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SINGLE LOCI AND HAPLOTYPES IN *CAPN1* AND *CAST* GENES ARE ASSOCIATED WITH GROWTH, BIOMETRICS, AND *IN VIVO* CARCASS TRAITS IN SANTA INÊS SHEEP

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Abstract

µ-calpain (*CAPN1*) and calpastatin (*CAST*) genes play key roles in protein turnover. The present study aimed to identify the variants in these genes associated with growth and ultrasound carcass traits in Santa Inês sheep. A sample of 192 no full sibling Santa Inês lambs was used. Fragments of the *CAST* and *CAPN1* genes were amplified and next-generation sequencing was performed in the MiSeq platform. Variants in the *CAPN1* and *CAST* sequences were then detected using bioinformatic tools. Withers and croup heights, body length, thoracic and croup widths, thoracic and leg girths, body depth, carcass fat score, rib eye area, fat thickness, body weights were recorded at weaning and at 140 days post-weaning, and average daily gain post-weaning was calculated. Both single-locus and haplotype association analyses were performed with the model as follows: farm (2 levels), year (4 levels), the month of birth (12 levels), and the covariate age of the animal. The fragments amplified included 4,514 bp between the 20th and 23rd exons of *CAST* as well as 3,927 bp between the 12th and 21st exons of *CAPN1*. In these regions, 58 (*CAST*) and 45 (*CAPN1*) variants were identified. In the *CAST* gene, the single-locus analysis revealed 22 suggestive additive effects (P<0.05) on several growth and carcass traits. Moreover, haplotype substitutions were associated with rib eye area (–0.689±0.290), average daily gain (–23.6±10.4), thoracic girth (–2.72±1.27), body length (–3.38±1.49), and leg girth (–2.84±1.37). Regarding the *CAPN1* gene, the single-locus analysis identified seven suggestive additive effects, while only one haplotype replacement effect on fat thickness (–0.0143±0.0053) was detected. The results of the present study suggest that variants in the *CAPN1* and *CAST* genes are associated with growth and ultrasound carcass traits in Santa Inês sheep, which may be a source of information to improve knowledge regarding the genetic control of these traits.

Key words: body weight, fat, lambs, muscle, selection

μ-calpain is an enzyme with proteolytic activity on muscle fibers, whereas calpastatin inhibits μ-calpain activity (Calvo et al., 2014). These enzymes have several key roles, such as cytoskeletal restructuring, cell cycle regulation, and apoptosis (Goll et al., 2003; Lebart and Benyamin, 2006). In addition, μ-calpain and calpastatin affect protein turnover, which consequently affects muscle growth (Dedieu et al., 2002). The *CAPN1* and *CAST* genes code the μ-calpain and calpastatin transcripts, respectively, and they have been evaluated as candidate genes in livestock (Ropka-Molik et al., 2017; Sun et al., 2018; Armstrong et al., 2018).

Several association studies with *CAST* variants in various sheep breeds have previously been performed (Nassiry et al., 2006; Byun et al., 2008; Chung and Davis, 2012; Khan et al., 2012; Yilmaz et al., 2014; Gorlov et al., 2016; Dagong et al., 2016; Jawasreh et al., 2017; Armstrong et al., 2018). These studies revealed the associations of *CAST* variants with growth, carcass, and meat traits in sheep. However, the effects of *CAST* variants on the growth and carcass traits of Santa Inês sheep remain unknown.

Notably, some associations have been reported between *CAPN1* variants and growth traits in livestock species such as cattle (Miquel et al., 2009), poultry (Felício et al., 2013), and swine (Ropka-Molik et al., 2017). However, similar associations in sheep remain unknown. In sheep, the calpain small subunit 1 (*CAPNS1* or *CAPN4*) has frequently been studied. Associations of *CAPNS1* variants with body weight (BW) and average daily gain (ADG) in Egyptian sheep breeds (Mahrous et al., 2016), yearling weight in Zel sheep (Dehnavi et al., 2012), and intramuscular fat in Polish Merino sheep (Grochowska et al., 2017) have been reported.

No previous association studies between *CAST* and *CAPN1* variants with either growth or carcass traits in Santa Inês sheep have been reported. Therefore, the current study aims to sequence fragments of *CAST* and *CAPN1* genes to perform association studies of growth and carcass ultrasound traits in Santa Inês sheep.

Material and methods

Population

The present study was conducted with the approval of the Ethical Committee for Animal Use from the Veterinary Medicine and Animal Science School of the Federal University of Bahia (UFBA) (protocol number 02/2010). A sample of 192 males Santa Inês was used in the current study. Seven unrelated sires were used to obtain 106 lambs, which were born between 2010 and 2012 at the Pedro Arle experimental farm of EMBRAPA Tabuleiros Costeiros, in Frei Paulo, Sergipe state. The smallest and largest half-sib families had 9 and 17 progenies, respectively. The remaining 86 lambs were born in 2014 and were further raised on the experimental farm of the UFBA in São Gonçalo dos Campos, Bahia state. However, pedigree control was not performed for this group because mating occurred at pasture, but only unrelated sires were used on this farm. Notably, no full siblings were used in the current study. A principal component analysis was additionally performed on variants in the *CAST*

and *CAPN1* genes, though no genetic structuration was observed (Figure 1), with eigenvalues for PC1 and PC2 of 15.9% and 11.9%, respectively. On both farms, the study animals received similar feeding, with water and mineral salt *ad libitum* and diets targeting a weight gain of 170 g/day, according to the NRC (2007).

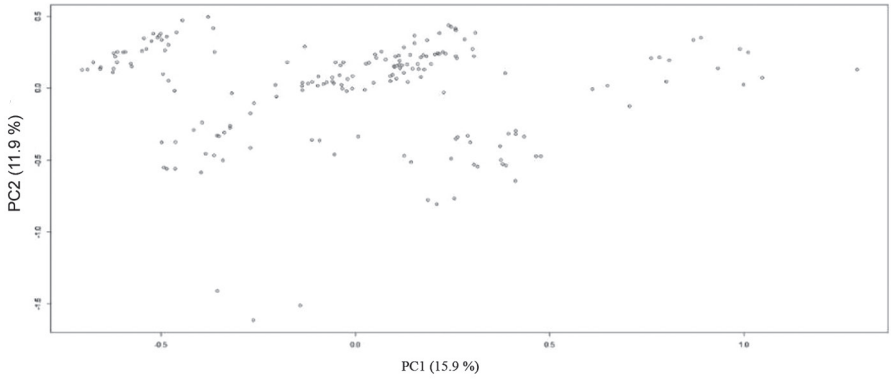

Figure 1. Scatter plot of the first two principal components (PC1 vs. PC2)

Phenotypes

The body weights at weaning (WW) (age ranging from 85 to 115 days) and post-weaning (PWW) (age ranging from 225 to 255 days) were recorded using an electronic scale. ADG was then calculated as follows:

$$ADG = \frac{PWW - WW}{140}$$

On the same day, the morphometric traits of lambs were measured using a tape and a measuring stick. These traits included withers (WH) (distance from the highest point of the thoracic vertebra to the ground) and croup (CH) heights (distance from the coxal tuberosity to the ground), body length (BL) (distance from supraglenoid tuberosity to sciatic tuberosity), thoracic (TW) (distance between the supraglenoid tuberosities) and croup (CW) (distance between the coxal tuberosities) widths, thoracic (TG) (contour of the thoracic cavity, just after the shoulder blades) and leg (LG) girths (mid-thigh contour), and body depth (BD) (distance from the thoracic vertebrae to the sternum). Moreover, carcass fat scores (CFS) were assessed on a scale of 1 to 5 (Moxham and Brownlie, 1976), with only one recorder assigning score values as follows: 1 (individual ribs are easily felt and no tissue can be felt over the ribs), 2 (individual ribs are easily felt, though some tissue is present), 3 (individual ribs can still be felt and one can feel more tissue over the ribs), 4 (one can only just feel ribs and there is fluid movement of tissue), and 5 (ribs cannot be felt and the tissue movement is very fluid). Furthermore, *in vivo* carcass ultrasonography was performed to obtain the rib eye area (REA) and fat thickness (FT) using an Aloka 500SSD ultrasound machine equipped with a 3.5-MHz linear transducer. The REA and FT

ultrasound images were recorded between the 12th and 13th ribs on the left side of the lambs. The length (A) and maximum depth (B) of the muscles were determined, while the REA was estimated using the equation:

$$\frac{A}{2} * \frac{B}{2} * \pi$$

Descriptive statistics of the traits assessed in the current study are presented in Table 1, which are similar to values previously recorded for Santa Inês sheep (Jucá et al., 2014, 2016).

Table 1. Sample size (N), mean and standard deviation (SD) of the traits in Santa Inês sheep

Traits	N	Mean	SD
WH (cm)	184	66.26	5.69
CH (cm)	184	66.93	5.64
BL (cm)	184	56.27	8.94
TW (cm)	184	17.77	2.11
CW (cm)	180	15.77	3.36
LG (cm)	184	40.47	8.23
TG (cm)	184	73.21	4.76
BD (cm)	180	25.23	2.13
WW (kg)	172	20.56	4.15
PWW (kg)	184	34.03	6.27
ADG (g)	171	136.99	62.11
REA (cm^2)	97	7.17	1.64
FT (cm)	99	0.20	0.04
CFS	181	2.32	0.37

WH – withers height, CH – croup height, BL – body length, TW – thoracic width, CW – croup width, LG – leg girth, TG – thoracic girth, BD – body depth, WW – weaning weight, PWW – post-weaning weight, ADG – average daily gain, REA – rib eye area, FT – fat thickness, CFS – carcass fat score.

Genotyping

Primer design was conducted depending on the gene sequence with the following access codes: *CAST* (Gene ID: 443364) and *CAPN1* (Gene ID: 443130). The software Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) was used to design the oligonucleotide sequences. NetPrimer (PREMIER Biosoft, Palo Alto, CA, USA) was used to test the quality of the sequences based on rating >90%, the melting temperature (Tm) of the sense and anti-sense primers varying only ±1°C, and the primers not having a dimer or cross dimer. In the *CAST* gene, the primers were as follows: 5'-*AAAAGCCAAAGAAGAGGATCG*-3' (forward) and 3'-*GGGAAACCACTTCAGAGACG*-5' (reverse); whereas the forward 5'-*TGTGCTGCGTTTCTTCTCAG*-3' and reverse 3'-*AAGGTCACCACTCCATCCAG*-5' primers were used in *CAPN1*.

The PCR conditions for both *CAST* and *CAPN1* genes are presented in Table 2. A 15 µL of solution was used in PCR analysis for both genes, which contained

0.3 mM of each primer, 7.5 µL of mix composed of Taq polymerase, optimized buffer and dNTP mixture (Emeraldamp Max Hs – Takara Bio, USA), and 100 ng of the template DNA. Amplification was performed using a Verity Thermal Cycler (Applied Biosystems, San Diego, USA). The amplification products were separated on 1% agarose gel and the amplified bands were stained with GelRed (Biotium, Fremont, USA). We used a pool of Santa Inês sheep DNA as a positive control to ensure that the primer design amplified the desired region.

Table 2. Polymerase chain reaction (PCR) conditions for *CAST* and *CAPN1* genes in Santa Inês sheep

PCR conditions	Initial denaturation	Denaturation	Annealing	Extension	Final extension
**CAST* (Step 1)	98°C/ 5 min	98°C/ 10 s (10 cycles)	63°C–58°C Δ-0.5°C/ 30 s	72ºC/ 4 min	72ºC/ 5 min
**CAST* (Step 2)		98°C/ 10 s (30 cycles)	58°C/30 s	72ºC/ 4 min	72ºC/ 5 min
CAPN1	98°C/ 5 min	98°C/ 10 s	58°C/30 s	72ºC/ 4 min	72ºC/ 5 min

*For the *CAST* gene, a touchdown PCR was performed.

The amplicons were purified with magnetic beads and the recommended volume of Agencourt AMPure XP (Beckman Coulter, Porterville, USA), thereby homogenizing the beads to bind to the amplified products. Immediately after this step, the samples were purified with 70% ethanol to remove contaminants. The pellet was then diluted and the beads were removed. The samples were diluted to 2 nM, considering the base pair size of the amplified products. Samples were quantified with a Qubit® fluorometer (Life Technologies, Carlsbad, USA), diluted to 0.2 ng/µl for library preparation, and subsequently sequenced. The Nextera® XT DNA sample preparation and the Nextera® XT index (Illumina, San Diego, USA) were used to prepare the library, with all steps being performed based on the Nextera XT protocol. Sequencing was performed on the MiSeq platform (Illumina, San Diego, USA) using the MiSeq Reagent Kit v2 (500 cycles).

Read quality was verified using FastQC software (www.bioinformatics.bbsrc.ac.uk/projects/). For the first round of data filtering, SeqyClean software version 1.3.12 (Zhbannikov et al., 2017) was used, adopting a quality parameter of 24 (Phred score) for each base and a minimum length of 50 bp. Subsequently, reads were aligned against the reference sheep genome deposited in the NCBI (version Oar_v4.0) using the Bowtie2 program (Langmead and Salzberg, 2012).

The detection of variants was performed based on the position in the reference sheep genome (Version Oar_v4.0). Subsequently, files from the sequence alignment map (SAM) format were converted to binary alignment map (BAM) format. The PCR duplicates were then removed, the sequences were sorted, and the index of the ordered file was constructed. Variant call was performed using the mpileup option of SAMtools, covering a value set for the quality of the mapping through the genome reference (-q20) and a filter quality ≥ 40 bases in the Phred (-Q40) scale. For the nomenclature of markers, we followed the recommendations of the Human Genome Variation Society (HGVS), using sheep genome version Oar_v4.0. Finally, the func-

tional annotation of variants was performed using the variant effect predictor (VEP) for the online annotation of Ensembl to identify the locations of mutations in various regions of the genome as well as the likely functional effects of the variants.

Hardy-Weinberg equilibrium and linkage disequilibrium analysis

A Hardy-Weinberg Equilibrium (HWE) test was performed for each variant by comparing the expected and observed heterozygosities with Haploview software (Barrett et al., 2005). In this test, a threshold of 5% was used to declare HWE. Moreover, Haploview was used to find linkage disequilibrium (LD) blocks. Criteria for the inclusion of variants in LD analysis were as follows: P>0.05 in the HWE test and minimal allelic frequency (MAF) higher than 1%.

Statistical analysis of the phenotypic dataset

An analysis of variance (ANOVA) was performed for each trait using the PROC MIXED procedure (SAS, 2011). The model

$$y_{ijkl} = \mu + F_i + Y_j + M_k + \alpha_{ijkl}(A) + \varepsilon_{ijkl}$$

was used, where y_{ijkl} is the value of the characteristic of interest, μ is the general average, F_i is the farm effect, Y_j is the effect of the year of birth, M_k is the month of birth, $\alpha_{ijkl}(A)$ is the effect of the covariable age of the animal at the time of the evaluation, and ε_{ijkl} is the residual term. Outliers (outside ±3 studentized residual) were removed.

Association analyses

For each locus, the additive and dominance effects were estimated using the software Qxpak 5 (Pérez-Enciso and Misztal, 2011). A total of 64 variants (39 in *CAST* and 25 in *CAPN1*) showed HWE (P>0.05) and MAF>1%, which were used in the single-locus analysis. Additionally, a haplotype association analysis was performed with the haplo.stat package (Lake et al., 2003). Only haplotypes with a frequency greater than 4% were used. In both single-locus and haplotype association analyses, the fixed effects included in the models were used in ANOVA. The significance level was calculated using Bonferroni correction.

Results

Variants in the *CAST* and *CAPN1* genes

A fragment of 4,108 bp, located between exons 20 (first base 93395596) and 23 (last base 93399703) of the *CAST* (Gene ID: 443364), was amplified. In this region, 48 intronic variants and one exon variant were identified (Table 3), all with MAF >1% and 39 variants in HWE (P>0.05). Notably, novel *CAST* variants were not found. In exon 20, the variant *rs413442067* is a synonymous variant since a *G/A* change does not alter the amino acid threonine in position 547 of the protein. The other *CAST* variants were present in introns 20, 21, and 22.

Table 3. Heterozygosity, Hardy-Weinberg equilibrium (HWE) p-value, and minor allelic frequency (MAF) of *CAST* variants in Santa Inês sheep

NCBI Number	HGVS name	Heterozygosity		HWE (P-value)	MAF	Region
		Observed	Predicted			
1	2	3	4	5	6	7
rs413442067	c.1462G>A	0.215	0.216	1.00E+00	0.123	Exon 20
rs424912630	c.1495+112G>A	0.136	0.154	2.56E-01	0.084	Intron 20
rs403339381	c.1495+132G>A	0.115	0.136	1.29E-01	0.073	Intron 20
rs414639908	c.1495+159G>A	0.476	0.484	9.13E-01	0.411	Intron 20
rs425997700	c.1495+167A>G	0.246	0.239	9.88E-01	0.139	Intron 20
rs415836747	c.1495+334G>A	0.168	0.179	5.50E-01	0.099	Intron 20
rs426993921	c.1495+437A>G	0.000	0.118	9.63E-20	0.063	Intron 20
rs409851241	c.1495+449A>G	0.377	0.500	9.00E-04	0.497	Intron 20
rs605481564	c.1495+464A>G	0.482	0.481	1.00E+00	0.403	Intron 20
rs417020700	c.1495+478T>C	0.497	0.473	6.12E-01	0.385	Intron 20
rs428230968	c.1495+486T>C	0.497	0.492	1.00E+00	0.437	Intron 20
rs406915912	c.1495+504T>C	0.497	0.486	8.90E-01	0.416	Intron 20
rs422402447	c.1495+535C>T	0.455	0.480	5.49E-01	0.401	Intron 20
rs160120782	c.1495+581T>C	0.382	0.500	1.60E-03	0.484	Intron 20
rs408105678	c.1495+728A>G	0.524	0.452	4.49E-02	0.346	Intron 20
rs419473804	c.1495+746A>G	0.450	0.419	4.07E-01	0.298	Intron 20
rs413404444	c.1495+877A>C	0.304	0.360	4.91E-02	0.236	Intron 20
rs399204438	c.1495+943A>G	0.539	0.500	3.64E-01	0.495	Intron 20
rs425885251	c.1495+1060T>C	0.545	0.500	2.93E-01	0.497	Intron 20
rs423099226	c.1495+1133G>A	0.497	0.444	1.39E-01	0.332	Intron 20
rs405663608	c.1495+1139G>A	0.215	0.208	1.00E+00	0.118	Intron 20
rs428213368	c.1496-1231T>C	0.429	0.410	6.69E-01	0.288	Intron 20
rs406867092	c.1496-1194A>G	0.393	0.346	9.45E-02	0.223	Intron 20
rs418210778	c.1496-1156T>C	0.487	0.498	8.42E-01	0.469	Intron 20
rs429530795	c.1496-1144T>C	0.539	0.454	1.46E-02	0.348	Intron 20
rs408051866	c.1496-1081C>A	0.094	0.109	2.31E-01	0.058	Intron 20
rs160120795	c.1496-1059G>A	0.398	0.500	6.40E-03	0.497	Intron 20
rs409258492	c.1496-1043C>G	0.403	0.500	1.01E-02	0.490	Intron 20
rs160120800	c.1496-971G>T	0.178	0.179	1.00E+00	0.099	Intron 20
rs399091954	c.1496-939A>G	0.152	0.166	3.88E-01	0.092	Intron 20
rs160120803	c.1496-820C>T	0.168	0.179	5.50E-01	0.099	Intron 20
rs400315475	c.1496-819G>A	0.503	0.442	8.71E-02	0.330	Intron 20
rs411571641	c.1496-810C>T	0.555	0.500	1.80E-01	0.497	Intron 20
rs422744326	c.1496-790G>A	0.225	0.231	8.77E-01	0.134	Intron 20
rs418161864	c.1496-638C>G	0.194	0.200	8.65E-01	0.113	Intron 20
rs161885177	c.1496-607A>T	0.534	0.459	3.52E-02	0.356	Intron 20

Table 3 – contd.

1	2	3	4	5	6	7
rs403866848	c.1496-533C>T	0.246	0.239	9.88E-01	0.139	Intron 20
rs415186098	c.1496-520C>T	0.309	0.357	9.18E-02	0.233	Intron 20
rs430517308	c.1496-458T>C	0.162	0.192	9.26E-02	0.107	Intron 20
rs409125240	c.1496-421A>G	0.120	0.140	1.56E-01	0.076	Intron 20
rs420247553	c.1496-381G>T	0.152	0.166	3.88E-01	0.092	Intron 20
rs427755483	c.1496-318C>T	0.241	0.235	1.00E+00	0.136	Intron 20
rs193637301	c.1496-217G>A	0.487	0.440	2.04E-01	0.327	Intron 20
rs421650487	c.1496-76C>T	0.136	0.154	2.56E-01	0.084	Intron 20
rs400201314	c.1547-70A>G	0.377	0.423	1.73E-01	0.304	Intron 21
rs411539518	c.1547-20C>T	0.236	0.231	1.00E+00	0.134	Intron 21
rs418818682	c.1618+232C>T	0.524	0.491	4.71E-01	0.435	Intron 22
rs401407818	c.1618+294A>C	0.021	0.051	1.00E-04	0.026	Intron 22
rs423886098	c.1619-179G>A	0.225	0.216	8.72E-01	0.123	Intron 22

For the *CAPN1* (Gene ID: 443130), a fragment of 3,927 bp between exon 12 (first base 42625107) and intron 20 (last base 42629033) was amplified. Forty-four intronic variants and one exon variant were identified (Table 4), all with MAF >1% and 25 in HWE (P>0.05). However, novel *CAPN1* variants were not identified. The synonymous variant *rs421035003* in exon 20 is a *C/T* substitution that does not change the amino acid asparagine in position 667 (see www.ensembl.org). Notably, other *CAPN1* variants were found in introns 14, 15, 16, 17, 18, 19, and 20.

Figure 2. Linkage disequilibrium blocks in the *CAST* gene in Santa Inês sheep

Table 4. Heterozygosity, Hardy-Weinberg equilibrium (HWE) p-value, and minor allelic frequency (MAF) of *CAPN1* variants in Santa Inês sheep

NCBI number	HGVS name	Heterozygosity		HWE (P-value)	MAF	Region
		Observed	Predicted			
rs417411045	c.1713+53G>A	0.071	0.164	1.86E-08	0.090	Intron 14
rs428375521	c.1713+131G>T	0.104	0.108	9.11E-01	0.057	Intron 14
rs407017992	c.1713+163C>T	0.333	0.345	7.73E-01	0.221	Intron 14
rs422192534	c.1713+242A>G	0.361	0.492	4.00E-04	0.437	Intron 14
rs400729075	c.1713+251T>C	0.350	0.487	2.00E-04	0.421	Intron 14
rs407944017	c.1714-246A>G	0.372	0.486	2.10E-03	0.415	Intron 14
rs419029128	c.1714-61C>T	0.306	0.495	3.34E-07	0.448	Intron 14
rs401662939	c.1714-35G>A	0.399	0.427	4.51E-01	0.309	Intron 14
rs162278939	c.1714-15G>A	0.213	0.255	5.72E-02	0.150	Intron 14
rs424143722	c.1780-17T>C	0.273	0.499	9.08E-10	0.481	Intron 15
rs398261375	c.1837+7G>A	0.377	0.423	1.88E-01	0.303	Intron 16
rs413668712	c.1837+73C>T	0.333	0.494	1.55E-05	0.445	Intron 16
rs424964941	c.1837+130T>C	0.339	0.489	4.79E-05	0.426	Intron 16
rs403309597	c.1837+166T>C	0.339	0.484	7.75E-05	0.410	Intron 16
rs410518425	c.1837+169G>A	0.355	0.491	3.00E-04	0.434	Intron 16
rs423441243	c.1837+182A>G	0.104	0.108	9.11E-01	0.057	Intron 16
rs417258958	c.1837+316G>A	0.470	0.464	1.00E+00	0.366	Intron 16
rs428514817	c.1838-473G>A	0.366	0.414	1.58E-01	0.292	Intron 16
rs403089766	c.1838-327G>A	0.148	0.339	2.93E-12	0.216	Intron 16
rs418303621	c.1838-209C>T	0.568	0.480	1.97E-02	0.399	Intron 16
rs161627773	c.1838-138C>T	0.246	0.224	3.32E-01	0.128	Intron 16
rs407863935	c.1838-16T>C	0.552	0.476	4.82E-02	0.391	Intron 16
rs419204865	c.1902+37G>A	0.333	0.292	8.66E-02	0.178	Intron 17
rs430177297	c.1902+76G>A	0.317	0.274	4.87E-02	0.164	Intron 17
rs408790217	c.1902+123G>A	0.355	0.357	1.00E+00	0.232	Intron 17
rs398427062	c.1903-187T>C	0.388	0.339	7.29E-02	0.216	Intron 17
rs161627780	c.1903-106G>C	0.454	0.379	1.07E-02	0.254	Intron 17
rs593794806	c.1903-102C>T	0.142	0.132	7.69E-01	0.071	Intron 17
rs420860201	c.1840-75T>C	0.328	0.303	3.98E-01	0.186	Intron 17
rs399366555	c.1840-6A>G	0.137	0.127	8.30E-01	0.068	Intron 17
rs1090899021	c.1909-81C>T	0.208	0.296	4.00E-04	0.180	Intron 18
rs406194123	c.1909-80G>C	0.246	0.326	2.80E-03	0.205	Intron 18
rs403005481	c.2043+163G>A	0.191	0.224	1.07E-01	0.128	Intron 19
rs418468486	c.2043+313T>C	0.415	0.489	5.36E-02	0.426	Intron 19
rs429532201	c.2043+377G>A	0.383	0.348	2.65E-01	0.224	Intron 19
rs403953588	c.2043+399A>G	0.541	0.488	2.03E-01	0.423	Intron 19
rs414993519	c.2044-244A>G	0.104	0.240	2.22E-10	0.139	Intron 19
rs430307080	c.2044-227T>C	0.481	0.486	9.85E-01	0.415	Intron 19
rs161627795	c.2044-110G>A	0.049	0.058	2.87E-01	0.030	Intron 19
rs427085960	c.2044-84G>A	0.273	0.329	4.02E-02	0.208	Intron 19
rs409655600	c.2044-56A>C	0.098	0.132	1.34E-02	0.071	Intron 19
rs421035003	c.2057C>T	0.404	0.425	6.04E-01	0.306	Exon 20
rs590844301	c.2160+32C>T	0.022	0.022	1.00E+00	0.011	Intron 20
rs410614126	c.2160+49T>G	0.421	0.446	5.17E-01	0.336	Intron 20
rs400201468	c.2161-60G>A	0.454	0.446	9.90E-01	0.336	Intron 20

NCBI – National Center for Biotechnology Information; HGVS – Human Genome Variation Society; HWE – Hardy-Weinberg equilibrium; MAF – minor allelic frequency.

Haplotype analysis identified five LD blocks in the *CAST* gene (Figure 2). Block-1 has 200 bp (*rs413442067*, *rs424912630*, *rs403339381*, *rs414639908*, and *rs425997700*); block-2 has 556 bp (*rs406915912*, *rs422402447*, *rs419473804*, *rs399204438*, and *rs425885251*); block-3 has 290 bp (*rs411571641*, *rs422744326*, *rs418161864*, *rs403866848*, and *rs415186098*); block-4 has 63 bp (*rs420247553* and *rs427755483*); and block-5 has 710 bp (*rs418818682* and *rs423886098*). For *CAPN1*, three LD blocks were identified (Figure 3). Block-1 has 175 bp (*rs417258958* and *rs428514817*), block-2 has 64 bp (*rs418468486* and *rs429532201*), and block-3 has 240 bp (*rs430307080* and *rs421035003*). The *CAST* and *CAPN1* haplotypes with frequency ≥1% are presented in Figure 4.

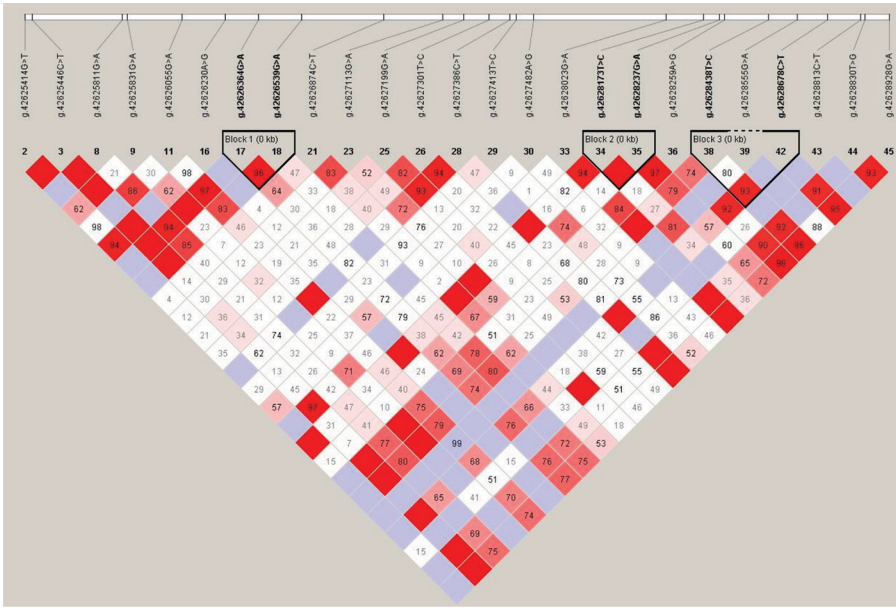

Figure 3. Linkage disequilibrium blocks in the *CAPN1* gene in Santa Inês sheep

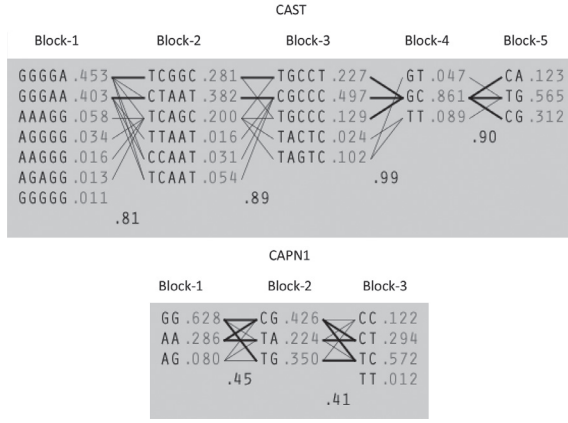

Figure 4. Haplotypes in the *CAST* and *CAPN1* genes with frequencies ≥1%

Association analysis

A single-locus association analysis was performed with 64 variants (39 in *CAST* and 25 in *CAPN1*). Consequently, a significance threshold of 0.0008 was used in this approach. At the Bonferroni threshold, only one variant in the *CAST* gene (*rs418818682*) was found to be in association with CFS (Table 5). This single-locus analysis also revealed the other 21 suggestive additive effects (P<0.05) of the *CAST* variants. Three *CAST* variants were associated with WW, and the differences between homozygotes were as follows: 3.53 kg (*rs428213368*), 2.46 kg (*rs400315475*), and 4.37 kg (*rs418161864*). Additionally, the *CAST* variant *rs418818682* was associated with PWW, while the difference between homozygotes was 2.41 kg.

Table 5. Additive effect (a) and standard error (SE) of polymorphisms associated with traits in Santa Inês sheep

Trait[1]	Variant	LRT[2]	P-value	A	SE	Region
			CAST			
WW	rs428213368	9.12	0.0025	–1.766	0.5748	Intron 20
WW	rs400315475	4.20	0.0405	–1.232	0.5969	Intron 20
WW	rs418161864	6.12	0.0133	2.183	0.8720	Intron 20
PWW	rs418818682	3.92	0.0476	1.203	0.6029	Intron 22
CFS	rs423099226	4.98	0.0256	–0.089	0.0396	Intron 20
CFS	rs428213368	5.11	0.0238	–0.092	0.0403	Intron 20
CFS	rs400315475	4.08	0.0434	–0.084	0.0412	Intron 20
CFS	rs415186098	4.24	0.0395	–0.085	0.0408	Intron 20
CFS	rs430517308	5.66	0.0173	–0.121	0.0505	Intron 20
CFS	rs418818682	12.17	0.0005*	0.123	0.0344	Intron 22
FT	rs403339381	4.81	0.0283	0.019	0.009	Intron 20
TG	rs419473804	4.42	0.0355	–1.004	0.4748	Intron 20
TW	rs414639908	8.51	0.0035	0.562	0.1903	Intron 20
TW	rs415836747	4.10	0.0428	0.643	0.3154	Intron 20
TW	rs605481564	4.84	0.0278	0.426	0.1926	Intron 20
TW	rs417020700	5.23	0.0222	0.454	0.1972	Intron 20
TW	rs406915912	6.17	0.0130	0.480	0.1917	Intron 20
TW	rs422402447	8.53	0.0035	0.550	0.1860	Intron 20
TW	rs399204438	6.54	0.0106	–0.508	0.1969	Intron 20
TW	rs425885251	5.73	0.0167	–0.478	0.1983	Intron 20
TW	rs411571641	4.18	0.0410	–0.414	0.2013	Intron 20
TW	rs418818682	8.11	0.0044	0.552	0.1917	Intron 22
			CAPN1			
BD	rs420860201	4.28	0.0386	1.6367	0.5496	Intron 17
WW	rs417258958	4.32	0.0377	–1.038	0.4953	Intron 16
CH	rs408790217	5.62	0.0177	1.396	0.5840	Intron 17
FT	rs408790217	4.51	0.0337	–0.016	0.008	Intron 17
REA	rs403953588	5.21	0.0225	0.409	0.177	Intron 19
REA	rs430307080	6.08	0.0136	0.422	0.168	Intron 19
WH	rs408790217	6.11	0.0135	1.304	0.5232	Intron 17

[1]WW – weaning weight; PWW – post-weaning weight; CFS – carcass fat score; FT – fat thickness; TG – thoracic girth; TW – thoracic width; BD – body depth; CH – croup height; WH – withers height; REA – rib eye area. [2]LRT – likelihood ratio test; *Significant effect at Bonferroni correction level.

For biometric traits, 11 *CAST* additive effects were found. Variant *rs419473804* was associated with TG, and the difference between homozygotes was 2.01 cm. Moreover, the additive effect of 10 *CAST* variants on TW was observed, with the difference between homozygotes ranging from 0.83 cm (*rs411571641*) to 1.29 cm (*rs415836747*). A total of six *CAST* variants demonstrated an additive effect on CFS, with the score differences between homozygotes ranging from 0.170 (*rs415186098*) to 0.246 (*rs418818682*). Moreover, an additive effect of the *CAST* variant *rs403339381* on an ultrasound image of FT was also observed, where the difference between homozygotes was 0.038 cm.

The single-locus analysis also revealed seven suggestive additive effects of *CAPN1* variants (Table 5). The *rs417258958* variant was associated with WW, and the difference between homozygotes was 2.08 kg. For morphometric traits, the *rs408790217* variant was associated with CH, WH, and FT, with differences between homozygotes of 2.79 cm, 2.61 cm, and 0.032 cm, respectively. Moreover, the *rs420860201* variant was associated with BD, with the difference between homozygotes being 3.27 cm. For carcass traits, the additive effect of the *CAPN1* variants *rs403953588* and *rs430307080* on REA were observed, with differences between homozygotes of 0.82 cm^2 and 0.84 cm^2, respectively.

Table 6. Regression coefficients (β) and standard errors (SE) of haplotype association analysis in Santa Inês sheep

Trait	Gene	Linkage disequilibrium	Haplotype replacement	β	SE	P-value
TG	*CAST*	Block-1	*GGGGA* by *AAAGG*	–2.7200	1.2700	0.033
REA	*CAST*	Block-2	*CTAAT* by *TCAAT*	–0.6890	0.2900	0.018
ADG	*CAST*	Block-3	*CGCCC* by *TGCCC*	–23.600	10.400	0.024
BL	*CAST*	Block-3	*CGCCC* by *TAGTC*	–3.3800	1.4900	0.025
LG	*CAST*	Block-3	*CGCCC* by *TAGTC*	–2.8400	1.3700	0.040
FT	*CAPN1*	Block-1	*GG* by *AG*	–0.0143	0.0053	0.008

TG – thoracic girth, REA – rib eye area, ADG – average daily gain, BL – body length, LG – leg girth, FT – fat thickness.

Five LD blocks were used in the haplotype association analysis (Figure 4). Therefore, a nominal P-value of 0.0063 was calculated using Bonferroni correction at 5%. While no significant association was found at a Bonferroni level ($P>0.0063$), six suggestive associations ($P<0.05$) were identified (Table 6). In the *CAST* gene, replacement of the haplotype *GGGGA* by *AAAGG* (block-1) was associated with a reduction of TG (–2.72±1.27). The replacement of haplotype *CTAAT* by *TCAAT* (block-2) was associated with a lower REA (–0.689±0.290). The replacement of *CGCCC* by *TGCCC* (block-3) was associated with a reduction of ADG (–23.6±10.4), while the replacement of *CGCCC* by *TAGTC* (block-3) was associated with both BL (–3.38±1.49) and LG (–2.84±1.37) reductions. In the *CAPN1* gene, the replacement of *GG* by *AG* (block-1) was associated with a reduction in FT (–0.0143±0.0053).

Discussion

Variants in the *CAST* and *CAPN1* genes

Some variants in Santa Inês sheep exhibited allelic frequencies similar to values reported in other sheep populations. For instance, the allelic frequencies of 0.88 (*G*) and 0.12 (*A*) were observed for the variant *rs413442067* in exon 20 of the *CAST* gene, while values of 0.93 (*G*) and 0.07 (*A*) were reported in both the IROA and MOOA populations (see https://www.ensembl.org). On the other hand, the synonymous variant *rs421035003* in exon 20 of *CAPN1* exhibited allelic frequencies of 0.31 for the *T* allele, though lower *T* frequencies were reported in both the IROA (0.05) and MOOA (0.14) populations (see https://www.ensembl.org). These differences may have resulted from the different selection processes for each sheep breed. Therefore, it is important to sequence the *CAPN1* and *CAST* genes in different sheep breeds to study their effects.

The present study was the first to sequence fragments of the *CAST* and *CAPN1* genes in Santa Inês sheep. While the *CAPN1* gene has been poorly studied in sheep, many previous studies of the *CAST* gene in sheep have used either PCR-SSCP (Nassiry et al., 2006; Dehnavi et al., 2012; Aali et al., 2017) or PCR-RFLP (Palmer et al., 1998; Sutikno et al., 2011; Dehnavi et al., 2012; Khan et al., 2012; Yilmaz et al., 2014; Gorlov et al., 2016; Jawasreh et al., 2017; Kumar et al., 2018) approaches; however, these studies did not sequence large fragments of these genes. Knight et al. (2012) was the only researcher to previously sequence large fragments of both *CAST* and *CAPN1* in sheep and reported 191 variants, which highlights the large genetic variability of these genes in sheep.

When compared to Knight et al. (2012), the present study sequenced a shorter area of *CAST* (from exons 20 to 23, including introns) and *CAPN1* (from exon 12 to intron 20) genes. Nevertheless, the present study was able to reveal that these genes have great variability in the Santa Inês breed, since 49 and 45 variants were found in *CAST* and *CAPN1* fragments, respectively (Tables 3 and 4). However, only one exon variant in each fragment was identified, which demonstrates the small variation in coding regions. This result was expected because, in the complete sequences of these genes available in the Ensembl database, there were 3,441 (*CAST*) and 647 (*CAPN1*) variants; however, only 44 (*CAST*) and 34 (*CAPN1*) were located in exons (see https://www.ensembl.org). Moreover, Knight et al. (2012) sequenced larger fragments of these genes in Australian sheep breeds and found 152 (*CAST*) and 39 (*CAPN1*) variants, though these were all present in the non-coding area. Notably, these genes play a crucial role in muscle growth, development, and atrophy (Kemp et al., 2013), while any non-synonymous mutation might have a nonviable effect on muscular traits for commercial purposes. The intronic variants reported here may also cause modifications in phenotypes, such as through small RNA expression (Sheng et al., 2011). Moreover, the intronic variants can be in LD with the causal variants not mapped in the current study.

Association analysis

The present study was the first to estimate the additive effect of both *CAST* and *CAPN1* variants on both growth and carcass traits in Santa Inês sheep. Previous

studies have demonstrated that μ-calpain is related to skeletal muscle mass growth (Kemp et al., 2013). Moreover, the suppression of calpastatin is known to increase the expression of μ-calpain, affecting cell proliferation, cell survival, and apoptotic pathways (Van-Ba et al., 2015). Therefore, a decrease in calpains activity may cause a reduction of muscular fiber degradation, thereby resulting in the further accumulation of muscle mass. Thus, the key role of μ-calpain and calpastatin in protein turnover supports the purported additive effects on the growth and carcass traits of Santa Inês sheep.

Previous studies have also reported an association between variants in the *CAST* gene with growth traits in sheep. Palmer et al. (1998) detected a variant in the exon 1C/1D by the digestion of a 622 bp PCR product, which has three genotypes named *MM*, *MN*, and *NN* and was named *CAST/MspI* (Palmer et al., 1998). This variant was associated with ADG by Khan et al. (2012), where *MN* showed a higher average value than *MM* in both Balkhi and Kajli sheep, while a similar effect on ADG was reported for both Awassi sheep (Jawasreh et al., 2017) and Salsk sheep (Gorlov et al., 2016). In Kıvırcık lambs, no difference was observed between *MM* and *MN* genotypes, though these two genotypes exhibited higher average WW, ADG, and FT than the *NN* genotype (Yilmaz et al., 2014). On the other hand, no differences ($P>0.05$) were observed between the three genotypes for BW in Indonesia local sheep (Sutikno et al., 2011) and yearling weight in Zel sheep (Dehnavi et al., 2012). In the same region of exon 1C/1D, Nassiry et al. (2006) reported another variant with three banding patterns using PCR-SSCP, which was associated with ADG in Kurdi sheep during the pre-weaning period. However, no association of this same SSCP variant was found for yearling weight in Zel sheep (Dehnavi et al., 2012). Moreover, the region between exons 1C/1D seems also to be in association with carcass traits because the *CAST/MspI* variant was associated with FT in Kıvırcık lambs (Yilmaz et al., 2014) and meat: bone ratio in Awassi sheep (Jawasreh et al., 2017).

Notably, variants in other *CAST* region may also be associated with growth traits in sheep. Byun et al. (2008) amplified 254 bp in exon 6 of Romney sheep and found three banding patterns forming six genotypes, which were found to be in association with birth weight and ADG in the pre-weaning period. Dagong et al. (2016) also amplified the same region in exon 6 and found an association with ADG in Thin Tail sheep. Chung and Davis (2012) amplified 1,323 bp between exons 24 and 25, finding three banding patterns with three genotypes, which demonstrated an additive effect on both birth weight in Polypay sheep and ADG in a mixed breed sheep population. However, no effect was found for both weaning weight and post-weaning weight (Chung and Davis, 2012). A PCR-RFLP analysis was also performed to amplify a fragment of intron 12 of the *CAST* gene (Greguła-Kania, 2012), where a variant with four banding patterns and eight genotypes was identified. However, only an insignificant association ($P>0.05$) with BW, ADG, and FT in two synthetic lines of lambs in Poland were found (Greguła-Kania, 2012). In addition, variant *rs404358363*, located in intron 2–3, was associated with birth weight and FT in Texel sheep (Armstrong et al., 2018).

The *CAST* intronic variants found in Santa Inês sheep are neither in splice sites nor encode small RNA transcripts. Therefore, LD with causal mutations may ex-

plain the observed association with *CAST* variants in the present study. In this context, variant *rs418818682* (associated with PWW, FCS, and TW) is at a distance of 295 bp from variant *rs596673812*, which is a missense variant in exon 24 that causes a tolerated amino acid replacement (Ser/Asn) at position 578 in calpastatin. Moreover, Chung and Davis (2012) reported a polymorphism between exons 24–25 that is associated with birth weight and pre-weaning ADG in sheep. While we were unable to study the complete *CAST* gene sequence, our results indicate that other regions of this gene require further investigation to more comprehensively understand these effects.

The present study revealed, for the first time, suggestive additive effects of variants in the calpain large subunit 1 (*CAPN1*) gene on growth and carcass traits in Santa Inês sheep. We found only two previous studies that tested *CAPN1* variants in association studies on sheep (Knight et al., 2012, 2014). In both studies, a panel with several variants (13 in *CAPN1*, 39 in *CAPN2*, and 19 in *CAPN3*) was evaluated, though no significant effects of *CAPN1* variants were found. However, some variants in the *CAPN2* gene were associated with meat tenderness (Knight et al., 2014) and FT (Knight et al., 2014). Notably, the calpain small subunit 1 (named *CAPNS1* or *CAPN4*) has been more intensively studied in sheep. Dehnavi et al. (2012) amplified a 190-bp fragment of the *CAPN4* gene in Iranian Zel sheep, which included the part of exon 5, all of intron 5, and part of exon 6. In this fragment, the authors identified two banding patterns named *AB* and *AA*, which were associated with yearling weight ($P<0.01$), with the *AB* genotype exhibiting a higher average yearling weight value (30.37 kg) than *AA* (27.91 kg). Posteriorly, Mahrous et al. (2016) sequenced this same variant in Barki, Rahmani, and Ossimi sheep breeds and found a *C>T* nucleotide substitution in intron 5, which was associated with BW, FW, and ADG being higher average values observed in the *TT* genotype. In Polish Merino sheep, an association of this same region of *CAPN4* with intramuscular fat, drip loss, and lightness (L*) of meat were also reported (Grochowska et al., 2017).

In bovines, *CAPN1* has been identified as an important candidate gene for tenderness (Hou et al., 2011; Ramayo-Caldas et al., 2016; Leal-Gutiérrez et al., 2018) and intramuscular fat (Cheong et al., 2008; Hou et al., 2011; Barendse, 2011). Moreover, some variants in this gene have been associated with growth traits in beef cattle. The *CAPN1* 316 is a cytosine/guanine (C>G) variant in exon 9 of bovine *CAPN1* gene and was associated with ADG and BW in Brangus cattle; however, no significant effect was found for FT and REA (Miquel et al., 2009). Moreover, Pratiwi et al. (2016) sequenced exons 5-6 of Bali cattle and found variants associated with rump thickness, rump FT, and marbling score. Additionally, variant *rs196951250* in intron 3 was associated with REA in Polish Large White pigs and FT in Duroc pigs (Ropka-Molik et al., 2017).

The variant *ss494474890*, located in intron 5 of the *CAPN1* gene, was associated with BW at 35 and 42 days of age, while also being associated with thigh weight, breast weight, and carcass weight in a chicken F2 population (Felício et al., 2013). In a previous study, a 212-bp fragment from the 3'UTR region was amplified by PCR-SSCP, and two alleles (*A1* and *A2*) were identified (Zhang et al., 2007). An additive effect was found for BW, carcass weight, breast muscle weight, abdominal

fat percentage, leg muscle weight, leg muscle percentage, and breast fiber density, being the *A1* allele associated with lower average values of abdominal fat percentage and higher average values of other traits (Zhang et al., 2007). Moreover, haplotypes formed with variants in exons 5, 6, and 16 of the *CAPN1* gene were associated with live weight, carcass weight, breast muscle weight, leg muscle weight, eviscerated percentage, and breast muscle fiber density in chicken (Zhang et al., 2008). Variants in *CAPN1* were also associated with broiler carcass traits such as breast muscle percentage (*g.30419210G<A*), abdominal fat weight (*g.30422873C<A*), leg muscle percentage, BW and carcass weight (*g.30425625G<A*) (Zhou et al., 2017).

Notably, some *CAPN1* variants observed in the present study fall within novel genes, whose function remains unknown. The variants *rs417258958*, *rs408790217*, *rs420860201*, *rs403953588*, and *rs430307080* all fall within the sequence of a novel gene (*ENSOARG00000022588*), whose transcript is a small RNA with 77 bp, whereas the variants *rs408790217*, *rs420860201*, *rs403953588*, and *rs430307080* fall within the sequence of another protein-coding gene (*ENSOARG00000013831*) with 11 exons and 494 amino acids. In sheep, only one miRNA (MI0025261) on chromosome 21 was reported on the miRbase.org database. However, 12 miRNAs that are highly expressed in the skeletal muscle of sheep can play a vital role in muscle development (Sheng et al., 2011). Therefore, studying the effects of both miRNA and alternative protein-coding can assist in clarifying the effects of these intronic variants on the growth and carcass characteristics of Santa Inês sheep.

The present study revealed several suggestive effects of variants in both *CAPN1* and *CAST* genes on many body traits in Santa Inês sheep. For selection purposes, a validation study using a different sample of Santa Inês sheep is necessary. Furthermore, sequencing a larger fragment of these genes would help to identify the causal mutations.

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