies (n = 3) had a gene expression that was 6.05- and 3.44-fold higher than the animals with one (n = 16) or no (n = 28) copies, respectively (Table 7). Finally, the lambs with one copy had a gene expression that was 1.76-fold lower than the animals with no copies.

Discussion

In this study, a GWAS performed for BCS, LW and GR traits in the Rasa Aragonesa sheep breed showed a single SNP rs425509273 associated with GR that overcame the genome-wise significance level. This SNP is located approximately 83 kb downstream from the *CYP7B1* gene in OAR9. This gene has been reported to be involved in the metabolism of endogenous oxysterols, which are key mediators of cholesterol and lipid homeostasis (Guillemot-Legris et al., 2016). Several studies have reported that *CYP7B1* inhibition leads to fatty liver (Dai et al., 2014) because of its role in the conversing cholesterol into bile acid pathways. Some studies have reported that *CYP7B1* expression is related to different physiological functions depending on the species and tissue where it is expressed (liver, reproductive tract and brain) (Stiles et al., 2009). In fact, loss of function mutations in the *CYP7B1* human gene causes spastic paraplegia 5A, a progressive neuropathy that is due to defects in cholesterol and neurosteroid metabolism (Stiles et al., 2009). In the reproductive tract, the CYP7B1 enzyme metabolises the androgens that antagonise oestrogen action so that mice without *CYP7B1* have abnormal prostates and ovaries (Stiles et al., 2009). We also identified at a chromosome-wise level of significance (FDR 10%) one, three and nine SNPs for BCS, LW and GR, respectively. For GR trait, SNP rs405607259 located in chromosome 7 was 21.6 kb from the *NPC2* gene, which is involved in the intracellular trafficking of cholesterol and other lipids (Kim et al., 2010). The *VRTN* and *SYNDIG1L* genes were near this SNP. *SYNDIG1L* has been reported in Landrace pigs to be a factor that affects final BW and back-fat thickness (Lee et al., 2018), whereas *VRTN* variants have been associated with the number of thoracic vertebrae in sheep (Cunyuan et al., 2019). The *ARHGAP24* gene was the only candidate gene found for the BCS at 210 kb from the significant SNP, which has been reported to be associated with pigs' growth performance (Meng et al., 2017). SNP rs422199592 in chromosome 13, related to LW, was 191.8 kb from *ZEB1* gene, which is associated with obesity in human and adipogenesis in mice (Gubelmann et al., 2014). In a study conducted by Tao et al., (2020), the GWAS of BW identified only the *AP-1 transcription factor subunit* (*FOSL2*) as a candidate gene. In the same way, Kizilaslan et al., (2022) identified several genes such as *PRDM2, PTGDR, PTPRG, KCND2, ZNF260, CPE, GRID2, SCD5, SPIDR, ZNF407, HCN3, TMEM50A, FKBP1A, TLE4, SP1, SLC44A1*, and *MYOM3* located on different chromosomes as putative quantitative trait loci (**QTL**) related to growth and linear type traits in Akkaraman sheep. It is noteworthy that most of the significant SNPs were found at very low frequencies, which could indicate a spurious association.

Because of the putative functional effect of the *CYP7B1* gene on body reserves, we decided to isolate the gene to look for the polymorphisms that could be involved in growth traits. Therefore, all the exons of the *CYP7B1* gene were isolated, as well as 996 bp of the promoter region. None of the 15 polymorphisms detected produced amino acid changes. Six polymorphisms were located in the promoter region, including a 22 bp insertion (indel (−58)) in the promoter at −58 bp from the TSS, as well as modifying transcrip-

tion factor-binding motifs. No TATA box-like sequences were found in the human *CYP7B1* gene promoter, whose transcription is activated through SP1-type elements (Wu et al., 1999). According to the literature, there is a CpG island of 1200 bp that includes exon 1 and 600 bp of the promoter region, and the region between bases −291 and +189 from the TSS is critical for its transcription. Accordingly, SNP5 (−63) was predicted to modify an SP1 consensus site and also the indel (−58). In this study, five *CYP7B1* polymorphisms were selected for validation due to their location near the TSS of the gene that could alter the transcription factor-binding sites and consequently the gene expression rate. The SNP association studies demonstrated that SNP3 (−114), SNP5 (−63), the indel (−58) and polyC (−25) were associated with GR, and the indel (−58) was also associated with BCS. These results were confirmed by the haplotype analysis and showed that animals carrying two copies of H2 (G-C-A-Ins22-C7 for SNP3 (−114)- SNP4 (−97)- SNP5 (−63)- indel (−58)- polyC (−25)) had lower GR compared to animals with one or no copies ($P < 0.05$). One outstanding finding was that the Ins22 and A alleles for the indel (−58) and SNP5 (−63) polymorphisms were, respectively, the alleles associated with a lower GR, and were predicted to modify the SP1 consensus sites. On the contrary, the H12/H12 diplotype implied a higher GR than diplotypes with one or no copies. H12 (G-C-G-N-C8) had the alleles of polymorphisms SNP3 (−114), SNP5 (−63), indel (−58) and polyC (−25), associated with an increased GR. According to the "Animal QTL database" (https://www.animalgenome.org/cgi-bin/QTLdb/index), no QTLs/eQTLs related to growth traits have been described in an interval of 10 Mb, being the *CYP7B1* gene located in the middle of this interval.

Because of the putative modification of the TF consensus sites, we further explored the *CYP7B1* expression by RT-qPCR. The statistical analysis considered the number of days of concentrate enriched with dl-α-tocopheryl acetate intake as a fixed effect because our previous work had demonstrated that vitamin E supplementation causes different responses in gene expression (González-Calvo et al., 2017). The present research work demonstrated that *CYP7B1* expression was affected by SNP5 (−63). The A allele caused a modification of SP1 and Adf-1 binding consensus sites, and could, thus, affect the gene expression rate. No effect was found for the indel (−58), but genotypes were unbalanced in the population studied with 28, 15 and 4 ewes being homozygous without the insertion, heterozygous and homozygous with the insertion, respectively. The haplotype analyses confirmed these results and showed that the *CYP7B1* expression appeared to vastly differ between animals with two copies of the H3 haplotype (n = 3; G-C-A-Ins22-C7) and the animals with one (n = 16) or no copies (n = 28). However, there were only three homozygous ewes for this haplotype. Furthermore, the *CYP7B1* expression was 1.76-fold higher in the ewes with no copies than in the animals with one copy. In this research, the animal model for validation differed from that of the association analysis, mainly because of the animals' age and sex. The gene expression studies used male lambs slaughtered at 75.20 ± 1.84 days, while the association studies included adult ewes managed according to the local traditional system. Despite these possible limitations, the results still demonstrated the involvement of the *CYP7B1* gene promoter in the variability of GR and BCS traits in the Rasa Aragonesa ewes. This is the first study demonstrating the association between some polymorphisms in the *CYP7B1* gene promoter and growth traits in sheep.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2023.100975.

Ethics approval

All the experimental procedures, including the care of animals and euthanasia, were performed in accordance with the guidelines of the European Union and with Spanish regulations for the use and care of animals in research, and were approved by the Animal Welfare Committee of the Centro de Investigación y Tecnología Agroalimentaria (CITA). Protocol number codes 2011–08 and 2009-01_MJT are for the animals used in the GWAS and the functional studies, respectively.

Data and model availability statement

None of the data were deposited in an official repository. The data/models that support the study findings are available from the authors upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

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Declaration of interest

None.

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