Characterisation of muscle calpain system SNPs and associations with production and beef quality traits in South African beef cattle following electrical stimulation and extended ageing

by

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Declaration

I, Annie Basson (UP student 02590190), declare that:

1. This research was original work conducted by me, as part of the requirements for the PhD (Animal Science) degree in the Department of Animal Science at the University of Pretoria.

2. I understand what plagiarism means and the rules and policies of the University of Pretoria in this regard and any work that was not original, was properly referenced within the text.

3. All protocols and procedures were conducted in an ethical manner and approved by the Agricultural Research Council, Animal Production Ethics Committee (ref no. APIEC11/025 and ARC AEC-I 2010 001) and the Animal Ethics Committee of the University of Pretoria (EC171114-161 and EC171114-162).

Signature 

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"This is where we've filled ourselves up with so many questions that they're overflowing and become answers."

Sir Terry Pratchett

Abstract

The genes of the muscle calpain system are the most important genes that affect beef tenderness and research on associations of these genotypes with beef quality traits in South African beef cattle is limited. Beef quality traits often have a large environmental component and it is important to minimize environmental biases for accurate analyses, while shorter distances between single nucleotide polymorphisms (SNPs) enable the identification of more informative variants across breeds. We therefore used the Illumina BovineHD (777K) SNP array to genotype bulls from five selected South African purebred beef breeds (Angus, Bonsmara, Brahman, Charolais and Nguni) for the *capn1*, *capn2*, *capn3* and *cast* genes. Detailed carcass and beef quality traits were determined, while maintaining a constant environment for finishing, carcass handling and sample analyses. Characterization of the calpain system genes revealed a high proportion of alleles favourable for tenderness in Bonsmara cattle (originally a Sanga-type composite) and an opportunity to improve the average frequency of tender alleles in Nguni cattle (Sanga, *Bos taurus africanus*). Extensive linkage was identified in the *cast* and *capn2* genes with regions of high linkage disequilibrium between breeds. This presents an opportunity for across-breed selection for candidate SNPs in overlapping haplotype blocks. The identification of candidate SNPs with mixed model association analyses (GCTA software) identified 62 putative associations with Warner-Bratzler shear force (WBSF) or myofibril fragment length (MFL), while only six of these SNPs (especially in the *cast* and *capn2* genes) affected these tenderness phenotypes over extended meat ageing periods. Most candidate SNPs (>80% of associations) affected certain stages of the meat ageing period and were unable to sustain the genetic effect over the entire meat ageing period of 20 days, confirming that the altered physiological mechanisms of extended aging are subject to effects from different genetic markers. Several quantitative trait nucleotides (QTNs) were identified in both the non-electrically stimulated (NS) and the electrically stimulated (ES) treatment groups using a different mixed model approach, more suited to these data (GAPIT package in R software). Associations of SNPs with tenderization, estimated by MFL at various stages of meat ageing, were closely linked to associations of SNPs with decreased calpastatin inhibitory effects on proteases (in linkage blocks). All four genes of the calpain system contained QTNs for calpastatin inhibition of proteases (ten in total) and the tenderizing effects of the SNPs in these genes were primarily found for MFL, where 13 QTNs were identified in both control and ES groups, with some SNPs sustaining effects for extended meat ageing periods. The effects of electrical stimulation on SNP associations with traits failed to demonstrate a universal effect across SNPs or traits. Although many SNP effects were only slightly affected by treatment, other SNP effects were observed in only one of the treatment groups, where electrical stimulation either enhanced or eliminated genetic associations. Novel QTNs for intermediary energy metabolism were identified, with four of these SNPs located in the *capn2* gene that could explain previous data linking pH to these genes. This provides the link between calpain protease activity (through energy metabolic pathways) and pH decline that will favour sarcoplasmic Ca^{2+} release, facilitating more rapid tenderization. These results validate the SNPs of the calpain-calpastatin system for selection to improve myofibril fragmentation in South African beef bulls and highlight the importance of tenderizing interventions, when determining genomic associations. Furthermore, these data confirmed the complex effects of calpain protease activity in cellular homeostasis that can also alter metabolic processes, though pleiotropic effects of these genetic markers.

Publications

International congress poster presentations

1. Basson, A., Frylinck, L., Anderson, J., van Heerden, S. M. & van Marle-Köster, E., 2016. Effects of SNPs in the calpain-1 and calpastatin genes on tenderness in South African crossbred beef cattle (Poster P04-36). Proceedings of the 62nd International Congress of Meat Science and Technology (ICoMST), Bangkok, Thailand, Book of Abstracts p. 57. http://icomst-proceedings.helsinki.fi/papers/2016_04_36.pdf

2. Basson, A., Frylinck, L. & Strydom, P. E., 2018a. Association of genomic markers (SNP) in the cast gene with tenderness in South African purebred beef cattle (Poster 1B30, #6505). Proceedings of the 64th International Congress of Meat Science and Technology (ICoMST), Melbourne, Australia, http://icomst-proceedings.helsinki.fi/papers/2018_03_11.pdf

3. Frylinck, L., Basson, A. & Strydom, P. E., 2018b. Association of genomic markers (SNP) in the capn-1 gene with tenderness in South African purebred beef cattle (Poster 1B31, #6520). Proceedings of the 64th International Congress of Meat Science and Technology (ICoMST), Melbourne, Australia, http://icomst-proceedings.helsinki.fi/papers/2018_03_12.pdf

4. Basson, A., Frylinck, L. & Strydom, P. E., 2019. Polymorphisms in the genes of glycolysis are associated with beef production and quality traits in South African purebred beef bulls (Poster #537). Proceedings of the 65th International Congress of Meat Science and Technology (ICoMST), Potsdam, Germany, http://icomst-proceedings.helsinki.fi/papers/2019_04_10.pdf

Peer-reviewed scientific articles (Chapter 4)

5. Basson, A., Strydom, P. E., van Marle-Köster, E., Webb, E. C. & Frylinck, L., 2022. Sustained effects of muscle calpain system genotypes on tenderness phenotypes of South African beef bulls during ageing up to 20 days. Animals 12(6)686.1-25. https://www.mdpi.com/2076-2615/12/6/686

Chapters to be prepared for peer-reviewed publication (Chapter 3, Chapter 5)

6. Characterization of the muscle calpain system genes in five South African purebred beef cattle breeds: The potential for selection across breeds.

7. Quantitative trait nucleotides of the muscle calpain system genes for myofibril fragmentation, calpastatin inhibition of proteases and energy metabolites in five South African beef breeds.

Popular publications

8. Basson, A. & Frylinck, L., 2018. Sag, sappig, eg-Suid-Afrikaans: Inheemse rasse toon genetiese potensiaal vir sagte vleis. Red Meat / Rooivleis 9(6):68-73. http://www.agriconnect.co.za/red-meat-digital-magazines

9. Basson, A., 2018. Genetika van vleisbeeste nagevors; Angus, Bonsmara, Brahman, Charolais & Nguni. RSG Landbou, 30 March 2018, (04:45), Radio Sonder Grense (RSG), 100 - 104 FM. http://www.elsenburg.com/radio-elsenburg/2018-03-30/rsg-landbou-vrydag-30-maart-2018.

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List of abbreviations

777K	Illumina® BovineHD SNP BeadChip (total 777,962 variants)
°C	degrees Celsius
µℓ	microliter
µM	micromolar (µmol/ℓ)
µm	micrometre
µmol	micromole
µmol/g	micromole per gram
a*	redness (+60=red, -60=green)
A*/G*/C*/T*	asterisk indicates alleles that were found to be favourable for tenderness
A2, A3	carcass classification (A = zero permanent incisors, 2 = lean to 3 = medium fatness)
A_{230} - A_{620}	absorbance at wavelengths varying between 230 - 620 nm
ADG	average daily gain
AGS	amyloglucosidase
An	Angus bulls
ANOVA	analysis of variance (a SAS procedure)
AOAC	Association of Official Analytical Chemists International
ARC-AP	Agricultural Research Council - Animal Production
ARC-BTP	Agricultural Research Council - Biotechnology Platform
ARC-OVI	Agricultural Research Council - Onderstepoort Veterinary Institute
ATP	adenosine triphosphate
b*	yellowness (+60=yellow, -60=blue)
Bh	Brahman bulls
BHT	butylated hydroxytoluene
BLINK	Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway
Bo	Bonsmara bulls
bp	base pairs
Br	breed effect in statistical analyses
BTA	*Bos taurus* autosome (see Chr)
BW	body weight, or final live weight before slaughter
C*	chroma or saturation index
C-3, C-6	three-carbon or six-carbon (substrates for glycolysis)
Ca^{2+}	calcium ions
$CaCl_2$	calcium chloride
CANP	calcium-activated neutral protease
CAPN	the calpain protein in humans
CAPN	the calpain gene in humans
capn	animal calpain protein
capn	animal calpain gene
capn1	the animal calpain-1 gene
capn2	the animal calpain-2 gene
capn3	the animal calpain-3 gene
capns	the animal gene for the small subunit of the calpain protease
CAST	the calpastatin protein in humans
CAST	the calpastatin gene in humans
cast	animal calpastatin protein
cast	animal calpastatin gene
CCW	cold carcass weight
CDP	calcium-dependent protease
Ch	Charolais bulls
Chr	chromosome (all calpain system genes were autosomal)
CIE	Commission Internationale de l'Éclairage

cm^3	cubic centimetre
$covar_{Br}$	fixed effect of breed in statistical analyses
CP	crude protein
CrP	creatine phosphate
CysPc	calpain-type cysteine protease conserved motif
D'	Lewontin's D-prime (measure of linkage disequilibrium)
d	days (post-mortem)
DAS	Department of Animal Science
DFD	dark, firm and dry (a condition of unbalanced glycogenolysis in meat) see PSE
dH_2O	distilled water
DM	dry matter
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
DTI-THRIP	Department of Trade and Industry-Technology and Human Resources for Industry Programme
EDTA	ethylene diamine tetra-acetic acid
EMA	eye muscle area
ES	electrical stimulation
FarmCPU	Fixed and random model Circulating Probability Unification software
FDR	False Discovery Rate
G6P	glucose 6-phosphate
G×E	interaction between genotype and environment that determines phenotype
g	gram
g	gravitational acceleration (9.81 m/s^2), conversion factor for kg to N [or random effect of all SNP in statistical analyses]
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAPIT	Genome Association and Prediction Integrated Tools software
GC score	Illumina® GenCall score ("no call" cut-off threshold for successful genotyping)
GCTA	Genome-wide Complex Trait Analysis software
Ge	genotype effect in statistical analyses
GeBr	interaction of breed with genotype in statistical analyses
GeTmt	interaction of treatment with genotype in statistical analyses
GLM	generalized linear model
GLUT4	the insulin-responsive glucose transporter 4 of skeletal muscle and adipose tissues
GWAS	genome-wide association study
h	hour
H1*/H2/H3	linkage block haplotype 1 (all favourable alleles) / haplotype 2 / haplotype 3 (more unfavourable alleles)
h_{ab}	hue angle or the fundamental colour / discoloration
HCW	hot (warm) carcass weight
HD	high-density (a term made redundant for microarrays, since the wide application of sequencing)
H*e*	expected heterozygosity
H*o*	observed heterozygosity
Hz	Hertz or the frequency of electrical current flow (cycles per second)
ICoMST	International Congress of Meat Science and Technology
IGF	insulin-like growth factor
IMF	intramuscular fat
kb	kilo-bases (base pairs)
kDa	kilo-Dalton
kg	kilogram (alternative unit for Warner-Bratzler shear force, see Newton)
kg/d	kilogram per day
L*	lightness (0=black, 100=white)
L6	6^{th} lumbar vertebra
LD	linkage disequilibrium
λ_{max}	the wavelength at which maximum absorbance occurs (e.g. 280 nm for protein)

LSM	least squares means
LTL	*longissimus thoracis et lumborum* (lumbar and thoracic portions of the muscle)
m^3/h	cubic meter per hour
MA/A1	minor allele (generally designated A1)
MAF	(empirical) minor allelic frequency
MAS	marker-assisted selection
MFL	myofibril fragment length
mg	milligram
MIT	Meat Industry Trust
MLM	Mixed Linear Model
MLMA	Mixed Linear Model Association
MLMM	Multi-locus Linear Mixed Model
mm	millimetre
mm^2	square millimetre
mm/min	millimetre per minute
ms	millisecond
MSA	Meat Standards Australia
N	Newton, SI unit for Warner-Bratzler shear force
$N_{2\ell}$	liquid nitrogen
NAS	Faculty of Natural & Agricultural Sciences
NCBI	National Center for Biotechnology Information (of the US National Library of Medicine)
ng/µℓ	nanogram per microliter
Ng	Nguni bulls
NIRS	near-infrared spectroscopy
nm	nanometre
NS	non-electrically stimulated (equivalent to the control in treatment groups)
NS + ES	pooled data for bulls in control plus ES treatment
PC	principal component
PCA	principal components analysis
PFK	phosphofructokinase
pH_u	ultimate tenderness
P_{HWdev}	probability value for the Hardy-Weinberg exact test
p*I*	pH at which the iso-electric point of muscle protein is reached
p*Ka*	$-\log_{10}$(dissociation constant), indicates the strength of acids
PKC	protein kinase C (Ca^{2+}-dependent protein kinase)
PSE	pale, soft and exudative (condition of unbalanced glycogenolysis in meat), see DFD
psi	alternative pressure unit (pounds of force per square inch)
QQ-plot	quantile-quantile plot
QTL	quantitative trait locus
QTN	quantitative trait nucleotide(s)
r^2	correlation between allele genotypes and different loci (measure of linkage disequilibrium)
r_g	genetic correlation coefficient
RMRDSA	Red Meat Research and Development South Africa
RSA	Republic of South Africa
RSG	Radio Sonder Grense
ryr1 (RyR)	ryanodine receptor gene 1 (the ryanodine receptor)
SAS	Statistical Analysis System (SAS Institute)
SCE	spectral component excluded (colour measurements)
SE	standard error
SL	sarcomere length
SNP	single nucleotide polymorphism
SNV	single nucleotide variant, or "SNP" that are not polymorphic
T°	temperature

T9 – T10	9th and 10th thoracic vertebrae
Tmt	treatment effect in statistical analyses
TnI	troponin I
TnT	troponin T
U	units (relative measurement of calpain system protein activity / inhibition)
UMD	University of Maryland Reference Genome (initiated in 2014)
UP	University of Pretoria
USA	United States of America
UTR	untranslated region
V	volt
VIA	video image analysis
VSGRM	variance-standardized genomic relationship matrix
w/w	weight per weight, or weight percentage
WBSF	Warner-Bratzler shear force
WHC	water-holding capacity
$ZnCl_2$	zinc chloride

Chapter 1: Introduction

Meat quality is a generic term referring to the characteristics (properties and perceptions) of meat and the most important components that contribute to the consumer's perception of a good cut of beef (eating quality) include tenderness, colour, texture, flavour and juiciness (Koohmaraie & Geesink, 2006). These characteristics interact with one another (Thompson, 2002; Koohmaraie & Geesink, 2006; Kemp & Parr, 2012), because the processes that establish the transition from muscle to meat, affect several of the quality attributes of beef simultaneously (Ouali *et al.*, 2006; Kemp *et al.*, 2010). It is therefore important that the complex, interacting factors that determine beef quality are understood when determining associations of genomic markers, to determine accurate phenotypic traits. Environmental factors should be defined and variation between ante– and post-mortem conditions should remain minimal, because factors that affect the complex progression of beef quality phenotypes, could alter their genetic associations with traits (Johnston & Graser, 2010; Mazzucco *et al.*, 2010; Gruber *et al.*, 2011).

Tenderness is a critically important component of meat quality, especially in beef, where it is of greater concern than in mutton or pork (Dransfield *et al.*, 1981; Koohmaraie *et al.*, 1991). Rather than being graded for beef quality attributes such as tenderness, South African beef is classified according to age, fatness and conformation that have a limited value in predicting the eating quality of the meat produced (Strydom, 2011). The major driver for beef producers is therefore to increase beef yield of carcasses that provide the best market price (Strydom *et al.*, 2015), with little incentive to improving beef quality. This is a worldwide trend where the last few decades has seen an increase in carcass mass (Terry *et al.*, 2020), in part due to improved nutrition and in part due to selection for larger carcasses. In recent years it has been proved that South African consumers are willing to pay a premium for an improved eating experience, for example Wagyu beef (Coleman, 2009; Roets, 2019). The ability to guarantee tenderness (and other beef quality traits) or to identify and predict significant toughness (Koohmaraie *et al.*, 1995; Hocquette *et al.*, 2012), will therefore be an important challenge to the South African beef industry. From the example of the Australian grading system, it is clear that these predictions of the complex interacting beef quality traits, require data of a similarly complex nature (Thompson, 2002).

Since beef quality traits generally have a large environmental component (Minick *et al.*, 2004; Smith *et al.*, 2007), interventions to accelerate and improve tenderness are commonly applied, such as electrical stimulation (ES), tenderstretch and meat ageing (Thompson *et al.*, 2006; Frylinck *et al.*, 2015; Mohan *et al.*, 2020). Considerable research is being conducted on technologies that can improve tenderization through disruption of muscle structural organization (Warner *et al.*, 2017, 2022), such as ultrasound (Bhargava *et al.*, 2021), pressure and exogenous proteases to increase tenderness and decrease the variation between carcasses (Warner *et al.*, 2017, 2022). Interventions that aim to accelerate meat ageing (Taylor & Cornell, 1985) such as electrical stimulation (Taylor & Cornell, 1985; Ferguson *et al.*, 2000; Johnston *et al.*, 2001) and tenderstretch (Thompson *et al.*, 2006; Wolcott *et al.*, 2009) are generally thought to ameliorate genetic effects of breed (or breed type) between carcasses. In order for genomic markers (SNPs) to be effective in selection (or prediction of beef quality), the research results need to be repeatable in commercial beef, result in an economically viable

increase in the quality of beef or decrease in tenderness (Dekkers, 2004), in the face of existing tenderizing interventions. Ultimately, the goal should be to produce guaranteed-tender beef, as soon as possible post-mortem, with minimal storage or meat ageing (Warner *et al.*, 2017). Due to the (cost) effectiveness of tenderizing interventions and the lack of predictive power of genetic markers between different beef breeds (Magolski *et al.*, 2013; Kim *et al.*, 2014), genomic selection in beef has not been widely used in commercial beef production (Georges *et al.*, 2019). In early research using a limited number of markers, it seemed that the genetic effects of SNP markers on traits were decreased by tenderizing interventions such as tenderstretch (Johnston & Graser, 2010; Robinson *et al.*, 2012) and ageing (Mazzucco *et al.*, 2010; Gruber *et al.*, 2011), or by growth promotants (King *et al.*, 2012; Robinson *et al.*, 2012). However, other authors have found that the interaction between genomic markers and meat ageing or electrical stimulation are additive, increasing the effects of markers on traits (Pinto *et al.*, 2011). These effects seemed to be SNP– or trait-dependent, with electrical stimulation preventing genetic effects on traits such as growth and fatness (Riley *et al.*, 2019), decreasing the associations with Warner-Bratzler shear force (WBSF) and trained panel sensory evaluation (Riley *et al.*, 2020a), but enhancing the associations between SNP and flavour scores (Riley *et al.*, 2020b). It therefore seems that variable meat ageing or electrical stimulation should not be considered equivalent (Morris *et al.*, 2006) in genomic association studies, because the physiological processes that govern the conversion of muscle to meat are accelerated by electrical stimulation, while the mechanisms of tenderization change as meat ageing progresses and can alter genomic associations (De Souza Fonseca *et al.*, 2022).

The investigation of genomic markers in different populations or for different traits is still an active area of research (Leal-Gutiérrez *et al.*, 2020; Gagaoua *et al.*, 2022). It is increasingly combined with other "–omics" investigations to elucidate the activity of genes (transcriptomics), the dynamics of their proteins and the functional properties of these proteins within cells (proteomics and metabolomics) to understand, not only the effect of genotypes, but also the complex physiological processes that determine the effectiveness of these genotypes. The genes of the muscle calpain system have been identified as the most important genes to affect beef tenderization, while few of these SNPs have been validated in South African beef cattle (Frylinck *et al.*, 2009). The calpain-calpastatin system was originally investigated for the *in situ* physiological functions of muscle development and myofibrillar protein degradation that is normally under strict control. Due to their diverse physiological functions in maintaining cellular homeostasis (Goll *et al.*, 2003; Sorimachi *et al.*, 2010, 2011; Kemp *et al.*, 2013; Ono *et al.*, 2016), these genes are known for their pleiotropic effects that go well beyond the degradation of proteins during post-mortem tenderization. The most extensively researched traits (established associations with the calpain-calpastatin system) are lipid content and composition of the carcass and traits related to animal growth and body conformation. SNPs of the calpain-cast system have been linked to total intramuscular fat content (Schenkel *et al.*, 2006; Zhang *et al.*, 2012; Iso-Touru *et al.*, 2018), fat depth (Collis *et al.*, 2012), marbling score (Cheong *et al.*, 2008; Li *et al.*, 2013) and fatty acid profile (Collis *et al.*, 2012; Hawken *et al.*, 2012; Zhang *et al.*, 2012), as well body weight (Zhang & Li, 2011; King *et al.*, 2012), body conformation (Gill *et al.*, 2009; Zhang & Li, 2011), lean yield (Juszczuk-Kubiak *et al.*, 2004b) and growth (King *et al.*, 2012). Additionally, extensive research has been conducted on the role of the calpains and calpastatin in determining several components of meat colour in both pork (Otto *et al.*, 2007; Ropka-Molik *et al.*, 2014) and beef, (Juszczuk-

Kubiak *et al.*, 2004b; Reardon *et al.*, 2010; Pinto *et al.*, 2011; Iso-Touru *et al.*, 2018) and they have been linked to the water-holding capacity of meat (Juszczuk-Kubiak *et al.*, 2004a; Cafe *et al.*, 2010; Ribeca *et al.*, 2013). The calpains affect beef eating quality (Casas *et al.*, 2006; Robinson *et al.*, 2012; Rezende *et al.*, 2021) and intramuscular connective tissue (Jiang *et al.*, 2009; Leal-Gutiérrez *et al.*, 2020). There has also been a drive to improve the efficiency of beef production and the calpain-calpastatin system SNPs have been linked to feed efficiency traits like residual feed intake (Riggs & Vaughn, 2015; Abo-Ismail *et al.*, 2018; Vaughn *et al.*, 2022) and feed conversion ratio (Ardicli *et al.*, 2019). The genes of the calpain-calpastatin system have been linked to reproductive traits like puberty (Collis *et al.*, 2012), pregnancy rate (Garcia *et al.*, 2006; Galliou *et al.*, 2020) days open and conception rate (Hill *et al.*, 2016; Obando, 2017), as well as environmental adaptation in taurine and Sanga-type breeds (Dikmen *et al.*, 2013, 2015; Kooverjee *et al.*, 2022).

Research aims and objectives

- To determine detailed, accurate phenotypes for growth, carcass and beef quality traits under carefully controlled conditions, to minimize variations in finishing, slaughter procedures, post-mortem carcass handling and laboratory analyses.
- To characterize the SNPs of the Illumina® BovineHD SNP BeadChip (total 777,962 variants) in South African purebred beef bulls (Chapter 3).
- To determine the level of linkage between SNPs of the genes of the calpain-cast system (134 SNPs of the reference transcripts) and the haplotype block structure of these genes, across and within breed types (Chapter 3).
- To validate the SNPs of the calpain-calpastatin system in South African purebred beef bulls for growth, carcass and beef quality traits, measured under carefully controlled protocols to minimize environmental variation. To determine (novel) associations of candidate SNPs with beef quality traits in South African beef cattle breeds (Chapters 4 and 5).
- To determine whether extended meat ageing diluted the genetic effects of genomic markers (SNPs), in a manner similar to breed differences that diminish as the meat ageing period increases (Chapter 4).
- To determine whether the accelerated meat ageing rate induced by electrical stimulation of carcasses, attenuated SNP effects on beef quality traits (Chapter 5).

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Chapter 2: Literature review

2.1. Beef tenderness

Increased tenderness during carcass ageing involves weakening of the myofibrillar structure of muscle fibres through degradation of myofibrillar and other proteins that disrupts the highly organized structure of skeletal muscle fibres (Culler *et al.*, 1978; Koohmaraie *et al.*, 1987; Koohmaraie, 1992a). This is achieved by proteases that must be endogenous (available and active in the sarcoplasm), able to mimic post-mortem changes *in vitro* and have access to myofibrils in meat (Koohmaraie, 1992a; Taylor & Koohmaraie, 1998). Calpains are sometimes considered as the only proteases of muscle that fit all of these criteria (Hopkins & Thompson, 2002; Geesink *et al.*, 2006; Koohmaraie & Geesink, 2006), but other proteolytic systems (Ouali & Valin, 1981; Herrera-Mendez *et al.*, 2006) and physical changes in muscle organization (Hwang *et al.*, 2003; Warner *et al.*, 2017) also make important contributions to tenderization (Table 2.1). Other tenderness attributes, in addition to structural organisation (measured by Warner-Bratzler shear force (WBSF) and myofibril fragment length (MFL)), include the intramuscular connective tissues and sarcomere length, affecting tenderness against the background of post-mortem glycolytic activity, pH decline and temperature changes (Bate-Smith, 1948). This includes what has often been called "background toughness" (connective tissue and muscle organization), processes of the toughening phase (rigor, sarcomere shortening) and proteolytic tenderization, which is a particularly important factor in the *longissimus* muscle (Koohmaraie *et al.*, 2002; Koohmaraie & Geesink, 2006). However, differences in tenderization in different muscles are under the control of different genes, as different quantitative trait loci (QTLs) for meat ageing were identified in *longissimus thoracis et lumborum vs*. *semitendinosus* muscles (Chang *et al.*, 2014).

The level of uncertainty of tenderness in the animal, before the meat is consumed, is unacceptably high (Koohmaraie *et al.*, 2003), with no accurate test to predict the eventual tenderness of beef from estimates in the live animal. Research must find a way to accurately predict tenderness (or select animals that consistently produce tender beef) and supply high quality meat to consumers (Meadus, 1998; Burrow *et al.*, 2001; Koohmaraie & Geesink, 2006). The grading system used by Meat Standards Australia (MSA) attempts to overcome these challenges through a complex model of predictive parameters, taking into account several animal, environmental, post-slaughter and cooking conditions that affect consumer eating quality of different cuts, but is still striving towards a more simplified method of prediction (Thompson, 2002; McGilchrist *et al.*, 2019). However, simpler models integrating molecular technologies or non-invasive measurements accurately predict some quality aspects, but generally fail to benefit the entire beef production chain (Berri *et al.*, 2019).

Another challenge in beef research is that, traditionally, the *longissimus thoracis et lumborum* (LTL) has been widely used as a standard because the muscle is easy to excise and offers a large sample size of convenient steaks (Shackelford *et al.*, 1995). The LTL has a relatively low collagen content (2.8%), making it less prone to toughening with increasing animal age (Harper, 1999), but is not representative of the collagen characteristics of the rest of the carcass (Li *et al.*, 2022b). It is suited to studying protease activities, in fact, the *longissimus* muscle is subject to a large proteolytic influence on tenderness (Koohmaraie & Geesink, 2006). It has relatively short sarcomere lengths (Dransfield, 1977), which could predispose it to cold-shortening, especially considering its

anatomical location on the surface of the carcass. Being a long muscle stretching almost the entire dorsal length of the carcass, gives it a high surface area and makes it highly susceptible to the immediate conditions that the carcass is exposed to, for example extreme chilling (Bendall, 1975), depending on the level of insulation from surface (subcutaneous) adipose tissue (Koohmaraie *et al.*, 1988). The LTL muscle is rich in white fibres (48%), with relatively similar proportions of intermediate (24%) and red (28%) muscle fibres (Seideman *et al.*, 1987). A strong correlation exists between objective and subjective tenderness measures of the *longissimus* muscle, but it does not accurately predict the tenderness of other muscles of the carcass (Schönfeldt & Strydom, 2011) and exaggerates the "toughness" of *Bos indicus* types (Shackelford *et al.*, 1995). With the advantages (or rather, convenience) of using this muscle and the large amount of data that is available for comparison with previous research, the meat science industry will most likely continue using the LTL for research purposes, despite its limitations.

Table 2.1. *Some of the most important factors that determine tenderness*

Factors that increase tenderness	**Reference(s)**
Protease activity, especially ↑calpain:↓calpastatin	(Shackelford *et al.*, 1991)
Myofibril fragmentation	(Culler *et al.*, 1978)
Longer meat ageing	(Hoagland *et al.*, 1917)
Carcass chilling rate	(Hoagland *et al.*, 1917; Savell *et al.*, 2005)
Electrical stimulation	(Harsham & Deatherage, 1951; Carse, 1973)
Intermediate glycolytic rate linked to interactions with temperature	(Newbold & Scopes, 1967) (Marsh *et al.*, 1987)
Factors that increase free, sarcoplasmic Ca^{2+} depletion of ATP addition of ions (e.g. $CaCl_2$)	(Takahashi *et al.*, 1987)
Gender (female / castrated *vs*. bulls)	(Wu *et al.*, 1985)
Antioxidants	(Rowe *et al.*, 2004)
Tenderstretch	(Hostetler *et al.*, 1972)
Muscle fibre disrupting technologies	(Parrish Jr., 1977)
Factors that decrease tenderness	
Rigor mortis	(Holburn, 1900; Newbold & Harris, 1972)
Older animal age (collagen cross-linkage)	(Hill, 1966; Sinex, 1968)
pH>6.0 @ temp <12°C	(Locker & Hagyard, 1963; Bendall & Taylor, 1972; Pearson & Young, 1989)
pH<6.0 @ temp >35°C	(Marsh, 1954; Dransfield, 1993)
Electrical stimulation (improperly applied)	(Dutson *et al.*, 1982; Hertzman *et al.*, 1993)
Short sarcomere lengths (extreme chilling)	(Locker, 1960)
β-adrenergic agonists	(Koohmaraie *et al.*, 1991a)
Poor temperament or excitability (stress)	(King *et al.*, 2006)
Protease inhibitors for example EDTA (Ca^{2+} chelator) and $ZnCl_2$	(Koohmaraie, 1989)
Bos indicus (compared to *Bos taurus*)	(Whipple *et al.*, 1990; Ferguson *et al.*, 2000)
Larger fibre diameter	(Ouali & Talmant, 1990; Crouse *et al.*, 1991)
Extensive production system (or low energy feeds)	(Esterhuizen *et al.*, 2008; Frylinck *et al.*, 2013)

2.1.1. Intramuscular connective tissue (collagen)

Muscle connective tissue (Bowman, 1840) is mainly composed of collagen (López-Bote, 2017) and, because it lends structural support to muscle tissues (Purslow, 2020), presents with a wide range of collagen proportions in meat (Purslow, 2017), depending on specific muscles' functional workload (Purslow, 1999). The collagen content and collagen solubility of *longissimus thoracis et lumborum* is similar between species in beef, pork or lamb (Li *et al.*, 2022b), where collagen of the perimysium accounts for 90% of intramuscular connective tissue (Koohmaraie *et al.*, 1991b) and this represents the robust collagen of beef, contributing to toughness (Purslow, 2018). Collagen facilitates muscle contractions by generating elastic forces, assisted by a small amount of elastin (Neuman & Milan, 1950), but also by absorbing force as its fibres transmit force between fascicles, forming structurally enforced cross-linkage within the working muscle (Tanzer, 1973; McCormick, 1994; Purslow, 2020). These cross-linkages are an important factor in tenderness.

An increase in age of animals does not result in an increase in total collagen content, but rather a decrease in the heat-labile proportion of total collagen (Hill, 1966; Schönfeldt & Strydom, 2011), where collagen fibres increase in thickness and organization with increasing animal age (Bailey & Shimokomaki, 1971; Purslow, 2005). This results in tougher meat, as collagen becomes more robust and less elastic (Jackson & Bentley, 1960; Sinex, 1968; Nishimura *et al.*, 1999) and was thought to be the only cause of meat toughness well into the 20th century (Meara, 1947). The number of heat-stable, covalent, cross-linkages of collagen fibres (Tanzer, 1973; McCormick, 1994; Harper, 1999) can have a large effect on the tenderness of the meat (Lepetit, 2007), due to the fact that innate proteases are not thought to significantly degrade connective tissue proteins during post-mortem meat ageing (Koohmaraie *et al.*, 1987). This not only leads to significantly tougher meat (Koohmaraie, 1992a; McCormick, 1994), but also a general decline in quality characteristics of meat produced by older animals (Perry & Thompson, 2005). The contribution of collagen to tenderness as a quality attribute of steaks is relatively large in muscles such as the *biceps femoris* and *semimembranosus* (Koohmaraie *et al.*, 2002) that have higher collagen levels and cross-linkages (Lepetit, 2007) and is closely linked to their functional workload (Purslow, 1999). Many researchers consider collagen to be a passive, "background toughness" effect on beef quality (Ouali *et al.*, 1992; Sentandreu *et al.*, 2002). However, the changes induced in collagen as a result of acidic condition (Meara, 1947), electrical stimulation (Savell *et al.*, 1977; Judge *et al.*, 1980; Mikołajczak *et al.*, 2019), post-mortem degradation (Stanton & Light, 1988; Nishimura *et al.*, 1996; Purslow, 2018) and heat (Tornberg, 2005), make it difficult to study its effects on tenderness using WBSF (Purslow, 2005, 2014). This technique generally includes ageing of steaks and cooking of meat, often by rapid grilling beyond levels where collagen denaturation occurs (Purslow, 2018).

Cooking meat, especially at higher temperatures (rapidly), results in increased tensile strength of connective tissues, coupled to shrinkage as a result of collagen denaturation (Hostetler & Landmann, 1968), which may occur at temperatures as low as 50°C for collagen of the endomysium (Bendall & Restall, 1983; Harper, 1999; Dominguez-Hernandez *et al.*, 2018). These attributes that could decrease meat quality, increase progressively above 64°C for collagen of the perimysium (Bendall & Restall, 1983; McCormick, 1994; Dominguez-Hernandez *et al.*, 2018), but will depend greatly on cooking methods and a range of poorly defined animal factors (Purslow, 2018). This innate inability to study the effects of collagen on tenderness using traditional methods

remains a challenge to research and the use of animals of a similar physiological age should limit phenotypic variation, allowing for more accurate genomic predictions. Although SNPs of the muscle calpain-calpastatin system failed to associate with collagen content or solubility in beef (Dunner *et al.*, 2013; Sevane *et al.*, 2014), sensory panel scores for connective tissue were linked to SNPs in the *capn3* (Jiang *et al.*, 2009) and *capn1* (Leal-Gutiérrez *et al.*, 2020) genes. However, genetic associations of SNPs with collagen, generally involve collagen content, rather than cross-linkage (Lei *et al.*, 2018) and alternative technologies are more likely to be applied by industry. Many new (often plant-based) proteases are being investigated as alternatives to tenderizing meat (Mohd Azmi *et al.*, 2023) with papain-like actions, but with fewer of the potential negative effects, like over-tenderizing beef causing an unpleasant mouth-feel (Gagaoua *et al.*, 2021). These include proteases with more controlled hydrolytic activities like actinidin and zingibain (Gagaoua *et al.*, 2021) and extracts from mushrooms, cashews, mango and cassava (among others), where collagen degrading proteases can have the potential to improve the tenderness of older carcasses (Bhat *et al.*, 2018b; Chanalia *et al.*, 2018).

2.1.2. Sarcomere length (carcass temperature and pH decline)

In addition to intramuscular connective tissue, the shortening of sarcomeres during the rigor phase can have overwhelming effects on tenderization of beef and is mainly determined by post-slaughter conditions, particularly the rate of temperature decline and its interaction with pH dynamics. The contribution of sarcomere length to tenderness as a quality attribute of steaks (Ertbjerg & Puolanne, 2017) is relatively large in muscles such as the *psoas major* that do not depend on protease activity for tenderization, compared to the *longissimus lumborum et thoracis* muscle, where proteolysis is the main contributor to tenderness (Koohmaraie *et al.*, 2002). However, sarcomere length is very long in the *psoas major* muscle, making it less prone to shortening, while sarcomere length is very short in the *longissimus thoracis et lumborum* muscle (Dransfield, 1977; Rhee *et al.*, 2004). Sarcomeres shorten during rigor (Marsh, 1954) that generally occurs within the first 24 hours post-mortem in beef (Wheeler & Koohmaraie, 1994; Koohmaraie *et al.*, 1996) and this has the potential to increase meat toughness (Locker, 1960). A greater risk exists in muscles where the sarcomere lengths are normally <2 µm (Koohmaraie & Geesink, 2006), such as the *longissimus thoracis et lumborum*, *semimembranosus* and *gluteus medius* muscles (Rhee *et al.*, 2004), but requires at least 20% shortening to affect beef quality (Marsh & Leet, 1966). Although sarcomere length can be a useful tenderness phenotype, variation in sarcomere lengths exists between muscles and breeds (Dransfield, 1977) and the conditions that predispose sarcomeres to excessive shortening, are generally only present when carcasses experiences extreme chilling conditions (such as rapid freezing), which can be accelerated in smaller, leaner carcasses (Locker & Hagyard, 1963). Cold-shortening occurs when carcasses cool too rapidly (Dransfield & Lockyer, 1985; Pearson & Young, 1989) and is a significant risk in smaller carcasses such as Nguni, especially when they are leaner (Strydom *et al.*, 2001; Frylinck *et al.*, 2009, 2015). This predisposition of altered sarcomere lengths in response to environmental conditions during the early post-mortem period (≤d 3), means that no associations of sarcomere length with the SNP have been identified in the calpain-calpastatin system (Cafe *et al.*, 2010b; Reardon *et al.*, 2010), including several other genes (Dunner *et al.*, 2013; Sevane *et al.*, 2014).

The observations of shrinkage during extreme chilling, coupled to toughness that can develop with slow chilling and is sometimes accompanied by sarcomere shortening, lead to the generation of an ideal pH : temperature window during the early post-mortem period, defining optimal chilling conditions prior to full rigor development. In this window, carcasses can be chilled (preserved) without adverse physical or biochemical effects on tenderization and is based on the original work of Locker & Hagyard (1963) and refined by Ferguson *et al*. (1999) and Thompson (2002). During the early post-mortem period, carcasses should be chilled fast enough to allow temperatures to decrease below 35°C by the time that the pH of the meat reaches 6, to avoid high rigor temperatures (Strydom & Rosenvold, 2014). At the same time, chilling should not be excessive, where carcass temperatures should remain above 10°C at the time when meat reaches pH 6, or full rigor (Thompson, 2002). These processes of temperature-related "toughening" should not vary significantly between carcasses of similar weight and fatness exposed to the same conditions (Koohmaraie & Geesink, 2006), where temperature has a large effect on the extent of shortening that takes place when muscles contract in response to the depletion of ATP energy, or rigor (Newbold & Harris, 1972). In commercial abattoirs, variation in carcass size and fatness is common and chiller facilities have to take a "one size fits all" approach, meaning that some carcasses will always run the risk of falling outside the optimal chilling window. Outside these optimal chilling conditions, beef quality phenotypes can be affected by a complex interplay of conditions that could confound correlations with genomic markers.

Heat-induced toughening occurs when carcasses are exposed to high rigor temperatures, which generally occurs when large carcasses are not chilled fast enough, or exposed to excessive electrical stimulation through the heat generated by muscular contractions (Strydom & Rosenvold, 2014). This is exacerbated by high energy finishing diets, resulting in a higher resting body temperature (greater metabolic heat) and increased carcass fatness (Dransfield, 1993; Simmons *et al.*, 1996; Strydom *et al.*, 2001). Although beef produced today is generally leaner than the twentieth century, the size of carcasses have increased by 60% over the last few decades (Strydom & Rosenvold, 2014; Terry *et al.*, 2020) due to selection for increased meat yield. High rigor temperatures lead to extreme pH decline (Simmons *et al.*, 1996), decreasing the duration of activity of the proteases (Dransfield *et al.*, 1992a) due to their accelerated inactivation (Dransfield, 1993, 1994a). The extreme intracellular conditions also result in protein denaturation and results in beef that is paler and exhibits surface exudate (Offer, 1991; Gutzke *et al.*, 2014; Warner *et al.*, 2014). Similar to cold-shortening, the altered (but very different) sarcoplasmic conditions (Marsh *et al.*, 1987) limit the degradation of the myofibrils and the tenderization of meat, during heat-induced toughening (Devine *et al.*, 1999). The negative impact of heat-toughening is not limited to tenderization, but a physical loss of carcass weight (water-holding capacity; WHC), poor appearance (WHC and colour) and problems that arise during processing of the meat and excess fluid (WHC) (Strydom & Rosenvold, 2014).

In contrast to the *longissimus thoracis et lumborum* muscle, the large muscular cuts of the hindquarters are more thermally inert, with a low surface area to volume ratio and are at greater risk of high-rigor toughening (Strydom & Rosenvold, 2014). The slower rate of cooling and elevated glycolytic rate (Tarrant & Mothersill, 1977) can result in paleness that can cause two-toning in primal cuts or individual steaks (Jacob & Hopkins, 2014; Kim *et al.*, 2014; Stufft *et al.*, 2017). The modification of sarcomere lengths through tenderstretch not only helps

prevent cold-shortening, but can also ameliorate the effects of high rigor temperatures on tenderness (Warner *et al.*, 2014), possibly through mechanisms beyond a simple prevention of shortening (Thompson, 2002). While feedlot finishing, carcass size and fatness offer little by way of interventions to alter heat production and thermal inertia of the carcass, proper application of electrical stimulation, enhanced chilling practices and the method of carcass suspension are practical solutions to mitigate the risks of carcasses being above 35°C at the onset of rigor (Strydom & Rosenvold, 2014).

2.2. Glycogen metabolism

Another notable factor that is temperature and pH-dependent and can have large modifying effects on tenderization (Warner *et al.*, 2010; Kemp & Parr, 2012; Hopkins *et al.*, 2014) is the level and rate of mobilisation of glycolytic substrates that drive several processes of beef quality. Animal factors that determine the balance between glycogenesis *vs*. glycogenolysis (muscle glycogen balance) in the ante-mortem period, such as nutritional status and stress (–susceptibility) (King *et al.*, 2006; Muchenje *et al.*, 2009) will determine the rate of pH decline post-slaughter, which is proportional to the amount of lactate build-up in meat (Newbold & Scopes, 1967). Alternatively therefore, post-mortem muscle energy metabolic conditions (during the ripening phase) and temperature are the most important factors that determine the conversion of muscle to meat and can have profound effects on general meat quality and tenderness in particular (Marsh *et al.*, 1987; Pike *et al.*, 1993).

In the live animal, excess glucose in metabolism is first used to stock the glycogen reserve of the liver (5 – 8% glycogen, 250 – 300 g in cattle), after which muscle glycogen is replenished (1% glycogen, equivalent to 1.8 – 2.2 kg glycogen in cattle). The nutritional status of animals will therefore affect the size of their muscle glycogen stores, particularly during the finishing phase (Perry & Thompson, 2005). For example, intensive *vs*. extensive production systems (or feedlot-finished *vs*. pasture-fed), can affect the metabolic status of muscle fibres and result in decreased energy metabolism post-mortem for animals raised on pasture (Frylinck *et al.*, 2013).

It is however also a highly volatile glucose store (López-Bote, 2017) that can be rapidly mobilized upon exposure to acute stress (epinephrine signal transduction) that rapidly leads to glycogenolysis and oxidation of metabolisable glucose as an energy fuel for muscle (Lacourt & Tarrant, 1985; López-Bote, 2017). Nutrition and stress (related to level of activity and temperament) will therefore affect the level of post-mortem anaerobic glycolysis in muscle, where basal levels are determined by fibre type and genotype (Ferguson & Gerrard, 2014). This can be modified by electrical stimulation (Ferguson *et al.*, 2000) that exhausts free ATP to activate glycolysis and prevents supercontraction (Koohmaraie, 1992a). Both excess and deficient muscle glycogen (glycolytic rate) can have detrimental effects on meat ageing and tenderization of carcasses (Marsh *et al.*, 1987), through changes in sarcoplasmic pH (Pösö & Puolanne, 2005).

With the glycogen level at slaughter predetermined, the rate of metabolic activity of muscle is notably regulated by temperature, where the reactions of glycolysis will slow as the carcass cools (Newbold & Scopes, 1967). Anaerobic glycolysis produces the relatively strong acid, lactic acid (pK_a = 3.7) that dissociates to its ionic form at cellular pH (lactate + H^+) and this will rapidly change the pH of the sarcoplasm (and free calcium ion (Ca^{2+}) release), which in turn affects the rate of proteolysis (that is also affected by temperature and substrate

availability). Calpains do not only respond to Ca^{2+}, but also have the ability to affect the balance of Ca^{2+} of the sarcoplasm, by interacting with sarcoplasmic reticulum proteins such as the ryanodine receptor (Singh *et al.*, 2004) and the plasma membrane Ca^{2+} pump (Bruce, 2018). Normally, pH will decrease from a value of ≃7.0 at death in the hot carcass (37°C), down to an ultimate pH of 5.5 – 5.6 (pH_u) (Callow, 1937) over 24 hours of chilling at 1°C, depending on carcass size and adiposity (Koohmaraie, 1992a). These processes are intimately linked to temperature (glycolysis ⇆ T° ⇆ pH ⇆ proteolysis).

Measurement of the concentration of glycolytic substrates, intermediates and products individually can be difficult to interpret, due to variable flux in the pathway. Glycolytic potential is a formula to estimate the activity of intermediary glucose metabolism in post-mortem muscle that results in lactate formation (Monin & Sellier, 1985; Hanson *et al.*, 2001) and can be used to predict some meat quality traits (Stempa & Bradley, 2021). The formula incorporates the most important 6-carbon glycolytic intermediates and the 3-carbon lactate already present per gram fresh tissue to give a more dynamic estimate of the state of glycolysis in meat (Monin & Sellier, 1985):

Glycolytic Potential (µmol/g muscle)

$$= 2 \times ([\text{ glycogen }] + [\text{ glucose 6-phosphate }] + [\text{ free glucose }]) + \text{lactate}$$

The reactions of anaerobic glucose metabolism and its link to pH decline, results in pleiotropic effects on beef quality that are linked to tenderness, water-holding capacity (WHC) and colour of beef through linked physiological processes (Poleti *et al.*, 2015; Marín-Garzón *et al.*, 2020). The glycolytic processes are associated to tenderness as biomarkers that facilitate tenderization and through ATP, pH and Ca^{2+} concentrations (Savell *et al.*, 2005; Warner *et al.*, 2010; King *et al.*, 2019) and their effects on proteases. The important role of calpains, especially *capn3* in growth and development (Nonneman & Koohmaraie, 1999) has been linked to altered distribution of muscle fibre types that will affect the metabolic processes within muscle fibres, but also altered function of the calpain system in different fibre types. Interestingly, calpastatin isoforms are differentially expressed in different muscle fibre types that could differentially affect the activity of calpains in different muscles (Bhat *et al.*, 2018a; Motter *et al.*, 2021). Selection for growth increases intermediary metabolic processes through-up regulation of the genes of metabolism in homeorhetic adaptation (Bernard *et al.*, 2009) and these common regulatory mechanisms of linked physiological processes (either through covalent modification or control of transcription and translation) could be an indirect link between the genes of the calpain system and intermediary metabolic processes (Mackay *et al.*, 2009; Zhu *et al.*, 2021).

Calpains can alter gene expression through effects on transcription factors (Ono *et al.*, 2016; Leal-Gutiérrez *et al.*, 2018; Guillocheau *et al.*, 2019). The calpains have been found to affect signal transduction pathways (Park *et al.*, 1993; Spinozzi *et al.*, 2021), including physiological effects on kinases and phosphatases (Goll *et al.*, 2003; Dargelos *et al.*, 2008) and cellular receptors (Spinozzi *et al.*, 2021). A notable substrate is the Ca^{2+}-dependent protein kinase subunits (PKC) that is normally responsible for transduction of β-adrenergic cellular signals, favouring glycogenolysis and gluconeogenesis (Goll *et al.*, 2003; Sorimachi & Ono, 2012). Glycogen phosphorylase (Purintrapiban *et al.*, 2001) is also a potential physiological substrate for calpain proteases and it would be of interest to determine whether calpain-calpastatin system genotypes are able to

affect the basal levels of glycogen in muscles. Associations of SNP in the calpain system genes with glycolytic potential have been established in pork (Sieczkowska *et al.*, 2010), while associations with pH has been established in pork (Otto *et al.*, 2007; Ropka-Molik *et al.*, 2014) and beef (Reardon *et al.*, 2010; Ardicli *et al.*, 2017) that implies (and was associated with) altered lactate production in meat (Krzęcio *et al.*, 2005) that is not necessarily coincidental to tenderizing effects.

The *capn3* gene has been found to interact with muscle-type phosphofructokinase (PFK) directly (Ojima *et al.*, 2011). Tenderization has been linked to the glycolytic enzyme and proteomic marker for tenderization (Sierra *et al.*, 2012; Zhao *et al.*, 2014), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and it is a substrate for calpain protease activity (Ono *et al.*, 2016), as well as creatine kinase (Purintrapiban *et al.*, 2001). Genotypes of the calpain-calpastatin system affected the expression of genes (protein concentration) of metabolic enzymes including mitochondrial energy metabolic processes (Ono *et al.*, 2016; Rosa *et al.*, 2018; Jahnke *et al.*, 2020) and together, these results suggests that calpains could play a physiological role in glycolysis *in situ* (Ono *et al.*, 2016) beyond the indirect link of post-mortem pH changes affecting enzyme activity.

2.3. Beef colour

Meat colour is the first quality aspect that is observed by the consumer (Mancini & Hunt, 2005) and is intimately linked to the processes of intermediary metabolism coupled to temperature and pH dynamics (Gagaoua *et al.*, 2020; Purslow *et al.*, 2021). Beef colour is determined by a complex array of factors including myoglobin oxygenation, mitochondrial oxidation, protein charge (pH_u), structural characteristics of myofibrils that determine light refraction and scattering, as well as the degree of proteolytic activity post-mortem (Purslow *et al.*, 2020). Normally, from a near-neutral pH (pH ≃7) anaerobic metabolism results in lactate build-up and a drop in pH to 5.5 (Bendall, 1973), which results in a colour change (McBee Jr. & Wiles, 1967), as the meat's pH approaches the proteins' iso-electric point (MacDougall, 1982; Lawrie, 1983). Structural changes will also affect the scattering of light from the surface of beef (Hughes *et al.*, 2020) and affect especially L* values (Hughes *et al.*, 2014b; Purslow *et al.*, 2020). This can be altered by processes of metabolism and stress (Gagaoua *et al.*, 2020; Purslow *et al.*, 2020), but also the proteolytic changes of beef during tenderization (Gagaoua *et al.*, 2020; Hughes *et al.*, 2020; Purslow *et al.*, 2020). This was confirmed by associations of muscle lightness and SNP in the calpain-calpastatin system (Juszczuk-Kubiak *et al.*, 2004a; Tizioto *et al.*, 2013; Ardicli *et al.*, 2017), where the tender alleles resulted in a favourable change in lightness in taurine (Melucci *et al.*, 2012; Gagaoua *et al.*, 2018) and crossbred beef (Mazzucco *et al.*, 2010; Reardon *et al.*, 2010).

Another important factor that determines the colour of meat is the state of oxygenation of myoglobin. In the ferrous state (Fe^{2+}), once meat is allowed to bloom (myoglobin binding the oxygen in air), oxymyoglobin has a red appearance. However, ferric iron (Fe^{3+}) in metmyoglobin cannot bind oxygen and has a brown appearance (Satoh & Shikama, 1981; Ouali *et al.*, 2006), while bacterial spoilage results in the formation of sulphmyoglobin, which has a green appearance (Nicol *et al.*, 1970; Andersson *et al.*, 1984). The important factors to consider here are oxidative stress (Gagaoua *et al.*, 2020), Fe^{2+} deficiency and muscle types (Matarneh *et al.*, 2017), for example the characteristically pale colour of the *semitendinosus* muscle and darker red colour of *biceps femoris* and *gluteus medius* (McKenna *et al.*, 2005). SNP in both the *capn1* gene and *cast* gene have been associated with

haem-iron content of beef (Casas *et al.*, 2014) and colour parameters, a*, b* and H_{ab} in meat. These included favourable effects of tender alleles for a* and b* in the *capn1* (Pinto *et al.*, 2011; Melucci *et al.*, 2012) and *cast* genes (Reardon *et al.*, 2010), favourable effects on b* (but not a*) in *capn1* (Ribeca *et al.*, 2013) and *cast* (Castro *et al.*, 2016), as well a favourable effects on H_{ab} in *capn1* (Li *et al.*, 2013) and *cast* (Castro *et al.*, 2016). These effects of calpain-calpastatin system SNP on colour parameters could potentially link to myoglobin subunits as substrates for calpains (Shima *et al.*, 2003; Rawlings *et al.*, 2008).

2.3.1. Dark, firm and dry (DFD) beef

When the pH of meat fails to decrease (pH_u > 6.0), due to insufficient anaerobic metabolism, the result is a darker colour (McBee Jr. & Wiles, 1967; Hughes *et al.*, 2014a; Matarneh *et al.*, 2021) and dark-cutting beef (or DFD, dark firm and dry beef) is an important challenge to the beef industry. It has been suggested that selection for tender genotypes in the *cast* gene could decrease DFD (Reardon *et al.*, 2010), as tender genotypes favour lighter meat (Juszczuk-Kubiak *et al.*, 2004a), although genome-wide analyses failed to associate calpain system genes with DFD directly (Lei *et al.*, 2021). Pasture-fed cattle are generally more susceptible to DFD than concentrate-finished beef cattle (Hughes *et al.*, 2014a), especially when cattle are grazed on pasture that fails to meet their energy requirements or for an insufficient length of time (Matarneh *et al.*, 2017; Ponnampalam *et al.*, 2017). This could contribute to deficient glycogen storage (Vestergaard *et al.*, 2000), but also lower fat coverage of the carcasses that result in more rapid cooling (Mancini, 2013), further limiting glycolytic activity. Decreased glycolytic capacity and increased risk of DFD can be exacerbated by handling and transport stress of extensively produced beef (Ferguson & Warner, 2008; Ponnampalam *et al.*, 2017; Clinquart *et al.*, 2022) and the tendency of pasture-fed beef to develop a greater proportion of oxidative muscle fibres (Vestergaard *et al.*, 2000; Ponnampalam *et al.*, 2017). Carcasses at a lower temperature during rigor and those from older animals generally produce a darker colour (Hughes *et al.*, 2014a), while some breeds are more likely to produce dark-cutting beef, for example Nguni cattle (Muchenje *et al.*, 2008; Neethling *et al.*, 2017).

However smaller-framed cattle can be successfully finished on high quality grazing by extending the feeding period compared to feedlot-finishing without significant loss of beef quality (Du Plessis & Hoffman, 2007), while a higher pH_u does not inevitably result in DFD (Wicks *et al.*, 2019). It is rather a result of an interplay of a complex range of production and animal factors (Dunne *et al.*, 2011; Neethling *et al.*, 2017), resulting in complex changes in beef quality that go beyond colour and water-holding (Duarte *et al.*, 2022).

When the ultimate pH does not approach the iso-electric point of muscle proteins (Tarrant & Sherington, 1980) that do not undergo significant denaturation, meat retains the colour of myoglobin and more light is absorbed by meat with a high pH_u (MacDougall, 1982), which appears darker (MacDougall & Rhodes, 1972) and exhibits altered bloom characteristics (Egbert & Cornforth, 1986). The retention of fluid in the intracellular fluid spaces also contributes to the dark colour and gives the meat a firm, almost fixed texture, because the myofibrillar structures do not shrink and organization is not disrupted by the normal processes of tenderization (Matarneh *et al.*, 2017). Although electrical stimulation was able to accelerate the decline in pH (Fjelkner-Modig & Rudérus, 1983a), it cannot improve the quality aspects of this beef, because of the lack of glycolytic intermediates (Dutson *et al.*, 1982; Fjelkner-Modig & Rudérus, 1983b). The lower glucose availability (Frylinck *et*

al., 2013), rather than the higher pH *per se* (Gill & Newton, 1979), makes the meat more susceptible to bacterial spoilage (Nicol *et al.*, 1970; Newton & Gill, 1981). This results from a decreased growth of lactic acid-producing bacteria utilising glucose substrates, which in turn allows for the propagation of harmful bacteria utilising amino acid metabolism, decreasing shelf-life of DFD beef (McPhail *et al.*, 2014; Souza Duarte, 2015; Shange *et al.*, 2019). The high pH of the meat prevents changes in the balance of ionic charges of the amino acids of DFD meat and intracellular fluid is not released from disrupted fluid spaces and the meat has a dry appearance (Matarneh *et al.*, 2017) due to increased WHC, discussed below.

2.3.2. Pale, soft and exudative (PSE) beef

Conversely, excessive glycogen metabolism, excessive Ca^{2+} leakage and rapid, extreme pH drop to reach a low ultimate pH ($pH_u \leq 5.4$) is associated with pale, soft and exudative meat (PSE). Cellular damage and protein denaturation (Scopes, 1964) increase light scattering increasing L* (Hughes *et al.*, 2020) and disruption of fluid spaces and intracellular fluid leakage results in surface droplet formation, giving the meat a characteristic light and watery appearance (Bendall & Wismer-Pedersen, 1962; MacDougall, 1982). It has been suggested that an interaction between the *cast* gene and defective ryanodine receptor gene in pork can exacerbate the fluid dynamic changes and protein degradation of PSE (Krzęcio *et al.*, 2005). The condition can be induced in normal carcasses, by exposing them to high rigor temperatures (Bendall & Wismer-Pedersen, 1962) described above, but is more often observed in stress-susceptible pigs (Doumit *et al.*, 2003).

2.4. Muscle fluid and water-holding capacity (WHC)

These conditions of altered beef colour through deviations in energy metabolic pathways, pH and temperature, clearly describes an additional interaction of these processes with the ability of meat to retain fluid. A large proportion of muscle tissue is composed of water (75%), which forms an integral part of the structure of muscle fibres (85% in myofibrils). Muscle "holds" 75% of its water and WHC refers to the ability of meat to retain fluid in the face of the biochemical and structural changes that occur post-mortem (bound water) that result in free drip (purge, exudate or weep) (Hamm, 1961; Hughes *et al.*, 2014b), as well as extrudable water that is lost when forces are applied to the meat, for example, during processing (Hamm, 1961). Two major mechanisms hold water within muscle or meat. The first is an osmotic capillary effect, where fluid is held in cellular spaces and sustained by cellular integrity and sarcomere volume (Offer & Trinick, 1983). Cellular compromise (e.g. freeze-thaw) and elastic pressure that shrinks these capillary spaces (e.g. rigor mortis), will force fluid out of the meat (Ertbjerg & Puolanne, 2017). The second factor is an electrostatic effect, where the charge of proteins (amino acids) attracts water through an osmotic pull (Hamm, 1961; Ertbjerg & Puolanne, 2017) that is minimal at the iso-electric point (p*I*) of meat and therefore interacts with the processes of metabolism and tenderization (Lawrie, 1983; Gault, 1985).

Water-holding capacity is not directly related to tenderness, but factors that affect WHC also affect tenderness, leading to a casual (or coincidental) relationship under certain conditions (Honikel, 2004; Huff-Lonergan & Lonergan, 2005). This is rooted in the extent of glycolysis that takes place in muscle post-mortem (and pH dynamics), which affects meat colour, WHC, juiciness, sarcomere length (and interacting with

temperature) and tenderness through overlapping mechanisms (Hughes *et al.*, 2014b; Purslow *et al.*, 2016). Associations of genetic markers in the calpain-calpastatin system with estimates of water-holding such as purge (Krzęcio *et al.*, 2005; Gandolfi *et al.*, 2011; Ribeca *et al.*, 2013), extrudable WHC (Krzęcio *et al.*, 2005; Tizioto *et al.*, 2013; Ropka-Molik *et al.*, 2014) and cooking loss (Juszczuk-Kubiak *et al.*, 2004a; Cafe *et al.*, 2010b; Ribeca *et al.*, 2013) of beef and pork could therefore more likely be a consequence of proteolysis.

Initially, rapid chilling is essential to limit the loss of fluid mass from the carcass (Schutte, 1933) and the percentage fluid loss can be increased by the excessive application of electrical stimulation (Savell *et al.*, 1978b). Rigor contraction decreases capillary forces holding water (Marsh, 1954), when spacing between fibrils increases as they "shrink" transversely forming drip channels and opening extracellular fluid spaces to allow osmosis (and membrane permeability), while the loss of structural organization (cytoskeletal proteolysis) decreases the ability to retain water (Honikel, 2004; Brewer, 2014; Hughes *et al.*, 2014b). The concept of WHC links to several processes that are associated with fluid loss, i.e. loss of saleable product and profit (Gault, 1985; Huff-Lonergan & Lonergan, 2005), including carcass mass loss, purge during meat ageing, freeze-thaw cycles, retail display and cooking, which can ultimately (potentially) affect the eating experience of the consumer (juiciness of the cooked meat). However, the relationship between WHC and juiciness scores is not simple and cooking losses and juiciness are not as clearly defined or understood as drip and purge (Purslow *et al.*, 2016; Hughes *et al.*, 2020).

2.5. Intramuscular fat

Intramuscular fat normally has limited direct effect on tenderness, contributing only 5% (Koohmaraie & Geesink, 2006) or 3 – 10% of (perceived) tenderness (Nishimura *et al.*, 1999). It plays a more important role in palatability, i.e. juiciness and flavour (McBee Jr. & Wiles, 1967; Warner *et al.*, 2010) in leaner meat (<8%), but could potentially disrupt the structure of connective tissue of the perimysium at high marbling levels (>8%) increasing tenderness (Nishimura *et al.*, 1999; Warner *et al.*, 2010). Although marbling is generally not appreciable in South African beef carcasses, carcass adiposity can have indirect consequences for post-mortem proteolysis through insulation effects or a lack thereof during cooling (Frylinck *et al.*, 2009) and electrical stimulation.

A moderate negative genetic association was identified (r_g = –0.17 to – 0.55) between intramuscular fat or marbling a score and WBSF (Bertrand *et al.*, 2001; Minick *et al.*, 2004), although this has been found to be a relatively strong, positive association in Simmental and *Bos indicus* composites (O'Connor *et al.*, 1997). A strong association (r_g = –0.82) between backfat thickness and day 7 post-mortem shear force was observed in Brahman cattle, which was moderate (r_g = –0.36) by day 14 post-mortem, with limited or no association with marbling (Smith *et al.*, 2007). Calpains have been linked to lipid metabolic enzyme transcription (Rosa *et al.*, 2018) and deposition of skeletal muscle (Kemp *et al.*, 2013) and body lipids (Ono *et al.*, 2016), with interactions between calpains and lipid metabolic pathways (Melloni *et al.*, 2000; Kramerova *et al.*, 2009). Furthermore, calpains facilitate increased insulin-mediated glucose uptake into adipocytes through GLUT4 (Paul *et al.*, 2003) that supplies substrate to adipogenesis.

Next to estimates of meat yield (growth and size), associations of the calpain-calpastatin system SNPs with lipid content and composition are of most interest to researchers. Associations with body fatness were

observed for SNPs in the *capn1* (Collis *et al.*, 2012; Zhang *et al.*, 2012) and *cast* genes (Schenkel *et al.*, 2005; Collis *et al.*, 2012), intramuscular fat in *capn1* (Barendse, 2011; Quintana, 2017) and *cast* (Iso-Touru *et al.*, 2018), while SNPs in the *capn1* (Cheong *et al.*, 2008; Li *et al.*, 2013; Sun *et al.*, 2018), *capn3* (Royer *et al.*, 2016) and *cast* genes (Royer *et al.*, 2016; Leal-Gutiérrez *et al.*, 2020) affected marbling. The genetic markers of these genes also correlated with measures of fat thickness in *Bos taurus* (Ardicli *et al.*, 2017; Abo-Ismail *et al.*, 2018), *Bos indicus* (Gomes *et al.*, 2013; Tizioto *et al.*, 2013) and composite beef (Collis *et al.*, 2012), mutton (Knight *et al.*, 2014) and pork (Ropka-Molik *et al.*, 2014), while fatty acid profile was different for the different genotypes of the calpain-calpastatin system markers (Zhang *et al.*, 2012; Dunner *et al.*, 2013).

2.6. Electrical stimulation

More than a century ago it was recognized that chilling of carcasses during ageing improved tenderness as the pH of the tissue declined (Hoagland *et al.*, 1917). At slaughter, the level, time and cycles of electrical stimulation are important modulators of the rate of energy depletion, determined by the rate of cooling of the carcass and is subject to modification by the fatness of the carcass and the insulatory properties of lipids (Koohmaraie *et al.*, 1988; Dransfield *et al.*, 1992b). Electrical stimulation can also overcome at least some of the age (Pike *et al.*, 1993) and/or breed-origin (Ferguson *et al.*, 2000) effects on tenderness under optimal chilling conditions, especially during the early phases of tenderization (Strydom & Frylinck, 2014). However, improper application of electrical stimulation (over-stimulation and/or insufficient chilling) could result in tougher beef (Bendall & Wismer-Pedersen, 1962; Dransfield, 1993) and it has been identified as an important risk factor in South African abattoirs, with ≥50% of commercial carcasses at risk of toughening as the result of high rigor temperatures (Hope-Jones & Strydom, 2021).

Even though it became known that electrical current can produce more tender meat in the middle of the 18th century (Franklin, 1769), a commercial devise for improving tenderness was only patented in the 20th century (Harsham & Deatherage, 1951), while the first scientific research was conducted more than two decades later (Carse, 1973). The major advantages of electrical stimulation are advanced *rigor mortis*, through enhancement of glycolysis by muscular contractions, accelerated fall in meat pH post-mortem (Carse, 1973; Chrystall & Hagyard, 1976) and decreased risk of cold-shortening (Davey *et al.*, 1976).

It became common practice to electrically stimulate carcasses to prevent cold-shortening (Davey *et al.*, 1976) when carcasses were rapidly chilled for hygienic purposes (Schutte, 1933), although chilling not only slows the reactions of exogenous enzymes (bacteriological), but also endogenous enzymes (that can result in decomposition) and chemical reactions such as the development of rancidity of fat (Heinze, 1990). The tenderizing effects of electrical stimulation on meat is not equivalent (of the same magnitude) in the different muscles of the carcass (Chrystall & Hagyard, 1976; Savell *et al.*, 1977; Johnston *et al.*, 2001), where the *Longissimus* muscle is especially predisposed to cold-shortening (Bendall, 1975) and can benefit more from electrical stimulation. Overall the research suggested that electrical stimulation made beef more tender (at least during early meat ageing), decreased variation between carcasses (Savell *et al.*, 1977), lead to favourable changes in colour (Savell *et al.*, 1978b; Eikelenboom *et al.*, 1985), but increased fluid and cooking losses (Savell *et al.*, 1978b), which could potentially decrease the juiciness of the final product (Bouton *et al.*, 1980).

The mechanism of the tenderizing effects (Harsham & Deatherage, 1951) and decreased variation in tenderness between carcasses (Savell *et al.*, 1977; Johnston *et al.*, 2001) at the same time of meat ageing (Savell *et al.*, 1978b) has been researched extensively. By activating motor neurons and muscular contractions, ATP and creatine phosphate of the sarcoplasm are exhausted, accelerating glycolysis and resulting in a build-up of lactate and rapid fall in pH (Carse, 1973; Bendall *et al.*, 1976; Davey *et al.*, 1976). Rigor mortis is therefore achieved within a few hours post-mortem instead of 24 h (Carse, 1973; Chrystall & Hagyard, 1976; Hopkins & Thompson, 2001) and, because heat is generated by muscular contraction itself, accelerates metabolic rate (Chrystall & Devine, 1978; Devine *et al.*, 1984) and carcasses must be chilled (Savell *et al.*, 1977; Takahashi *et al.*, 1984). If carcasses remain warm (above 35°C at pH greater than 6.0), high-rigor temperature or heat-toughening can compromise tenderness (Dransfield, 1993; Simmons *et al.*, 1996; Strydom & Rosenvold, 2014). Meat was found to be more tender after electrical stimulation in part due to increased enzymatic degradation of fibrils that could involve altered function of lysozymes (Savell *et al.*, 1977; Pommier *et al.*, 1987), but is generally ascribed to calpains (Ducastaing *et al.*, 1985), while physical and structural changes also contribute to tenderization (Hwang *et al.*, 2003). Much of the early experimental (theoretical) work on electrical stimulation used relatively high voltage stimulation (not practical for worker safety in abattoirs) and a large part of tenderizing effects were ascribed to physical damage to meat, which disrupts the structural organization of muscle fibres (Dutson *et al.*, 1977; Savell *et al.*, 1977, 1978a; Sorinmade *et al.*, 1982) and increases collagen solubility (Judge *et al.*, 1980) to make meat more tender. The true advantage of electrical stimulation is to accelerate the onset of rigor by depleting muscle ATP, activating anaerobic metabolism and allowing free Ca^{2+} to favour proteolysis (Strydom *et al.*, 2005; Simmons *et al.*, 2008). The efficacy of electrical stimulation in tenderization therefore lies in its ability to accelerate (improve) anaerobic metabolism (Fabiansson & Reuterswärd, 1985) and myofibril fragmentation (Uytterhaegen *et al.*, 1992), but has no advantage over extended meat ageing (Taylor & Cornell, 1985), as the favourable effects of ES are transient (Lepetit *et al.*, 1986; Dransfield *et al.*, 1992b; Strydom *et al.*, 2005).

The nutritional status of animals (glycogen status of muscle fibres) determines the efficacy of electrical stimulation on advancing rigor and tenderization (Dutson *et al.*, 1982; Fabiansson *et al.*, 1985) and differences in the energy status of muscle fibres could alter the effect of electrical stimulation on genomic association analyses. Limited data is available on the effects of electrical stimulation on the association of SNPs with traits. It was initially determined that genomic effects on carcass traits were not additive to the effects of electrical stimulation (Riley *et al.*, 2019, 2020a), due to the decrease in variation between carcasses (Riley *et al.*, 2019). These results were consistent with previous observations that environmental effects can attenuate genomic associations, including ageing (Mazzucco *et al.*, 2010; Gruber *et al.*, 2011), tenderstretch (Johnston & Graser, 2010; Robinson *et al.*, 2012) and application of growth promotants (King *et al.*, 2012). However, for some SNP and some traits, interventions such as meat ageing (Pinto *et al.*, 2011) and electrical stimulation increase the number of associations observed between SNPs and traits (Pinto *et al.*, 2011; Riley *et al.*, 2020b). Together these data suggest that the interactions with the peri-mortem environment (G×E) are more complex and could be specific to traits or SNPs, but more likely involve more complex interactions of the interrelated traits that integrate genomic and phenomic data with proteomics and metabolomics (Gagaoua, 2021; Gagaoua *et al.*, 2022; Li *et al.*, 2022a; Warner *et al.*, 2022).

2.7. Proteolytic capacity

Although calpain activity is normally under strict control *in situ*, ischaemia coupled to continued metabolism results in several changes within muscle fibres of the carcass that activate several proteases. These include changes in pH and the intracellular fluid free Ca^{2+} concentrations, where pH 5.5 and 200 µM Ca^{2+} increases calpain activity in the thousand-fold range (Takahashi, 1996). In 1972 it was recognized that myofibrils (especially Z-lines) are subject to Ca^{2+}-dependent proteolysis at near-physiological, neutral pH (Busch *et al.*, 1972), by endogenous protein fraction protease(s), which disrupt the structural organization of the sarcomere in post-mortem muscle (Koohmaraie *et al.*, 1987; Koohmaraie, 1992b; Barkhouse *et al.*, 1996). This involves degradation of myofibrils to disrupt the highly-organized structure of muscle fibres, where the proteolytic rate is intimately linked to myofibril fragmentation, which in turn is closely associated with tenderness (Seideman *et al.*, 1987; Taylor & Koohmaraie, 1998; Geesink *et al.*, 2006), but also depends on factors such as sarcomere length and collagen solubility. For the *Longissimus lumborum et thoracis* muscle however, myofibril fragment length is the best predictor of tenderness (Culler *et al.*, 1978), due to its large proteolytic capacity (Berge *et al.*, 1993; Koohmaraie & Geesink, 2006).

It was only in 1992 that it was established (although still not widely accepted) that calpains could be extensively responsible for this proteolysis (Koohmaraie, 1992a), while in 2006 direct proof was given that calpain-1 (and no other protease to any great extent) induces the proteolytic changes of tenderness (Geesink *et al.*, 2006), at least in the first week post-mortem. Calpastatin is more variable than calpains (Smith *et al.*, 2009) and higher inhibitory function of calpastatin has been linked to factors that induce "tough" meat such as β-adrenergic agonist (Koohmaraie *et al.*, 1991a) treatment, *Bos indicus* breed type and callipyge-affected muscles (Goll *et al.*, 1998). It became clear that calpain-1 and calpastatin played a crucial role in the rate at which myofibrils degrade in the first 14 days post-mortem (Koohmaraie *et al.*, 1987; Geesink & Koohmaraie, 1999), after which stage calpain-2 activity could potentially lead to further improvements in tenderness (Colle & Doumit, 2017) in muscles or breed types that exhibit delayed tenderization. Limited activity of calpain-1 can however continue up to 42 days post-mortem and calpain-2 until 70 days post-mortem, where tenderization continues at a reduced rate, with continued sarcoplasmic protein degradation (Phelps *et al.*, 2016).

2.7.1. Post-mortem muscle protein degradation

When post-mortem muscle loses blood flow and oxygen supply, ATP levels are gradually depleted as oxidative phosphorylation is shut down in the absence of oxygen supply and the relative inefficient anaerobic metabolic processes continue by forming lactate, as muscle turns to meat. Due to the steep concentration gradient for Ca^{2+} into the sarcoplasm from organelles such as the sarcoplasmic reticulum, the free Ca^{2+} concentrations of the cytosol rises dramatically. It was proposed that this mechanism directly induced myofibril fragmentation in the "calcium theory of meat tenderization" (Takahashi *et al.*, 1987). The addition of $CaCl_2$ enhanced fragmentation, while $ZnCl_2$ prevented it (Taylor & Etherington, 1991). Zinc is however a potent inhibitor of calpains, through enhanced calpastatin activity (Koohmaraie, 1989) and the *in vitro* results attributing effects to Ca^{2+} directly (Takahashi, 1996), do not correspond to changes observed in carcasses (Geesink *et al.*, 2001). The extreme rise in free Ca^{2+} concentrations in the intracellular fluid leads to the activation of calpains,

which do not extensively degrade proteins (to small peptides and amino acids), but rather result in the formation of relatively large peptides through limited cleavage of proteins (Goll *et al.*, 1998) disrupting the innate structure of muscle fibres. Approximately half of the tenderization occurs within the first 24 hours post-mortem (Dransfield, 1994b). Proteins that are very rapidly degraded include troponin T (TnT), desmin, vinculin, talin, spectrin, nebulin, and connectin/titin (Goll *et al.*, 1998). Many of these (connectin/titin, nebulin and troponin T) have a crucial role in maintaining the structural integrity and organization of the myofibrils. Slower degradation of troponin I (TnI), filamin, C-protein, dystrophin, and tropomyosin occurs, while α-actinin and M protein are degraded very slowly (Goll *et al.*, 1998).

The meat from *Bos taurus* cattle is generally (on average) more tender than meat from *Bos indicus* cattle (Whipple *et al.*, 1990; Shackelford *et al.*, 1995; O'Connor *et al.*, 1997; Strydom *et al.*, 2016), due to greater proteolytic activity of *Bos taurus* muscle post-mortem (Shackelford *et al.*, 1991). This is the result of greater activity of calpastatin (relative to calpain) in *Bos indicus* muscle post-mortem, rather than decreased calpain activity (Whipple *et al.*, 1990; Shackelford *et al.*, 1991; O'Connor *et al.*, 1997). This results in less myofibril fragmentation (Whipple *et al.*, 1990) due to lower degradation of myofibrillar proteins during the meat ageing period (Wright *et al.*, 2018) and decreased tenderness (increased WBSF) with a small contribution from decreased fatness of their carcasses (Shackelford *et al.*, 1991) and potentially greater toughness from low collagen solubility (Riley *et al.*, 2005). This tendency towards tougher meat in animals with a strong *Bos indicus* composition, even with best practices in the peri-mortem period (Robinson *et al.*, 2001), can be overcome by electrical stimulation (Ferguson *et al.*, 2000; Gursansky *et al.*, 2010) and extending the meat ageing period of meat to allow for more proteolysis (Watanabe *et al.*, 1996; O'Connor *et al.*, 1997). This can also be attributed to differences in the proportion of fibre types, related to glycogen content and post-rigor metabolism, which in turn influence the ultimate tenderness that is reached (Thompson *et al.*, 2006). *Bos indicus* have decreased proteolytic capacity, i.e. their meat will necessarily be tougher, although this effect can be partially overcome by electrical stimulation (Ferguson *et al.*, 2000) when cold-shortening is a risk (Strydom *et al.*, 2005). This is also evident from the distribution of markers in the calpain system, where the allelic frequencies of *Bos indicus* cattle (Brahman), differ significantly from *Bos taurus* cattle in which many of the markers were developed (White *et al.*, 2005). For example, the widely used *capn1-316* marker exhibited an allele frequency of 20 – 24% for the favourable allele in *Bos taurus* crosses, but only 1% in Brahman, where no "tender homozygotes" were present (Casas *et al.*, 2005; White *et al.*, 2005). Also, the "tender" *capn1-530* allele was predominant (86%) in *Bos taurus* crosses, this allele was completely fixed with only homozygotes in Brahman (Casas *et al.*, 2005; White *et al.*, 2005).

Four potential proteolytic systems could be responsible for tenderness through post-mortem proteolysis that establishes the ultimate tenderness of beef. The calpains are the most extensively researched system and includes the inhibitor calpastatin, while lysosomal cathepsins, ubiquitin-linked proteasomes and the caspases of apoptosis have all been linked to proteolytic tenderization. The relative importance of each system is the subject of debate (Kemp & Parr, 2012) and it seems that they interact at some levels directly, but also indirectly through cellular conditions (e.g. by temperature, pH and free Ca^{2+} concentration).

2.7.2. The calpain system

Calpains are calcium ion-dependent, intracellular fluid, non-lysosomal cysteine endopeptidases / proteases / proteinases / proteolytic enzymes that occur in almost all eukaryotes (Table 2.2). In evolution, calpains seem to be the oldest of the cysteine proteases (Sorimachi *et al.*, 2011a) and they are calcium binding proteins and cysteine proteases, all in one (Campbell & Davies, 2012). The calpain system is conserved in most vertebrates, phytocalpains (C02.019) occur in plants and even bacteria produce calpains or calpain-like proteases (Sorimachi *et al.*, 2011b).

Table 2.2. *Nomenclature in the calpain system*

Calpains	**Description**	**Comment**
protease clan CA	the papain superfamily of proteases (cysteine proteinases with structures similar to papain) the calpain family of endopeptidases, distantly similar to papain	(Rawlings & Barrett, 1993, 2013; Rawlings *et al.*, 2018)
family C2	– Ca^{2+} as cofactor – function best at "neutral" pH (7.2 – 8.2) – without the requirement for signal transduction pathways – mainly located cytosolic (some mitochondrial)	generally contains two copies of the CysPc domain
CANP or CDP	originally called Calcium-Activated Neutral Protease or Calcium-Dependent Protease	discovered in 1964 (Guroff, 1964)
calpain#	Calcium-dependent papain-like enzyme	
calpain-1 (C02.001)	EC 3.4.22.52	originally μ-calpain (μCL, μ80K)
calpain-2 (C02.002)	EC 3.4.22.53	originally m-calpain (mCL, m80K)
calpain-3 (C02.004)	EC 3.4.22.54	originally p94 (nCL-1, n-CANP)
CAPN*	the human calpain gene, e.g. *CAPN1*	human gene for the large subunit of calpain-1
CAPN	the human calpain enzyme, e.g. CAPN1	the large subunit of the protease, calpain-1
capn	the calpain gene in other species, e.g. *capn1*	gene for the large subunit of calpain-1
capn	the calpain enzyme in other species, e.g. capn1	the large subunit of the protease, calpain-1
capns	gene encoding the small subunit of calpain (in animals)	originally *capn4* (heterodimerises with native capn1 and capn2)
Calpastatin	**Description**	**Comment**
protease inhibitor clan II	Clan II = Inhibitor (9th clan, I), being relatively unstructured	(Rawlings *et al.*, 2004, 2018)
family I27	the calpastatin inhibitor family (27th family)	inhibitors of C2 family of peptidases
calpastatin# (LI27.001)	inhibitor of calpains, with the "L" prefix for compound inhibitors (a few inhibitor units per protein molecule)	several isoforms exist from a single gene
CAST*	human gene for calpastatin	one gene encodes several inhibitory peptides
CAST	the human calpastatin peptide(s)	
cast	the calpastatin gene in other species	one gene encodes several inhibitory peptides
cast	the calpastatin peptide inhibitor in other species	

* standardized nomenclature from Croall & Ersfeld (2007); #calpain and calpastatin from Murachi *et al*. (1981).

In muscle, calpains are concentrated at the Z-disk (Parameswaran & Sharma, 2012), which is an important site for myofibril degradation (Koohmaraie & Geesink, 2006). The activity of calpains is increasingly activated as ATP is exhausted and Ca^{2+} sequestration (1 µM free Ca^{2+} concentrations) is no longer possible and Ca^{2+} leaks (≤100 µM free Ca^{2+} concentrations) from the sarcoplasmic reticulum and mitochondria (Koohmaraie, 1992a).

2.7.2.1. Calpain-1

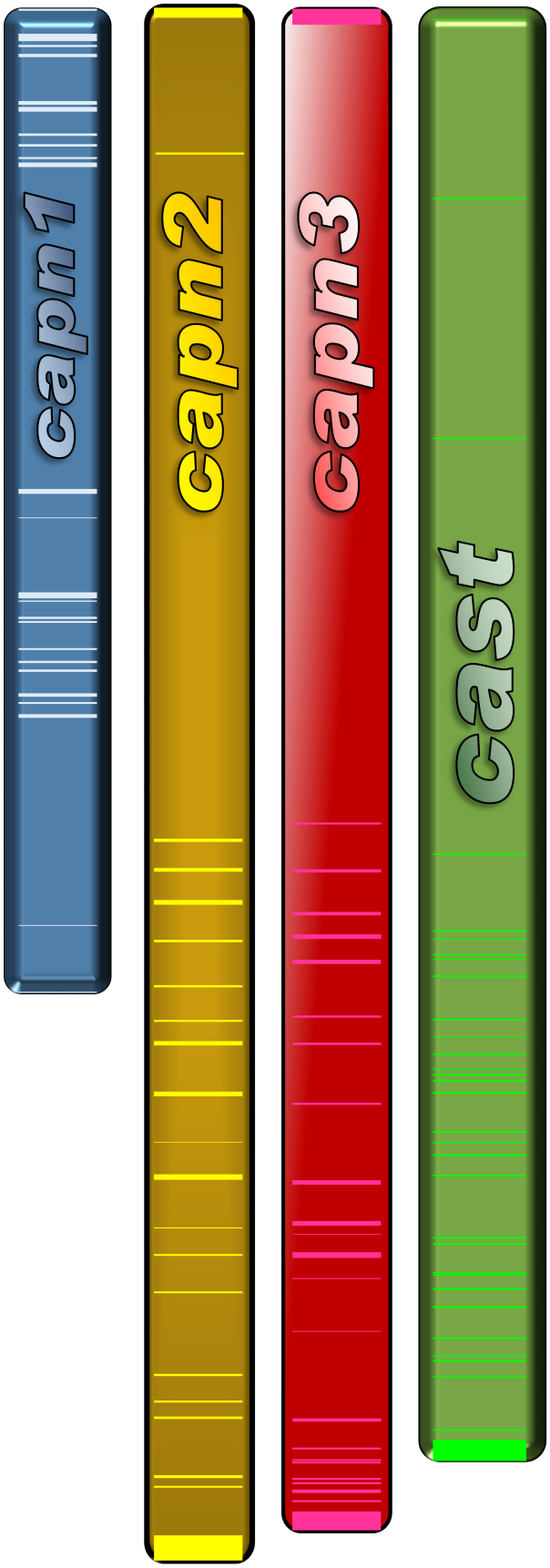
The gene for the large subunit of calpain, *capn1* (Figure 2.1) is located on bovine chromosome 29 (Chr29), corresponding to a site for the QTL for tenderness (Smith *et al.*, 2000). It is commonly accepted that calpain-1 activity (in terms of its level and duration of activity) is almost exclusively responsible for proteolytic tenderness of beef and myofibril fragmentation of early meat ageing (Koohmaraie, 1992a; Taylor & Koohmaraie, 1998; Geesink *et al.*, 2006). Research of the last decade focused primarily on the physiology and genomics of this enzyme and its inhibitor, calpastatin. The other calpains and other proteolytic systems could also have a small effect on beef tenderness (≤ 10%) during the meat ageing process (Kemp *et al.*, 2010).

Calpain-1 activity is generally highest ≤3 days post-mortem, from where activity rapidly decreases (Boehm *et al.*, 1998) as it is subject to some degradation itself (Kemp *et al.*, 2010) and gradually decreases during meat ageing (Koohmaraie, 1992a; Boehm *et al.*, 1998; Koohmaraie & Geesink, 2006). Much of the research on the effects of polymorphisms in the *capn1* gene (and *cast* gene) has focused on beef where these associations are common. Correlations of tenderness with the calpain system have not been as clearly established in mutton or pork (Kemp *et al.*, 2010), but probably also because tenderness is not such a great concern in these species. An association between relative activity of calpain-1 per calpastatin inhibition has been established in lamb *Longissimus lumborum et thoracis* muscle, although sarcomere length can make a significant contribution to tenderness (Veiseth *et al.*, 2004). Calpain-1 is subject to some autolysis (large subunit 80 kDa → 78 kDa → 76 kDa) in the presence of the increased Ca^{2+} of post-mortem muscle, which in turn decreases the level of Ca^{2+} required for half-maximum activity even though some activity is eventually lost through autolysis (Koohmaraie & Geesink, 2006).

2.7.2.2. Calpain-2

The calpain-2 gene (Figure 2.1) is located on Chr16 (Zimin *et al.*, 2009). Although calpain-2 (m-calpain) is generally not significantly active in early-tenderization (due to its greater Ca^{2+} requirement) its activity persists for longer in muscle during meat ageing (Kemp *et al.*, 2010) and its activity remains relatively stable for 14 days post-mortem (Dransfield *et al.*, 1992a; Koohmaraie, 1992a; Koohmaraie & Geesink, 2006).

Calpain-2 is also subject to autolysis in the presence of the increased Ca^{2+} of post-mortem muscle (large subunit 80 kDa → 78 kDa), which decreases the Ca^{2+} concentration for half-maximum activity (Koohmaraie & Geesink, 2006). Depending on the specific muscle, the active autolyzed calpain-2 could still be present in muscle up to 84 days post-mortem and it is possible that this late meat ageing calpain activity could tenderize beef after 14 d ageing, when ultimate tenderness has not been reached yet (Colle & Doumit, 2017).



***capn1* gene (NCBI 281661)**

on Chr29 (AC_000186.1), 51,505,224 bp

Location = 44,059,778 – 44,104,001

Length: 44,223 bp

24 exons

***capn2* gene (NCBI 281662)**

on Chr16 (AC_000173.1) 81,724,487 bp

Location: 27,775,837 – 27,845,845

Length 70,008 bp

21 exons

***capn3* gene (NCBI 281663)**

on Chr10 (AC_000167.1) 104,605,016 bp

Location 37,823,064 – 37,891,571

Length 68,507 bp

24 exons

***cast* gene (NCBI 281039)**

on Chr7 (AC_000164.1) 112,638,689 bp

Location: 98,431,182 – 98,594,904

Length: 163,722 bp

32 exons

Figure 2.1. *The bovine muscle calpain system genes*:* capn1*,* capn2*,* capn3 *and* cast.

*The *capn* genes (introns and exons) drawn to scale, with lighter shaded areas indicating exons of the genes. The *cast* gene is drawn to ⅛ scale of the *capn* genes. bp – base pairs; NCBI – National Center for Biotechnology Information of the US National Library of Medicine. From the NCBI Genome Data Viewer (http://www.ncbi.nlm.nih.gov) using the Bos_taurus_UMD_3.1.1 transcript (NCBI, 2021).

2.7.2.3. Calpain-3

The calpain-3 gene is located on Chr10 (Figure 2.1) and is important in muscle development and growth in cattle and sheep (Nonneman & Koohmaraie, 1999). Calpain-3 (single 94 kDa protein) has a limited ability to affect tenderness (Geesink *et al.*, 2005, 2006) and the extent of the contribution to tenderness still needs to be elucidated (Lian *et al.*, 2013). It is not subject to autolysis in bovine and ovine muscles post-mortem and its activity is relatively stable (Boehm *et al.*, 1998) and not affected by calpastatin (Koohmaraie & Geesink, 2006).

Calpain-3 binds myofibrillar connectin/titin, making it difficult to extract from muscle (Koohmaraie & Geesink, 2006; Ono *et al.*, 2016) and substrates include connectin/titin, nebulin, TnT and desmin (Kemp *et al.*, 2010). Although these substrates correspond to tenderness, knockout mice exhibited normal post-mortem proteolysis and calpain-3 failed to correlate with shear force in pigs, suggesting it has minimal importance in ultimate tenderness (Kemp *et al.*, 2010). Calpain-3 is subject to rapid autolysis *in vitro* at physiological Ca^{2+} concentrations (Sorimachi & Ono, 2012) and in murine species *in vivo*.

2.7.2.4. Calpastatin

The calpastatin gene (Figure 2.1) is located on Chr7 (Bishop *et al.*, 1993) and a single gene codes several peptides through alternative splicing (Geesink *et al.*, 1998). Although several isoforms are present, the most common form of calpastatin in muscle has 4 inhibitory domains that also require Ca^{2+} for inhibition of calpains (Koohmaraie & Geesink, 2006). Calpastatin is activated by concentrations of free Ca^{2+} lower than those required to activate calpains (Koohmaraie, 1992a), which could explain the better predictive value of calpastatin for tenderness found in some research (Koohmaraie *et al.*, 1995; Kemp *et al.*, 2010).

At 24 h post-mortem, calpastatin accounts for 40% of the variation in tenderness, at which stage it is itself subject to proteolysis (Kemp *et al.*, 2010), resulting in the formation of inhibitory calpastatin fragments in post-mortem muscle (Boehm *et al.*, 1998). However, post-mortem conditions in muscle alter calpastatin function to relatively rapidly decrease its action (Boehm *et al.*, 1998), allowing for continued calpain activity (Koohmaraie, 1992a). Both the pH decline and chilling affect the ability of calpastatin to inhibit calpains. At 25°C and pH 7.5, 87% of calpain activity is inhibited, while this decreases to only 55% at pH 5.7. If the temperature at pH 7.5 is decreased to 5°C, only 59% inhibition of calpain activity by calpastatin occurs, which is decreased to only 6% as pH drops to 5.7.

Proteolysis of calpastatin by calpain fails to lead to complete loss of activity (Koohmaraie & Geesink, 2006), because the smaller calpastatin fragments retain inhibitory function (Boehm *et al.*, 1998), and this could contribute to the highly variable nature of post-mortem calpastatin activity equivalent to a 5-fold difference between maximum *vs.* minimum inhibition observed in Brahman steers (Smith *et al.*, 2009). Factors that increase calpastatin activity are associated with tougher meat as an indirect result of decreased post-mortem proteolytic (calpain-1) activity, e.g. treatment with β-adrenergic agonists (Koohmaraie *et al.*, 1991a) and the callipyge genotype (Kemp *et al.*, 2010). The increase in calpastatin induced by different β-agonists was reflected by the decrease in myofibril fragmentation and increase in WBSF (Strydom *et al.*, 2009). The callipyge phenotype in sheep (defective myostatin) involves over-expression of calpastatin, but is also linked to decreased caspase activity, which can compromise early meat ageing (Kemp & Parr, 2012).

The occurrence of PSE-like meat from normal pigs, or normal pork from stress-susceptible pigs could possibly be ascribed to an interaction between the *cast* and *ryr1* genes and their proteins (directly and/or indirectly), with Ca^{2+} homeostasis as a common link between the two (Singh *et al.*, 2004; Krzęcio *et al.*, 2005). These polymorphisms and their interactions were linked to WHC, drip and pH (Krzęcio *et al.*, 2005).

2.7.3. The muscle calpain-calpastatin system in homeostasis

The *in situ* functions of calpains are poorly defined (Spinozzi *et al.*, 2021), while the substrates that have been identified for calpains (many of which are physiologically active) implies a role for these proteases in homeostatic processes. The calpains interact with hormonal function, including impaired insulin action (Sreenan *et al.*, 2001; Yuasa *et al.*, 2016), glucagon, insulin-like growth factor (IGF)-binding proteins (Ghosh *et al.*, 2005) and somatostatin (Rawlings *et al.*, 2008). This could even suggest a possible contribution of the calpains to the complex homeorhetic responses during skeletal muscle growth and development that function beyond a simple proteolytic effect. However, it remains to be established whether these protein substrates make a significant change to cellular metabolic processes of the living animal and are confounded by associations and interactions between individual cellular processes (similar to the post-mortem period).

Next to tenderness traits, the most extensive association analyses of SNPs of the calpain-calpastatin system involve traits associated with growth and carcass meat yield. The totality of evidence linking these genes to growth, suggests that the effects of calpains (and calpastatin) on these traits go beyond a simple effect on protein turnover (Goll *et al.*, 1998). Especially since the effects of the genomic markers on growth is not consistently negative for alleles that are favourable for tenderness (Cafe *et al.*, 2010a; King *et al.*, 2012; Ardicli *et al.*, 2019). The SNPs have been extensively associated with measures of body weight during growth in taurine (Saatchi *et al.*, 2014; Howard *et al.*, 2015; Michenet *et al.*, 2016) and indicine cattle (Royer *et al.*, 2016; Martínez *et al.*, 2017) and body weight at slaughter in taurine (Zhang & Li, 2011; Ardicli *et al.*, 2017) and indicine cattle (Collis *et al.*, 2012; King *et al.*, 2012; Royer *et al.*, 2016). These can be intimately linked to associations with carcass weight in taurine (Bailey, 2013; Ardicli *et al.*, 2017) and indicine cattle (King *et al.*, 2012; Fernandes Jr. *et al.*, 2016; Royer *et al.*, 2016). The genotypes of the calpain-calpastatin system were linked to estimates of growth (gain) (Gomes *et al.*, 2013; Abo-Ismail *et al.*, 2018; Ardicli *et al.*, 2019; Fadhil & Zülkadir, 2021) and resulted in an increase in lean beef yield (Juszczuk-Kubiak *et al.*, 2004b; Royer *et al.*, 2016) and indirect estimates of lean meat yield (Gomes *et al.*, 2013; Tizioto *et al.*, 2013; Ardicli *et al.*, 2017).

These effects on growth and yield are closely linked to altered metabolism (Li *et al.*, 2022a), while energy and lipid metabolism (and hormone signalling) were closely linked to feed efficiency (Mota *et al.*, 2022). The SNPs in *capn* and *cast* genes have been associated with feed efficiency in several studies (Gomes *et al.*, 2013; Riggs & Vaughn, 2015; Obando, 2017; Abo-Ismail *et al.*, 2018; Ardicli *et al.*, 2019) and selection for improved efficiency did not alter WBSF or muscle calpain activity, although slightly higher calpastatin activity was noted (McDonagh *et al.*, 2001).

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Chapter 3: Characterization of the muscle calpain system genes in five South African purebred beef cattle breeds: The potential for selection across breeds

This thesis chapter will be prepared for publication in a scientific, peer-reviewed journal.

Characterization of the muscle calpain system genes in five South African purebred beef cattle breeds: The potential for selection across breeds

Simple summary

Selection for improved beef quality and accelerated ageing will not only result in a better product that can be guaranteed for the consumer, it will also lead to more efficient beef production, decreasing the time required for storage and refrigeration of meat during ageing. Although South African indigenous beef cattle have been characterized using SNP arrays of varying density, limited information is available on the genes associated with beef quality in the calpain system of South African beef. Coupled with other interventions and technologies that accelerate the conversion of muscle to meat, selection for improved beef quality traits may decrease the impact on the environment, making it more sustainable with a lower carbon-footprint. With rising costs and uncertainty of energy supply, accelerated ageing and decreased storage time will increase profit and decrease the probability of meat spoilage due to disruptions in the cold-chain. The 777K Illumina® BovineHD SNP BeadChip provides sufficient density and high linkage in the calpain-calpastatin system genes (the most important for tenderization), to identify candidate SNPs for selection across breeds. Indigenous Sanga breeds such as the Nguni will become increasingly more important as climate change moves the focus from purely yield-driven beef production, towards the use of adaptable cattle that can sustain production of quality beef in harsh environments.

Abstract

In order to characterize the conserved genes (*capn1*, *capn2*, *capn3* and *cast*) in selected South African cattle breeds, beef bulls were genotyped using the Illumina® BovineHD SNP BeadChip (777K). The goal was to determine whether this high-throughput generic SNP array provided sufficiently dense coverage of these genes, because lower density arrays may fail to identify sufficient informative SNPs in the calpain-system genes in Sanga-type or indicine cattle. Five representative beef cattle breeds were chosen; an indigenous Sanga-type (Nguni), an indigenous composite breed (Bonsmara), *Bos taurus* breeds (Angus and Charolais) and *Bos indicus* (Brahman) that are commonly used in cross-breeding for commercial beef production in South Africa. The higher density of the genotypes (lower distance between SNPs), eliminated some of the disadvantages of using lower density SNP arrays that may contain limited informative SNPs for these genes, in some breeds. Our results confirmed breed differences in allelic frequencies in the calpain-system genes, especially Brahman, although the 777K Illumina® BovineHD SNP BeadChip should provide sufficient informative SNPs per gene for breeds, including Brahman and Sanga-types, to determine multibreed associations with beef production and quality traits. Differences between breed types were detected in haplotype block analyses, where high linkage disequilibrium (in especially *capn2* and *cast*) demonstrated that candidate SNPs could be applied across breeds, through linkage between overlapping haplotype blocks.

Keywords: indigenous beef; calpain-calpastatin system genes; allele frequency; linkage; haplotype blocks; across-breed selection

3.1. Introduction

After the first publication of a comprehensive bovine genome linkage map that included *cast* gene sequence information (Barendse *et al.*, 1994; Bishop *et al.*, 1994) and rapid annotation of genes (Womack, 1998; Itoh *et al.*, 2003; Williams, 2005), a large number of studies were aimed at identifying the candidate single nucleotide polymorphisms (SNPs) for a simple genomic merit for beef quality traits, for marker-assisted selection (MAS). With the mapping of the genes for calpastatin (*cast*) (Bishop *et al.*, 1993) and the large subunit of calpain-1 (*capn1*) (Smith *et al.*, 2000), they became the main focus of research on SNPs that determine beef quality, especially tenderness. With improved mapping and annotation and advancement of sequencing technologies and software, the information from a variety of genetic maps were collated (Ihara *et al.*, 2004; Itoh *et al.*, 2005; Snelling *et al.*, 2005), coinciding with more diverse SNP association analyses of more complex traits in beef (Casas *et al.*, 2005). The integration of high-resolution sequencing with existing bovine genetic maps (Wind *et al.*, 2005; Snelling *et al.*, 2007) culminated in the publication of the first whole-genome assemblies (The Bovine Genome Sequencing & Analysis Consortium, 2009; Zimin *et al.*, 2009) containing millions of SNPs in 2009 (The Bovine HapMap Consortium, 2009) and an increase in the number of association studies linking calpain system SNPs to a variety of economically important phenotypes.

Identification of a single SNP that explained significant variance in tenderness in the *capn1* (Page *et al.*, 2002) and *cast* (Barendse, 2002) genes was originally thought of as the key to selection for tenderness. However, it quickly became clear that these SNPs were not universally effective in all cattle populations (Page *et al.*, 2004; Casas *et al.*, 2005; White *et al.*, 2005), with variation between breed types (*Bos taurus* and *Bos indicus*) (Johnston & Graser, 2010) and even between individual herds of the same breed (Williams *et al.*, 2009; Lee *et al.*, 2014, 2019; Pereira *et al.*, 2022), much like the physiological processes themselves (Wheeler *et al.*, 1990; Whipple *et al.*, 1990; Koohmaraie *et al.*, 2003). In addition, the gains in beef quality derived from favourable genotypes, generally identified in improved taurine cattle, may be too small to be economically viable (Lee *et al.*, 2019; Leal-Gutiérrez *et al.*, 2020).

Despite the quantitative nature of beef quality traits (such as tenderness) and the lack of genetic diversity, i.e. low minor allelic frequency (MAF) for many of the commonly studied SNPs (Johnston & Graser, 2010; Tait Jr. *et al.*, 2013), research has continued to use these "classic" SNPs for association analyses. Markers such as *capn1-316*(–T2), *capn1-4751*(–T3), *capn1-530*, *cast-C/T*(*–2859*; T1) and *UoG-cast*(*–282*) were validated with varying effects for taurine (Rincker *et al.*, 2006; Gruber *et al.*, 2011), indicine (Van Eenennaam *et al.*, 2007) and crossbred beef (Johnston & Graser, 2010) and are still being characterised and investigated for beef quality (Iguácel *et al.*, 2019; Lee *et al.*, 2019, 2022; Pereira *et al.*, 2022) in search of a simple, universal solution to selection for beef production. Although effective in targeted populations or research herds, they cannot simply be applied to a commercial setting or to other populations such as South African beef cattle or crossbred beef (Allan & Smith, 2008; Van Marle-Köster *et al.*, 2013; Picard *et al.*, 2015).

A major challenge in the beef industry, compared to the dairy industry, where genomic tools have been successfully applied for some time (Van Raden *et al.*, 2009), is that beef production is characterized by a large number of breeds (and breed types) that are often used in crossbreeding (Scholtz, 2005). Beef quality traits are also generally very expensive (and time-consuming) to quantify and to raise and finish cattle to slaughter age on

grain diets, requires large inputs. This, coupled to slaughter, packaging and storage of aged beef samples that are often complex and expensive to determine accurately, can limit the number of phenotypes measured in genomic studies of a large sample size. During the past decade there has been a drive to genetically characterize South African beef cattle (especially indigenous breeds) using high-throughput arrays (Qwabe *et al.*, 2013). Generic SNP arrays (with evenly distributed, rather than custom-selected SNPs) of lower density that were developed for genotyping taurine dairy cattle can provide insufficient coverage (SNP density) for characterization of informative SNP in South African indigenous cattle or indicine breeds (Makina *et al.*, 2015; Zwane *et al.*, 2016). Most genotyping of South African cattle has employed lower density high-throughput arrays up to 150K and the high cost of denser array genotyping or sequencing is an important limiting factor in these data (Mrode *et al.*, 2019).

The Illumina® BovineHD SNP BeadChip analyses ≃777K variants that are uniformly distributed across the genome, approximately 3.4 kb apart (2.1 – 3.7 kb in the calpain-calpastatin system genes) (Illumina® Data Sheet, 2015). It offers greater coverage of the calpain system genes, through a larger number of SNPs that are more densely distributed over greater proportion of these genes (compared to lower density arrays). During development of the array, variants from improved beef and dairy taurine breeds, indicine breeds, crossbred *Bos taurus* × *Bos indicus* cattle and African taurine (important in Sanga ancestry) were considered for inclusion in the array and validated (total 28 breeds, hybrids and other bovids). The greater number of SNP per gene that were not exclusively identified from taurine cattle should improve the estimates of linkage disequilibrium (LD) and haplotype block structure across breeds and improve the power of association analyses across breeds (Lund *et al.*, 2014; Pérez O'Brien *et al.*, 2014a), i.e. decreased ascertainment bias. To our knowledge, this is the first study to characterize the genes of the calpain-calpastatin system, using high-density (777K) true genotyping in South African beef cattle. Although the 777K Illumina® BovineHD SNP BeadChip is a step in the right direction, advances in sequencing technology mean that the genetic variation between individuals can be mapped in much greater detail once regions with candidate SNPs are identified (redefining the concept of high-definition genotyping once more) (Braz *et al.*, 2018; Bedhane *et al.*, 2019; Wang *et al.*, 2020), including almost 20,000 variants in these causal genes (EVA, 2021).

Genotyping was therefore conducted using a standard commercial, distributed SNP array, the BovineHD SNP BeadChip (777K) (Illumina® BovineHD SNP BeadChip), to determine whether this method of genotyping would identify sufficient informative SNPs to characterize the calpain-calpastatin system genes, with a view to multibreed association analyses. Purebred bulls were chosen to determine the potential usefulness of this high-throughput array in *Bos indicus* (Brahman) and indigenous Sanga-types (Nguni and the composite breed Bonsmara), compared to commonly-researched taurine breeds (Angus and Charolais). We propose that the higher density of genotypes could allow across-breed application of this array, taking advantage of the LD in these genes to search for candidate SNPs for selection across breeds, including *Bos taurus africanus*. The study was conducted using a relatively small sample size, because the cost of HD genotyping and detailed phenotypic analyses was prohibitive and the results should be considered within these constraints.

3.2. Materials and methods

3.2.1. Animals

Purebred South African beef bulls (n = 166) were sourced from different reputable breeders and feedlot-finished from 9 months old up to 12 months old (descriptive statistics of these bulls are summarised in Chapter 4, Table 4.1). Bulls were finished to achieve a carcass class of A2/A3, i.e. zero incisors and lean to medium fatness (Anonymous, 1990, 2015), before slaughter through captive bolt stunning and exsanguination (detailed in Addendum A). All procedures were approved by the Animal Ethics Committee of the University of Pretoria (EC171114-161) and the Ethics Committee of the Animal Production Institute of the Agricultural Research Council (ARC AEC-I 2010 001).

Beef breeds were chosen to be representative of specific types used in South Africa as purebred animals or for crossbreeding. Angus (n = 27) were selected to be representative of British *Bos taurus*, Charolais (n = 34) as European (continental) *Bos taurus* and Brahman (n = 35) as Zebu type *Bos indicus*. Two indigenous breeds were included in the study, Bonsmara (n = 35); a South African composite breed originally created with a large Sanga contribution and Nguni (n = 35); a Sanga breed or *Bos taurus africanus*. The relatively small number of animals per breed used here, would capture only a fraction of the possible genetic variation within breeds and these results will need to be confirmed in the larger populations. This is especially important since the individual breeds are characterised by subpopulations like Red– *vs*. Black Angus breeds and possible genetic variation between different ecotypes of Nguni cattle. These experimental bulls would however be sufficient to test the principle of using the 777K array to characterise different South African breeds (data in this chapter) and to determine the effect of extended ageing on genomic effects that may differ between breeds with different tenderizing rates (Chapter 4).

3.2.2. Genotyping

Shortly after slaughter, muscle or tail hair samples were collected for the extraction of genomic DNA using the NucleoSpin® Tissue kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). All analyses of muscle were determined on the *longissimus thoracis et lumborum* muscle, between the lumbar (L6) and thoracic region (T10). DNA was extracted following four, repeating cycles of freezing in liquid nitrogen (–196°C) and thawing at 56°C, before the addition of proteinase, in order to improve DNA yield (following the manufacturer's instructions). All DNA extraction, quality control and genotyping were conducted at the Agricultural Research Council, Animal Genetics (Irene, South Africa) and the Biotechnology Platform (Pretoria, South Africa).

Microvolume spectrophotometry quality control was performed after overnight stabilization of the extracted DNA at 4°C using a NanoDrop 2000/2000c Spectrophotometer and DNA yield determined using Qubit® 3.0 fluorometric analysis (Thermo Fisher Scientific, Waltham, MA, USA) and ethidium bromide agarose gel electrophoresis (to determine possible fragmentation). A few samples with low double-stranded DNA (dsDNA) concentration were concentrated by evaporative heat (56°C), while several samples were diluted to achieve the normalized range of 37 – 70 ng/µL before genotyping.

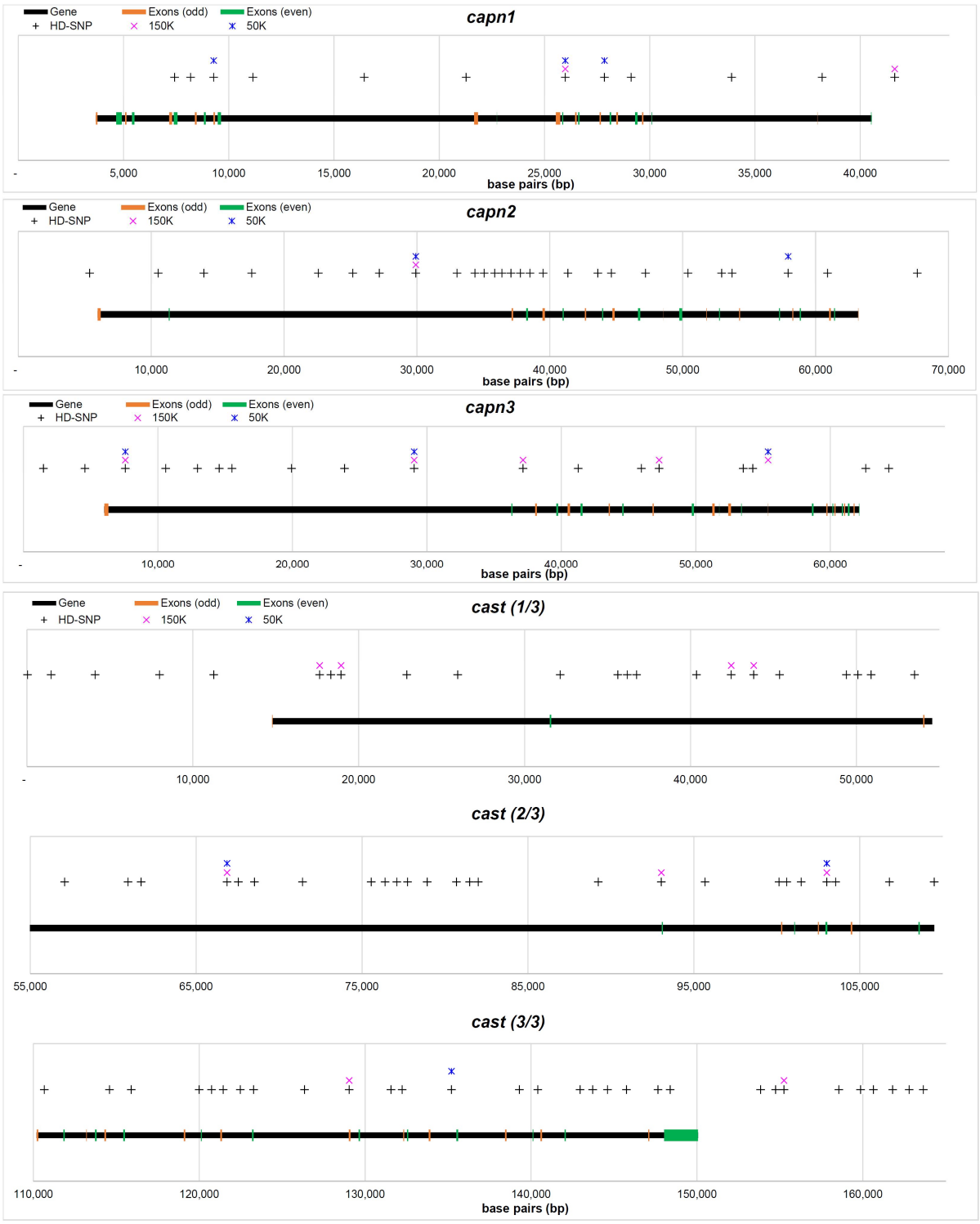

Figure 3.1. *The extent of the transcripts and genes of the muscle calpain system and the single nucleotide variants of the Illumina® BovineHD SNP BeadChip (777K) included in analyses, compared to the BovineSNP50 BeadChip and GGP Bovine 150K arrays.*

All samples were successfully genotyped using the BovineHD SNP BeadChip (Illumina®, San Diego, CA, USA), which identified 777,962 variants in the SNP array. Intensities were captured using the HiScanSQ platform using the Illumina® iScan Control Software (iCS, Illumina®, USA) and analysed using Genome Studio Software Version 2.0.4 (Illumina®, USA). The four genes of the muscle calpain system are the calpain-1 gene (*capn1*) on

chromosome 29, the calpain-2 gene (*capn2*) on chromosome 16, the calpain-3 gene (*capn3*) on chromosome 10 and the calpastatin gene (*cast*) on chromosome 7. A total of 114 variants were identified within the bounds of these genes (see Addendum A, Table 1), as determined by the Genome Data Viewer of the National Center for Biotechnology Information (NCBI, 2021). Using these sequencing transcripts, SNPs that fell within a region of between five (*capn1*) and 14 (*cast*) kilobases from the boundaries of these genes (±8%) were also included in the analyses (Figure 3.1), bringing the total number of SNPs to 144.

3.2.3. Data analyses

PLINK (Purcell *et al.*, 2007) and Genome Studio (Illumina®, USA) software were used to apply quality control measures to the total number of variants of these genes (detailed in Addendum A) and to record basic genetic information from individual samples and from individual variants (Table 3.1).

Table 3.1. *Summary of genomic data for all variants in the muscle calpain system in South African purebred beef bulls genotyped with the Illumina® BovineHD SNP BeadChip (777K).*

Gene	Call rate (SNP)[1]	Heterozygosity [2]		MAF [2]	LD# (D')	LD (r^2)	Distance between SNPs [3]
		Ho	He				
capn1	99.80%	0.36 (0.27 - 0.48)	0.44 (0.31 - 0.50)	35.5% (19.6 - 49.1)	0.69	0.26	3.11
capn2	99.40%	0.33 (0.07 - 0.45)	0.37 (0.06 - 0.50)	28.6% (3.3 - 47.9)	0.92	0.42	2.40
capn3	99.73%	0.30 (0.08 - 0.46)	0.32 (0.09 - 0.49)	22.8% (4.8 - 44.0)	0.63	0.20	3.70
cast	99.66%	0.31 (0.01 - 0.42)	0.33 (0.01 - 0.49)	21.7% (17.3 - 41.5%)	0.84	0.48	2.15

[1] Overall genotyping rate before quality control 99.63% (22,244 total measurements, 83 missing genotypes); [2] Numbers in parentheses represent the range of values calculated; [3] Mean distance between consecutive SNPs (kb).
Ho – observed heterozygosity; He – expected heterozygosity; LD (D') – linkage disequilibrium expressed as Lewontin's D-prime; LD (r^2) – LD as allele association; MAF – minor allelic frequency; SNPs – single nucleotide polymorphisms (or variants)

To determine stratification between breeds, principal components analysis (PCA) was conducted (Patterson *et al.*, 2006) with the full dataset of genotypes of the 777K SNPs of the Illumina® BovineHD SNP BeadChip. Quality control and PCA analyses were conducted using PLINK and visualized using Microsoft Excel and Orange 2.7 data mining software (Slovenia). Data from all 735,293 autosomal variants were loaded in PLINK and subjected to quality control, with a 99.10% total genotyping rate. None of the bulls was removed according to the individual call rate threshold, while 25,891 variants were removed due to the variant call threshold (0.05). A further 34,443 variants failed the Hardy-Weinberg exact test ($<1\times10^{-6}$) and 47,851 variants exhibited MAF <5%. This left 627,108 SNPs that were used to generate a variance-standardized genomic relationship matrix. These data were used to perform PCA, using autosomal SNPs (42,669 non-autosomal or unassigned) to determine the clustering of breeds.

Allelic frequencies of the calpain-calpastatin genes were determined using PLINK and these data were used to determine MAF. For each locus, "tender alleles" were identified as those that favoured phenotypes that are linked to more tender beef, using the weighted mean for each genotype. The first criterion was the genotype that favoured low Warner-Bratzler shear force (WBSF) and if no clear difference was identified, the second

criterion was a short myofibril fragment length (MFL). For some SNPs low calpastatin inhibitory function or finally, high calpain protease activity was used to identify tender alleles at each locus and indicated with an asterisk as the favourable allele (A*/C*/G*/T*) and more detail on the determination of these phenotypes are supplied in Addendum A, Section 4. When considering the designation of "tender alleles", it is important to remember that not all the SNPs will necessarily significantly associate with tenderness phenotypes, even though the genes are functionally linked to the calpain protease system in muscle tissue and tenderization (and generally form part of quantitative trait loci for tenderness). However, the researchers feel confident that the designation of alleles as "tender" will not compromise the conclusions drawn from favourable allelic frequencies.

Analyses of linkage disequilibrium (LD) expressed as Lewontin's D-prime (D') were visualized using Haploview 4.2 (Barrett *et al.*, 2005) (Broad Institute, USA), for pooled data from all breeds and within each breed. Pairwise analysis of linkage between adjacent SNPs (Gabriel *et al.*, 2002) was conducted using the default settings of the software program (r^2 threshold = 0.8), where at least 95% of the loci within each haplotype are in strong LD (with 98% upper confidence interval) This technique corrects for some of the limitations of using D' (Lewontin, 1964, 1995) and data is also expressed as the allelic association, or r-squared (r^2) that represents a more accurate estimate of LD (Hill, 1974).

3.3. Results

3.3.1. Breed differentiation

The largest genetic difference between breeds was observed for Brahman (*Bos indicus*) compared to taurine-types (*Bos taurus* and *Bos taurus africanus* or Sanga-types). Indicine cattle segregated along the first principal component, PC1 (29.34% of variance), while taurine breeds remained undefined under the first PC (Addendum B, Figure S1 A). Considering PC2 (11.42% of variance), Nguni stratified as a distinct cluster from Angus, with another cluster for the remaining breeds (Addendum B, Figure S1 B), while PC3 (5.34% of variance) was able to more clearly define the two taurine breeds (Angus and Charolais) and the two Sanga-types (Nguni and the composite Bonsmara) (Addendum B, Figure S1 C).

After segregation of indicine cattle (Brahman), combination of PC1 and PC2 clearly defined *Bos taurus africanus* (Nguni) from *Bos taurus* (Angus and Charolais), with the Sanga composite (Bonsmara) in an intermediate position between African taurine and European taurine breeds (Figure 3.2 A). The intermediate position of Bonsmara between Sanga and taurine cattle (Figure 3.2 B) is due to the origin of this composite breed combining predominantly Sanga with taurine ancestry (62% Afrikaner, 19% Shorthorn and 19% Hereford). In order to differentiate between the continental *vs.* British taurine breeds that are most closely related as breed types, the third principal component, PC3 was required to cluster the variation between Charolais and Angus into separated groupings (Figure 3.2 C). As PC4 was used in the genetic association models (Chapters 4 and 5), it is included here with the clustering of breed types (Figure 3.2 D).

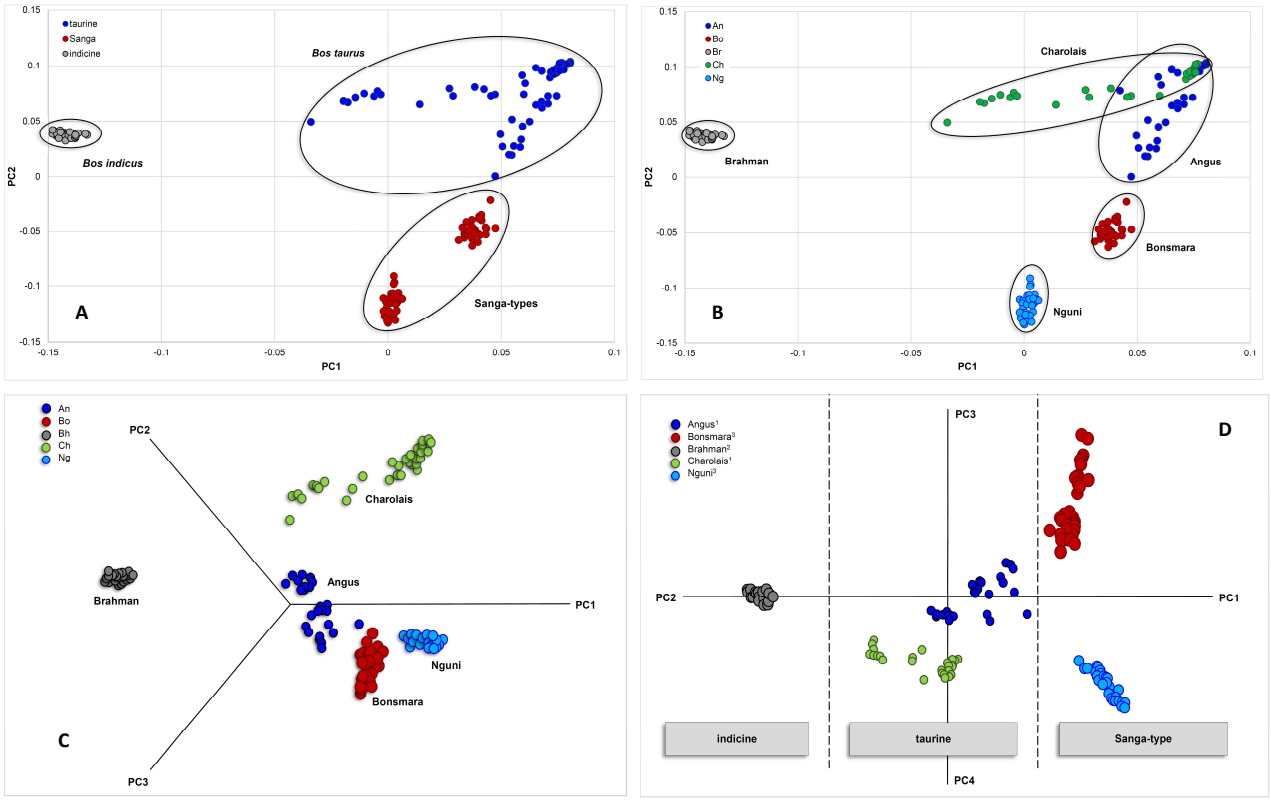

Figure 3.2. *Stratification of South African purebred bulls (n = 166) through principal components analysis (PCA) of 777K Illumina® BovineHD SNP BeadChip using principal components: PC1 (29.34%), PC2 (11.42%), PC3 (5.34%) and PC4 (4.45%); An - Angus [1]; Bo - Bonsmara [3]; Bh - Brahman [2]; Ch - Charolais [1]; Ng - Nguni [3]; representative of 1: taurine, 2: indicine and 3: Sanga-type cattle;* ***A****: Clusters of major breed types using PC1 and PC2;* ***B****: Partial segregation of breeds using PC1 and PC2;* ***C****: Clusters of individual breeds using PC1, PC2 and PC3;* ***D****: Breed and type differentiation using four principal components.*

3.3.2. Allelic frequencies (minor and tender alleles)

Within the genes of the calpain-calpastatin system, 83 missing genotypes were identified, or 0.37% (Table 3.1). The mean distance between single nucleotide polymorphisms (SNPs) was 2.15 kb in *cast*, 3.70 kb in *capn3*, 2.40 kb in *capn2* and 3.11 kb in *capn1*. The minor allelic frequency (MAF) for these four genes was 24.4%, with 8.7% homozygous A1A1 genotypes, 31.6% A1A2 and 59.7% A2A2 homozygous bulls, when A1 was designated the minor allele from pooled data (Addendum B, Table S1).

The A1 (minor) allele from pooled data was generally the minor allele within each of the breeds, with some exceptions. This explains the small differences in mean values between pooled data for A1 (Addendum B, Table S2) and mean values calculated within each breed for the true minor allele (Table 3.2). The MAF ranged between 17.6% in Angus to 27.9% in Charolais. When pooled data was separated by breed and by gene, differences in allelic frequency was particularly evident in Brahman for the three calpain genes (Addendum B, Table S2).

Table 3.2. *Summary of minor allelic frequency (MAF) of SNPs in the muscle calpain system genes in South African beef cattle breeds, genotyped using the Illumina® BovineHD SNP BeadChip (777K).*

Gene [1]	Pooled MAF (n = 166)	Angus [2] (n = 27)	Bonsmara [2] (n = 35)	Brahman [2] (n = 35)	Charolais [2] (n = 34)	Nguni [2] (n = 35)
***cast* (77 SNP)**	21.6%	15.4%	14.9%	24.3%	29.9%	21.9%
***capn3* (18 SNP)**	22.7%	23.1%	22.8%	12.9%	22.4%	21.1%
***capn2* (28 SNP)**	28.5%	15.1%	36.4%	27.2%	25.1%	22.1%
***capn1* (11 SNP)**	35.5%	29.5%	27.4%	15.8%	29.4%	26.9%
Total (134 SNP)	24.4%	17. 6%	21.4%	27.9%	27.9%	22.3%

[1] Number of single nucleotide polymorphisms (SNPs) or variants is quoted in parenthesis for each gene or all genes; [2] A1 designated for each breed separately.

The frequency of alleles favourable for tenderness traits (or tender alleles) in Angus was generally high (Table 3.3), especially for the *capn1* gene, where the frequency of tender alleles was 10% greater than the weighted mean for pooled data, while calpastatin allelic frequency was also higher than average. The greatest proportion of tender alleles in the most important genes for tenderization was however found in Bonsmara cattle, where the frequency was 8.4% higher in the *cast* gene and 13.4% greater in the *capn1* gene. Nguni generally exhibited tender allele frequencies slightly higher than the mean, except in the *capn1* gene the where tender allele frequencies were 11% higher. The indigenous breed types therefore exhibited the genetic potential to produce tender beef. Charolais had an 8% lower proportion of tender alleles in the calpastatin gene, but generally, tender allelic frequencies were similar to the mean of the pooled data. Brahman generally exhibited a lack of tender alleles in the genes for the calpains. Although allele frequencies in *cast* was not greatly different from the pooled mean, in the *capn2* gene tender allelic frequency was almost 7.5% lower, 13.5% lower in *capn3* and almost 35% lower in *capn1*. These results were not unexpected and confirm the results from the PCA, where the first PC was able to segregate Brahman cattle as unrelated to taurine and African taurine cattle.

3.3.3. Linkage disequilibrium

Measures of linkage disequilibrium (LD) between SNPs were evaluated within genes following quality control of variants. Higher levels of LD, expressed as Lewontin's D-prime (D') or allelic association (r^2), were observed for the *cast* and *capn2* genes, with greater levels of diversity (moderate LD) in the *capn1* and *capn3* genes. The high level of LD in the *capn2* gene (D' = 0.91; r^2 = 0.42) and the *cast* gene (D' = 0.84; r^2 = 0.48) suggests that selection has altered the recombination within these genes, causing greater association between different alleles of their SNPs. This was particularly high (D' $\geq$ 0.95; $r^2 \geq$ 0.55) in the Angus, Bonsmara and Nguni breeds, where a non-random association of a very high proportion of alleles for both these genes was evident. The LD was moderate in the *capn1* gene (D' = 0.69; r^2 = 0.26) and *capn3* gene (D' = 0.61; r^2 = 0.20), where breed differences in LD was more prevalent (Table 3.4 and Addendum B, Table S3 and S4), suggesting an opportunity for selection exists within these genes that could be applied across breeds for overlapping regions of high LD.

Table 3.3. *Allelic frequency of SNPs in the muscle calpain system favourable for tenderness, stratified by breed in South African beef breeds, genotyped using the Illumina® BovineHD SNP BeadChip (777K).*

SNV	Chr	Position	[SNP]	Tender allele	Pooled data	Angus	Bonsmara	Brahman	Charolais	Nguni
7_721	7	98,431,222	[T/G]	T*	58.5%	81.5%	58.6%	54.3%	57.8%	45.7%
7_722	7	98,432,644	[T/C]	T*	57.5%	81.5%	58.6%	51.4%	55.9%	45.7%
7_723	7	98,435,292	[T/C]	C*	57.5%	81.5%	58.6%	51.4%	55.9%	45.7%
7_724	7	98,439,177	[A/G]	A*	17.2%	3.7%	31.4%	0.0%	17.7%	30.0%
7_725	7	98,442,449	[T/C]	C*	57.5%	81.5%	58.6%	51.4%	55.9%	45.7%
7_726	7	98,448,831	[A/G]	A*	80.7%	85.2%	91.4%	67.1%	85.3%	75.7%
7_727	7	98,449,505	[T/C]	C*	80.7%	85.2%	91.4%	67.1%	85.3%	75.7%
7_728	7	98,450,117	[T/C]	C*	57.5%	81.5%	58.6%	51.4%	55.9%	45.7%
7_729	7	98,454,085	[A/G]	A*	64.5%	81.5%	58.6%	74.3%	60.3%	51.4%
7_730	7	98,457,153	[T/C]	C*	23.2%	3.7%	32.9%	15.7%	29.4%	30.0%
7_731	7	98,463,330	[A/G]	A*	13.6%	1.9%	31.4%	0.0%	4.4%	27.1%
7_732	7	98,466,806	[T/C]	T*	64.5%	81.5%	58.6%	74.3%	60.3%	51.4%
7_733	7	98,467,371	[T/C]	C*	64.8%	81.5%	60.0%	74.3%	60.3%	51.4%
7_734	7	98,467,934	[T/G]	T*	64.8%	81.5%	60.0%	74.3%	60.3%	51.4%
7_735	7	98,471,546	[A/G]	G*	64.5%	81.5%	58.6%	74.3%	60.3%	51.4%
7_736	7	98,473,634	[T/G]	G*	27.7%	16.7%	40.0%	10.0%	23.5%	45.7%
7_737	7	98,474,995	[T/G]	G*	25.0%	16.7%	40.0%	21.4%	14.7%	30.0%
7_738	7	98,476,556	[T/C]	C*	32.8%	16.7%	40.0%	25.7%	29.4%	48.6%
7_739	7	98,480,585	[T/C]	T*	79.2%	83.3%	90.0%	77.1%	67.7%	78.6%
7_740	7	98,481,274	[A/C]	C*	79.2%	83.3%	90.0%	77.1%	67.7%	78.6%
7_349	7	98,482,074	[T/C]	T*	81.6%	83.3%	90.0%	87.1%	69.1%	78.6%
7_741	7	98,484,691	[A/C]	C*	78.3%	82.0%	87.9%	78.1%	67.2%	77.9%
7_742	7	98,488,255	[T/C]	C*	80.1%	83.3%	90.0%	80.0%	69.1%	78.6%
7_743	7	98,492,079	[A/G]	A*	85.8%	83.3%	91.4%	84.3%	71.2%	97.1%
7_744	7	98,492,868	[A/G]	G*	85.8%	83.3%	91.4%	84.3%	72.1%	97.1%
7_901	7	98,498,047	[T/G]	G*	88.3%	83.3%	91.4%	94.3%	73.5%	97.1%
7_746	7	98,498,729	[A/G]	G*	88.3%	83.3%	91.4%	94.3%	73.5%	97.1%
7_747	7	98,499,702	[A/C]	C*	78.6%	83.3%	90.0%	74.3%	64.7%	81.4%
7_748	7	98,502,599	[T/C]	T*	82.2%	83.3%	90.0%	87.1%	69.1%	81.4%
7_749	7	98,506,739	[A/G]	G*	82.8%	83.3%	91.4%	87.1%	70.6%	81.4%
7_750	7	98,507,574	[T/G]	G*	79.2%	83.3%	90.0%	77.1%	64.7%	81.4%
7_751	7	98,508,282	[A/C]	A*	78.6%	83.3%	90.0%	74.3%	64.7%	81.4%
7_752	7	98,508,931	[T/C]	T*	78.6%	83.3%	90.0%	74.3%	64.7%	81.4%
7_753	7	98,510,114	[T/C]	C*	99.4%	100.0%	100.0%	98.6%	98.5%	100.0%
7_754	7	98,511,880	[T/G]	T*	86.1%	87.0%	97.1%	82.9%	79.4%	84.3%
7_755	7	98,512,675	[A/G]	A*	78.6%	83.3%	90.0%	74.3%	64.7%	81.4%
7_756	7	98,513,190	[A/G]	G*	78.6%	83.3%	90.0%	74.3%	64.7%	81.4%
7_757	7	98,520,428	[T/C]	T*	78.5%	83.3%	90.0%	75.0%	62.5%	81.4%
7_758	7	98,524,220	[A/G]	G*	78.6%	83.3%	90.0%	74.3%	64.7%	81.4%
7_759	7	98,526,859	[T/C]	T*	92.7%	96.3%	92.9%	92.6%	85.3%	97.1%
7_760	7	98,531,321	[A/G]	A*	79.2%	85.2%	90.0%	74.3%	66.2%	81.4%
7_761	7	98,531,781	[A/G]	G*	9.0%	14.8%	8.6%	5.7%	14.7%	2.9%
7_762	7	98,532,654	[T/C]	T*	79.5%	85.2%	90.0%	75.7%	66.2%	81.4%
7_670	7	98,534,197	[A/G]	A*	79.2%	85.2%	90.0%	74.3%	66.2%	81.4%
7_763	7	98,534,736	[T/C]	C*	82.9%	85.2%	88.6%	78.8%	80.9%	81.4%
7_764	7	98,537,976	[T/C]	T*	93.4%	88.9%	98.6%	90.0%	88.2%	100.0%
7_765	7	98,540,675	[T/C]	C*	93.1%	88.9%	98.6%	91.4%	85.3%	100.0%

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SNV	Chr	Position	[SNP]	Tender allele	Pooled data	Angus	Bonsmara	Brahman	Charolais	Nguni
7_766	7	98,541,844	[T/G]	G*	79.5%	85.2%	91.4%	74.3%	66.2%	81.4%
7_767	7	98,545,774	[T/C]	T*	83.1%	85.2%	91.4%	80.0%	77.9%	81.4%
7_768	7	98,547,086	[T/C]	T*	79.5%	85.2%	91.4%	74.3%	66.2%	81.4%
7_769	7	98,551,183	[A/G]	G*	84.2%	96.3%	92.9%	75.7%	76.6%	81.4%
7_770	7	98,551,927	[T/C]	T*	79.4%	85.2%	91.4%	75.0%	64.7%	81.4%
7_771	7	98,552,632	[A/C]	C*	79.2%	85.2%	91.4%	74.3%	64.7%	81.4%
7_772	7	98,553,659	[T/C]	C*	79.2%	85.2%	91.4%	74.3%	64.7%	81.4%
7_773	7	98,554,459	[A/G]	A*	79.8%	85.2%	91.4%	75.7%	66.2%	81.4%
7_774	7	98,557,529	[A/G]	A*	15.4%	3.7%	7.1%	22.9%	22.1%	18.6%
7_775	7	98,560,223	[A/G]	A*	81.6%	85.2%	91.4%	81.4%	69.1%	81.4%
7_776	7	98,562,742	[A/G]	A*	74.7%	59.3%	90.0%	76.5%	62.1%	81.4%
7_777	7	98,563,418	[T/C]	T*	79.4%	86.5%	91.4%	74.3%	64.7%	81.4%
7_116	7	98,566,391	[A/G]	A*	83.4%	96.3%	92.9%	74.3%	75.0%	81.4%
7_778	7	98,570,487	[T/G]	G*	77.4%	85.2%	91.4%	64.3%	66.2%	81.4%
7_779	7	98,571,597	[T/G]	G*	77.6%	85.2%	91.4%	64.3%	66.7%	81.4%
7_780	7	98,574,139	[T/C]	C*	77.4%	85.2%	91.4%	65.7%	64.7%	81.4%
7_781	7	98,574,903	[T/C]	C*	77.4%	85.2%	91.4%	65.7%	64.7%	81.4%
7_782	7	98,575,799	[T/G]	T*	77.6%	85.2%	91.4%	65.7%	64.7%	82.4%
7_783	7	98,576,940	[T/C]	T*	82.5%	85.2%	91.4%	78.6%	76.5%	81.4%
7_784	7	98,578,836	[A/G]	G*	78.9%	85.2%	91.4%	62.9%	75.0%	81.4%
7_350	7	98,579,574	[A/G]	G*	78.3%	85.2%	91.4%	68.6%	66.2%	81.4%
7_786	7	98,585,027	[T/C]	C*	76.8%	85.2%	91.4%	62.9%	64.7%	81.4%
7_787	7	98,585,930	[T/C]	C*	77.0%	85.2%	91.4%	62.9%	65.2%	81.4%
7_788	7	98,586,431	[A/C]	A*	78.9%	85.2%	91.4%	70.0%	67.7%	81.4%
7_789	7	98,589,740	[T/C]	T*	73.5%	61.1%	90.0%	68.6%	63.2%	81.4%
7_790	7	98,591,048	[T/C]	C*	82.5%	85.2%	91.4%	78.6%	76.5%	81.4%
7_791	7	98,591,826	[T/C]	T*	74.2%	59.3%	90.0%	64.3%	72.7%	81.4%
7_792	7	98,592,979	[T/C]	T*	79.7%	85.2%	91.4%	76.5%	64.7%	81.4%
7_793	7	98,593,984	[T/C]	T*	80.1%	85.2%	91.4%	67.1%	76.5%	81.4%
7_794	7	98,594,835	[T/C]	C*	14.4%	10.0%	6.1%	19.7%	16.7%	18.6%
					71.3%	**75.2%**	**79.6%**	**67.2%**	**62.9%**	**72.4%**

SNV	Chr	Position	[SNP]	Tender allele	Pooled data	Angus	Bonsmara	Brahman	Charolais	Nguni
10_712	10	37,824,551	[A/G]	G*	9.6%	20.4%	8.6%	1.4%	19.1%	1.4%
10_347	10	37,827,639	[A/G]	A*	76.2%	90.7%	98.6%	15.7%	88.2%	91.4%
10_350	10	37,830,642	[T/C]	C*	83.1%	79.6%	85.7%	80.0%	77.9%	91.4%
10_713	10	37,833,640	[A/G]	A*	9.3%	20.4%	8.6%	0.0%	19.1%	1.4%
10_714	10	37,836,009	[T/C]	T*	81.3%	77.8%	84.3%	75.7%	76.5%	91.4%
10_715	10	37,837,623	[T/C]	T*	4.8%	1.9%	7.1%	0.0%	13.2%	1.4%
10_716	10	37,838,570	[A/G]	A*	83.1%	79.6%	85.7%	80.0%	77.9%	91.4%
10_718	10	37,843,006	[T/G]	T*	32.5%	16.7%	60.0%	22.9%	19.1%	40.0%
10_719	10	37,846,946	[A/G]	G*	43.4%	37.0%	68.6%	24.3%	36.8%	48.6%
10_293	10	37,852,123	[T/C]	T*	35.8%	50.0%	30.0%	5.7%	48.5%	48.6%
10_720	10	37,860,216	[A/G]	G*	35.5%	38.9%	50.0%	21.4%	27.9%	40.0%
10_721	10	37,864,319	[A/G]	G*	56.0%	79.6%	50.0%	21.4%	73.5%	61.4%
10_722	10	37,869,021	[A/G]	A*	43.7%	59.3%	51.4%	41.4%	32.4%	37.1%
10_723	10	37,870,339	[T/C]	C*	81.9%	90.7%	71.4%	97.1%	69.1%	82.9%
10_725	10	37,876,596	[T/C]	T*	13.9%	22.2%	15.7%	2.9%	13.2%	17.1%
10_726	10	37,877,303	[A/G]	G*	15.1%	22.2%	15.7%	2.9%	13.2%	22.9%
10_727	10	37,885,702	[T/G]	T*	12.0%	22.2%	14.7%	1.5%	9.7%	14.1%
10_728	10	37,887,422	[T/C]	C*	13.9%	22.2%	15.7%	2.9%	13.2%	17.1%
					40.6%	**46.2%**	**45.7%**	**27.6%**	**40.5%**	**44.4%**

SNV	Chr	Position	[SNP]	Tender allele	Pooled data	Angus	Bonsmara	Brahman	Charolais	Nguni
16_758	16	27,781,212	[T/C]	T*	33.8%	13.0%	48.5%	61.4%	22.7%	18.6%
16_759	16	27,786,368	[A/G]	G*	47.6%	27.8%	61.4%	65.7%	39.7%	38.6%
16_760	16	27,789,803	[A/G]	A*	57.6%	81.5%	42.9%	34.3%	70.3%	65.7%
16_761	16	27,793,409	[T/C]	T*	56.1%	80.8%	41.2%	41.4%	57.6%	65.7%
16_762	16	27,798,419	[A/G]	A*	12.8%	19.2%	4.5%	0.0%	36.4%	5.9%
16_763	16	27,801,014	[T/C]	T*	52.1%	74.1%	38.6%	34.3%	57.4%	61.4%
16_764	16	27,803,013	[A/G]	G*	20.8%	14.8%	42.9%	8.6%	17.7%	18.6%
16_216	16	27,805,751	[A/G]	G*	31.0%	18.5%	57.1%	22.9%	20.6%	32.9%
16_765	16	27,808,861	[A/G]	A*	67.2%	85.2%	48.6%	55.7%	76.5%	74.3%
16_766	16	27,810,205	[A/G]	A*	54.5%	79.6%	41.4%	34.3%	57.4%	65.7%
16_767	16	27,810,903	[T/C]	T*	67.8%	85.2%	48.6%	58.6%	76.5%	74.3%
16_768	16	27,811,695	[T/C]	C*	21.7%	14.8%	42.9%	12.9%	17.7%	18.6%
16_769	16	27,812,241	[T/C]	C*	21.7%	14.8%	42.9%	12.9%	17.7%	18.6%
16_416	16	27,812,915	[T/C]	C*	26.5%	14.8%	51.4%	18.6%	19.1%	25.7%
16_770	16	27,813,621	[A/G]	A*	63.6%	81.5%	42.9%	55.7%	75.0%	67.1%
16_771	16	27,814,339	[A/G]	A*	67.2%	85.2%	48.6%	57.1%	75.0%	74.3%
16_772	16	27,815,334	[A/G]	A*	52.1%	74.1%	38.6%	34.3%	57.4%	61.4%
16_773	16	27,817,199	[T/C]	T*	27.7%	14.8%	42.9%	34.3%	25.0%	18.6%
16_774	16	27,819,457	[A/G]	G*	95.8%	94.4%	97.1%	100.0%	86.8%	100.0%
16_775	16	27,820,470	[T/C]	T*	27.7%	14.8%	42.9%	34.3%	25.0%	18.6%
16_776	16	27,823,039	[A/G]	G*	65.7%	85.2%	57.1%	48.6%	60.3%	81.4%
16_777	16	27,826,235	[T/C]	T*	19.9%	14.8%	42.7%	11.4%	13.6%	15.6%
16_778	16	27,828,773	[A/G]	G*	20.8%	14.8%	42.9%	11.4%	14.7%	18.6%
16_779	16	27,829,558	[T/C]	C*	96.7%	98.1%	98.6%	100.0%	86.8%	100.0%
16_727	16	27,833,776	[A/C]	C*	96.8%	100.0%	100.0%	87.9%	96.3%	100.0%
16_780	16	27,836,737	[A/G]	A*	79.2%	94.4%	85.7%	57.1%	76.5%	85.7%
16_781	16	27,843,498	[T/C]	C*	28.9%	9.3%	48.6%	40.0%	16.2%	25.7%
					48.6%	**52.1%**	**51.9%**	**42.0%**	**48.0%**	**50.1%**

SNV	Chr	Position	[SNP]	Tender allele	Pooled data	Angus	Bonsmara	Brahman	Charolais	Nguni
29_183	29	44,067,207	[T/C]	C*	46.4%	55.6%	64.3%	24.3%	60.3%	30.0%
29_184	29	44,067,968	[A/G]	G*	66.3%	81.5%	82.9%	25.7%	67.7%	77.1%
29_316	29	44,069,063	[C/G]	C*	19.6%	25.9%	18.6%	0.0%	7.4%	47.1%
29_185	29	44,070,926	[A/G]	A*	28.3%	25.9%	48.6%	0.0%	8.8%	57.1%
29_186	29	44,076,213	[A/C]	C*	61.9%	72.2%	73.5%	20.0%	71.2%	75.7%
29_187	29	44,081,056	[T/C]	T*	62.7%	77.8%	87.1%	10.0%	68.2%	74.3%
29_370	29	44,085,769	[A/G]	A*	23.5%	37.0%	32.9%	11.4%	35.3%	4.3%
29_4751	29	44,087,629	[T/C]	C*	50.9%	59.3%	65.7%	10.0%	51.5%	70.0%
29_188	29	44,088,897	[A/G]	A*	58.7%	63.0%	67.1%	24.3%	55.9%	84.3%
29_189	29	44,093,671	[T/C]	T*	63.3%	77.8%	77.1%	21.4%	74.2%	70.0%
29_190	29	44,097,970	[A/G]	G*	59.6%	70.4%	75.7%	10.0%	75.0%	70.0%
29_191	29	44,101,417	[T/C]	T*	68.1%	77.8%	77.1%	32.9%	75.0%	80.0%
					50.8%	**60.3%**	**64.2%**	**15.8%**	**54.2%**	**61.7%**

Chr - chromosome; [SNP] - alleles of the variant; SNV - single nucleotide variant coded as CHR_last 3 digits of Illumina name; SNV that failed quality control (MAF<0.05, failed Hardy-Weinberg exact test) are highlighted in red; deviation of >10% from data pooled for breeds were highlighted in grey and greater than 20% in yellow; Where the allele designated A1 in pooled data was not the minor allele in the specific breed, text was edited to bold and underlined.

Table 3.4. *Average linkage disequilibrium of the muscle calpain system genes (*capn1*,* capn2*,* capn3 *and* cast *genes) in South African beef breeds genotyped using the Illumina® BovineHD SNP BeadChip (777K).*

Genes		Pooled data	Angus	Bonsmara	Brahman	Charolais	Nguni
ALL	D'	0.81	0.93	0.95	0.87	0.82	0.93
	(r^2)	(0.41)	(0.60)	(0.53)	(0.40)	(0.44)	(0.52)
cast	D'	0.84	0.98	0.98	0.87	0.86	0.98
	(r^2)	(0.48)	(0.76)	(0.60)	(0.46)	(0.52)	(0.60)
capn3	D'	0.61	0.79	0.83	0.80	0.77	0.68
	(r^2)	(0.20)	(0.23)	(0.29)	(0.22)	(0.34)	(0.23)
capn2	D'	0.91	0.95	0.98	0.90	0.84	0.96
	(r^2)	(0.42)	(0.57)	(0.59)	(0.30)	(0.40)	(0.56)
capn1	D'	0.69	0.76	0.86	0.85	0.65	0.83
	(r^2)	(0.26)	(0.25)	(0.31)	(0.37)	(0.22)	(0.38)

D' – mean linkage disequilibrium expressed as Lewontin's D-prime; Pooled data - weighted mean linkage disequilibrium for all breeds; r^2 – mean linkage disequilibrium expressed as allele association.

3.3.4. Haplotype blocks

The high level of linkage observed in the genes of the calpain-calpastatin system was associated with extensive haplotype blocks (Figure 3.3), while the structure of these blocks was different between the different breeds (Addendum B, Table S5). Although uniformity was observed within some genes for some breeds, limiting the opportunities for selection because of low genetic variance within genes, diversity existed in others, where the opportunity exists to increase the frequency of alleles that are favourable for beef quality traits.

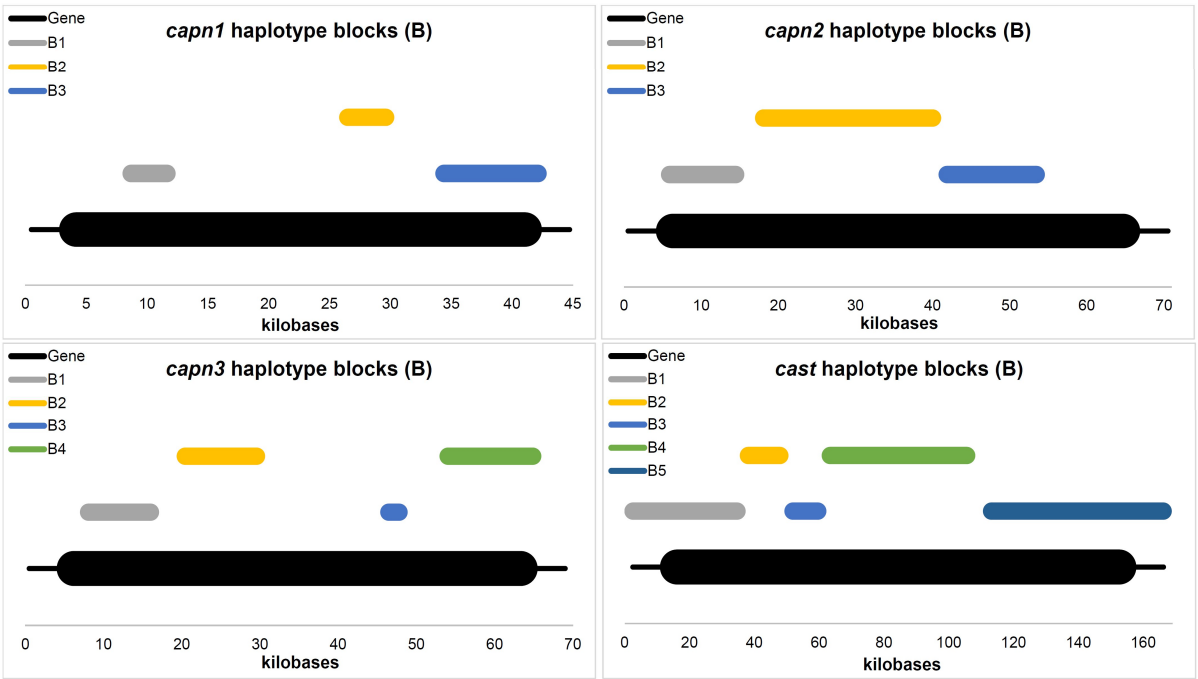

Figure 3.3. *Haplotype blocks of the muscle calpain system genes in South African beef breeds genotyped using the Illumina® BovineHD SNP BeadChip (777K); B1-B5 – Haplotype blocks identified using pairwise analysis in Haploview 4.2* (Gabriel *et al.*, 2002; Barrett *et al.*, 2005)*; thick black lines represent coding regions of the genes; thin black lines represent the reference sequence, ±8% of the gene's length* (NCBI, 2021).

3.3.4.1. The calpastatin gene

Most of the regions upstream and downstream of the *cast* gene, were part of haplotype blocks that extended into the 5'-untranslated region (UTR) and 3'-UTR regions of the genes respectively (Addendum B, Table S5). This means that the regions that are responsible for control of the activity of the *cast* gene (the functional regions of the gene) were linked to the structural (or coding) regions of the gene through LD. Within breeds, the 5'-UTR exhibited fragmented haplotype blocks in Bonsmara, Charolais, and Nguni that were not continuous with the coding regions, but were linked to coding regions of the gene in Angus and Brahman. Large haplotype blocks extending into the coding regions were identified in the 3'-UTR of all breeds except Brahman.

With *cast* generally considered the most important gene controlling tenderization of beef, it is interesting to note that all 68 SNPs within the gene of Angus cattle formed part of a single haplotype block. The only exceptions were SNPs that failed quality control (were not polymorphic) and *cast_794*, downstream of the cast gene. Bonsmara block structure was very similar to Angus, with a haplotype block upstream of the gene, including all five SNPs of the reference sequence, while a large haplotype block included all the other (67) SNPs that passed quality control, except the downstream SNP, *7_794*. Nguni haplotype blocks were more fragmented, especially in the regions upstream and toward the 5'-part of the gene, although all SNPs formed part of haplotype blocks (B1 – B4). Haplotype block B1 (5'-UTR) and haplotype block B3 (98,450,117 – 98,476,556) could be potential sites for selection, where the frequency of favourable alleles was generally 10% lower than the pooled average and enough variation occurred to improve quality through increasing the proportion of favourable alleles of the *cast* gene for Nguni. The 3'-UTR, up to almost halfway down the *cast* gene transcript of Charolais, was relatively fragmented with four haplotype blocks, while tender allelic frequencies were, at least, 10% below the pooled average for many SNPs. This demonstrates an opportunity to improve the *cast* gene in the South African Charolais breed. The haplotype blocks of Brahman were distributed throughout the gene and this suggests that a greater level of recombination would occur, while less selection for tenderization occurred in this breed. Except for the fragmented nature of the haplotype blocks in Brahman, the extensive LD of the *cast* gene exhibited the most promise for identification of candidate SNPs that could be applied across shared or overlapping haplotype blocks of different breeds.

3.3.4.2. The calpain-2 gene

Extensive LD was also observed in the *capn2* gene. In Sanga-types (Bonsmara and Nguni) one large haplotype block spanning the entire gene was identified that indicates very low levels of recombination of the gene in these breeds (Addendum B, Table S5). Within the three other breeds, two haplotype blocks were identified spanning a large portion of the gene; the first haplotype block (B1) between 15 – 18 SNPs long and a second (B2) shorter block of between 3 – 6 SNPs.

Extensive linkage and overlapping of haplotype block B1 in all five breeds represent an opportunity for selection, including a relatively small number of candidate SNPs in this gene that are linked through LD. Although LD indicated that the inheritance of the gene was influenced by selection, this was not a directional change for beef quality traits, with an allelic frequency that is almost equally distributed between alleles. This could be due to traits other than beef quality that have been selected for and are affected by genotypes in the gene.

3.3.4.3. The calpain-1 and calpain-3 genes

Although haplotype blocks were identified in the *capn1* and *capn3* genes (Addendum B, Table S5), the overlap between blocks in different breeds was limited, while only a single haplotype block of limited extent was identified in Brahman (for both genes). A more diverse SNP panel will be required for selection in these genes, especially if Brahman were included as a breed targeted for selection, considering the larger deviations in allelic distribution for these genes.

3.4. Discussion

Genomic selection is a relatively new concept in beef production compared to dairy production, where genomic selection has been successfully applied for some years (Georges *et al.*, 2019). In addition to the constraints of the cost of high density true genotyping or sequencing and the challenges of collecting accurate beef quality phenotypes, another major challenge to the application of genomic selection in beef cattle is a lack of variation in some genomic markers in the calpain-calpastatin system for tenderness phenotypes in some populations. Several researchers have proved that selection in breeds and herds has already resulted in an increase in the favourable alleles of the calpain-calpastatin system, to the level of being essentially fixed for candidate SNPs of the *cast* gene (Morris *et al.*, 2006; Van Eenennaam *et al.*, 2007; Frylinck *et al.*, 2009; Robinson *et al.*, 2012) and the *capn1* gene (Corva *et al.*, 2007; Williams *et al.*, 2009; Iso-Touru *et al.*, 2018). Conversely, candidate SNPs have been found to be almost fixed for the unfavourable alleles in the *capn1* gene in improved taurine (Ekerljung *et al.*, 2012), taurine crosses (Van Eenennaam *et al.*, 2007), indicine (Smith *et al.*, 2009) and African taurine cattle (Frylinck *et al.*, 2009), for candidate SNPs. It remains to be seen whether these generalizations can be applied to African cattle breeds, as the financial cost of detailed genotyping and phenomics has been a major challenge to performing accurate association analyses. The Illumina® BovineHD SNP BeadChip represents an opportunity to identify candidate SNPs in the calpain-calpastatin system in South African indigenous cattle that is well below the 14 kb threshold identified for indicine breeds (Pérez O'Brien *et al.*, 2014a) and should include sufficient informative SNPs in these genes to accurately determine LD, with the aim to perform association analyses in future.

3.4.1. Breeds and their differentiation

Population stratification can be visualized using principal components analysis (PCA), by capturing the variation between individuals from the variance-standardized relationship matrix, i.e. a multi-variate analysis of population structure or relatedness (Patterson *et al.*, 2006). These matrices generate principal components (PC) that each, consecutively capture a smaller proportion of the genetic diversity within (for example) each breed. By plotting these values for each individual, tight clusters emerge for homogenous groups (or breeds), while more diffuse clustering could indicate that sub-populations (or ecotypes) are present within these open clusters (Anderson *et al.*, 2010), such as those previously identified in Bonsmara and Nguni.

The large genetic difference between Brahman and taurine breeds was not unexpected (McKay *et al.*, 2008). Brahman cattle are a popular choice for crossbreeding in South Africa, because of their adaptation to tropical and subtropical conditions, while indicine cattle produce beef that is very different from improved

taurine beef cattle breeds (Wheeler *et al.*, 1990; Whipple *et al.*, 1990). Although the physiological mechanisms that determine the conversion of muscle to meat are similar, many inherent differences within muscle result in lower quality beef. Brahman cattle generally produce tougher beef (Whipple *et al.*, 1990; Shackelford *et al.*, 1995; O'Connor *et al.*, 1997), where Warner-Bratzler shear force (WBSF) values are not only higher (Robinson *et al.*, 2001), but also more variable than taurine cattle (Robinson *et al.*, 2001), especially in the *Longissimus* muscle most often used in research (Shackelford *et al.*, 1995). This is mainly the result of a greater inhibitory effect of calpastatin, rather than decreased protease degradation directly (Whipple *et al.*, 1990; Shackelford *et al.*, 1991; O'Connor *et al.*, 1997). The adaptive advantages of using Brahman in crossbreeding, therefore comes with a compromise; adaptation is achieved at the expense of beef quality (Warner *et al.*, 2022). These physiological differences can be linked to the differences in tender allelic frequencies in the calpain-calpastatin system, although only a moderate reduction in tender allelic frequency was observed in the *cast* gene, with the most noticeable reduction in tender alleles in the *capn1* gene. This genetic difference not only explains the increased toughness of beef from indicine cattle, but can also be linked to the variability in tenderization (Gruber *et al.*, 2011).

Nguni did not cluster in an intermediate position between indicine and taurine types, but rather relatively closer to taurine cattle, but distinct from other breeds. Nguni cattle have a greater proportion of taurine than indicine ancestry (Makina *et al.*, 2016). Nguni cattle in this research was a relatively homogenous group and the characteristics of the population that is identified here could not be representative of different breed ecotypes, or even all South African Nguni (Wang *et al.*, 2015) and will need to be confirmed in a larger sample. Nguni cattle are generally small– to medium-framed, tropically adapted indigenous South African *Bos taurus africanus* cattle, with origins in Southern and Eastern Africa (Bester *et al.*, 2001; Scholtz *et al.*, 2017). Due to their cultural and ceremonial importance in communal farming (that makes up a relatively large portion of the South African beef herd), selection focused more on appearance, temperament and adaptability than on Western perceptions of beef quality traits that are important in improved breeds (Vimiso *et al.*, 2012). Nguni does however have the ability to produce high quality beef (Casey *et al.*, 1990; Swanepoel *et al.*, 1990; Strydom, 2008), outperforming *Bos taurus* breeds (re–)productively under extensive systems (Du Plessis *et al.*, 2006). With intensive feedlotting (commonly used for finishing beef in South Africa), Nguni produce beef that is in most respects of similar quality to improved taurine cattle, but of higher quality than indicine cattle (Strydom *et al.*, 2000, 2008, 2011). Nguni have high levels of myofibril fragmentation (Strydom *et al.*, 2000, 2011), resulting from increased calpain protease activity (Strydom *et al.*, 2011). Nguni have the genetic potential to produce tender beef (Frylinck *et al.*, 2009), but their smaller carcasses could be at risk of cold-shortening in conventional chilling systems designed for larger framed beef (Strydom *et al.*, 2001; Frylinck *et al.*, 2009, 2015) and are currently not favoured in the yield-driven market (Chingala *et al.*, 2017). The breed is however used in crossbreeding, to allow for improved adaptive and reproductive performance, in crosses with larger improved taurine breeds (Ramsay *et al.*, 2000; Scholtz & Theunissen, 2010; Strydom & Hope-Jones, 2022). Unlike crossbreeding with Brahman, Nguni crossbred cattle do not suffer a significant loss of beef quality that can be linked to their closer relationship to taurine than indicine cattle. The Nguni cattle in this experiment exhibited favourable allelic frequencies in the calpain-calpastatin system genes that were above-average, and were second only to Bonsmara cattle in the

capn1 gene. As climate change-induced heat and drought continues to place increasing pressure on agriculture (Kooverjee *et al.*, 2022; Thornton *et al.*, 2022), their smaller frame size and reduced maintenance cost (Bester *et al.*, 2001) could play to their advantage in a commercial feedlotting setting.

The Bonsmara breed was originally a medium– to large-framed composite breed with 62% Sanga (Afrikaner) and 38% taurine that shared some of the adaptive traits of Sanga cattle such as a smooth coat and tick resistance (Marufu *et al.*, 2011). It is therefore the most popular breed in South African feedlotting (Ramsay *et al.*, 2006), often used in crossbreeding with Angus. The large proportion of Sanga ancestry in the creation of the Bonsmara breed, explains why they clustered as a Sanga-type, intermediate between Sanga and taurine breeds, but seemed more closely related to Angus as an improved taurine breed than Sanga. Bonsmara cattle have previously been found to be an indigenous breed that is more like improved European taurine cattle, than African taurine or Sanga cattle (Makina *et al.*, 2016) and generally exhibited favourable allelic frequencies similar to or higher than Angus. This genetic potential to produce high quality beef in an intensive production setting, relatively large carcasses and adaptation to (sub-)tropical environments, make them a popular breed in South African beef production (Horst, 1983).

3.4.2. Allelic frequencies

The autosomal SNPs were successfully genotyped in all breeds using the 777K Illumina® SNP BeadChip, with only 0.90% missing genotypes. For all the autosomal SNPs of the 777K Illumina® SNP BeadChip, minor allelic frequency (MAF) was 0.27 and is in agreement with studies using sparser arrays in South African cattle (Makina *et al.*, 2015; Bosman *et al.*, 2017; Dlamini *et al.*, 2022). The MAF was similar in Angus and Bonsmara breeds (0.24), but lower in *Bos indicus* (0.17), which is consistent with results from Espigolan *et al*. (2013). Frequencies were however higher than those first reported using 50K in South African breeds (Qwabe *et al.*, 2013; King *et al.*, 2022). Our data is consistent with observations of considerable variation in the calpain-calpastatin system genes of minor (and tender) allelic frequencies between breeds (Wientjes *et al.*, 2017), particularly for indicine compared to taurine cattle. Although the Illumina® BovineHD (777K) SNP BeadChip resulted in much improved coverage of the genes of the calpain-calpastatin system with 134 SNPs, compared to the 12 SNPs and 18 SNPs of the 50K and 150K arrays respectively, this still represents less than 1% of all the potential variants in these genes (plus the 8% region up– and downstream of the coding sequences). Almost 19,000 variants have been identified in the calpain-calpastatin system genes (EVA, 2021; NCBI, 2021), with more variants (1,982) in the 3'- than the 5'-UTR (938). Of these potential variants, 8,158 occur in *cast*, 2,823 in *capn3*, 4,158 in *capn2* and 3,569 in *capn1* that are on average between 12 bp (*capn1*) to 24 bp (*capn3*) apart (NCBI, 2021). Therefore, although the 777K Illumina® SNP BeadChip would capture more of the variation in these genes than lower density genotyping arrays, ideally whole-genome sequence data of regions that are rich in candidate SNPs for selection for beef quality traits, should be investigated in future (Gershoni *et al.*, 2022).

Angus cattle can be considered an improved taurine breed, with decreased heterogeneity in functional genes because of selection for beef quality traits (Iso-Touru *et al.*, 2018), which will also result in more loci fixed for favourable alleles and high LD (Mackay *et al.*, 2009; Walsh & Lynch, 2018). This is especially true because alleles favourable for tenderness were also found to be favourable for other beef quality traits that could be

altered through selection (Saucedo Uriarte *et al.*, 2021; Kowalczyk *et al.*, 2022). This seems to be the case for the *cast* gene of Angus and Bonsmara cattle in this study, where already-high frequency of favourable (tender) alleles, limits the opportunity for selection for beef quality within these genes. This was demonstrated in South African crossbred beef, where high frequency of tender alleles in the *cast* gene (two SNPs) was accompanied by a lack of association with tenderness phenotypes up to day 14 post-mortem (Frylinck *et al.*, 2009). For Bonsmara cattle in our study, 58 of the 77 SNPs exhibited a favourable allelic frequency of greater than 85% of which 37 SNPs were greater than 90%. Alternative genes, such as those of intermediary metabolism and enzyme control should therefore be investigated for beef quality in these breeds (Warner *et al.*, 2022). It does however indicate that selection goals for improved quality were effective in changing the population genetics of these breeds, for the most important candidate gene for beef tenderness, *cast*. Similarly, Angus and Bonsmara cattle favourable allele frequency of the *capn2* and *capn3* genes was the highest, or second highest of the different breeds. Bonsmara cattle had a 13.4% greater percentage of favourable alleles in the *capn1* gene than the pooled mean indicating that this gene (especially) has been improved in Bonsmara.

The Nguni cattle generally had favourable allelic frequencies that were greater than Brahman and Charolais, and similar to those observed in Angus and Bonsmara. This confirms previous observations that adapted indigenous breeds exhibited allele frequencies closer to improved temperate breeds than tropically-adapted (indicine) breeds (Blecha *et al.*, 2019), even though Nguni cattle have a relatively large proportion (30%) indicine admixture (Makina *et al.*, 2016). It is consistent with observations that indigenous cattle breeds have the genetic potential in the *capn* and *cast* genes to produce tender beef (Frylinck *et al.*, 2009; Avilés *et al.*, 2013b) and is highlighted by the fact that Nguni had a favourable allele frequency much greater than any other breed for the important candidate SNPs, *capn1-316* and *capn1-4751*. They do however still possess the genetic diversity within these genes, to enable targeted selection, to improve the proportion of favourable alleles in the calpain-calpastatin system genes. This should lead to improvement of tenderness (and other beef quality traits), while maintaining the diversity in adaptive genes, that must be conserved (Biscarini *et al.*, 2015; Mwai *et al.*, 2015).

The large genetic distance between Brahman and the taurine breeds like Angus (that generally produce high quality beef) were confirmed by the PCA and expected differences in beef quality by the variation in tender allelic frequencies. Here, Brahman demonstrated many allelic distributions, which differ from the taurine-types, for example where minor alleles (A1) of taurine were most abundant in indicine (A2). Brahman is well-know for variation in allelic frequency in the *cast* (Casas *et al.*, 2006; Johnston & Graser, 2010) and *capn1* genes (Curi *et al.*, 2010; Johnston & Graser, 2010) and the differential characterization often presents as a low proportion of tender alleles (Casas *et al.*, 2005; White *et al.*, 2005) similar to those recorded in this experiment. This was the case in all four genes here, but especially the *capn1* gene. Although the nature of the associations between SNPs and traits remain the same in indicine cattle, i.e. favourable alleles are the same (Saucedo Uriarte *et al.*, 2021; Kowalczyk *et al.*, 2022), very low allelic frequencies limit the opportunity for selection. As an example, these Brahman were fixed for the unfavourable allele for the *capn1-316* marker, while *capn1-4751* C* allele accounted for only 10% of alleles, compared to between 51.5% – 70.0% in the other breeds.

Charolais are among the top ten breeds finished under intensive, feedlotting in South Africa (Ramsay *et al.*, 2006) and provides a favourable cross with Nguni cattle (Bester *et al.*, 2001; Strydom & Hope-Jones, 2022). Although Charolais exhibited a large proportion of favourable alleles for beef quality in the *capn1* gene, the tender allele frequency was similar to the pooled mean for *capn2* and *capn3*, while the *cast* gene was found to have a very low frequency of favourable alleles that was even lower than Brahman. These results were not consistent with previous observations of SNPs in the *cast* gene where the favourable allelic frequency was generally greater than 50% (Ekerljung *et al.*, 2012; Li *et al.*, 2013) and often greater than 70% (Williams *et al.*, 2009; Allais *et al.*, 2011; Avilés *et al.*, 2013a). However, large variation in the frequencies of SNPs have been observed in the *capn1* gene of different Charolais herds, where the frequency of the favourable alleles varied between less than 10%, up to 83% for *capn1-316* (Williams *et al.*, 2009; Allais *et al.*, 2011).

3.4.3. Linkage disequilibrium and haplotypes

Genomic selection in beef cattle is changing the genetic variation of selected breeds or herds (Misztal *et al.*, 2021) and could result in more uniformity in beef production (Iung *et al.*, 2020), which is a favourable outcome from a production point of view, consistently supplying consumers with a quality product. This should however be weighed against the loss of genetic diversity, with growing concerns globally about indigenous breeds that will become increasingly important in a changing environment (Biscarini *et al.*, 2015; Dlamini *et al.*, 2022). The high LD of genes in the muscle calpain system (the *capn* genes and *cast*) could be the result of selection for improved beef quality (Zhang *et al.*, 2012) and the *cast* gene has been identified as a region where selection has resulted in high LD and decreased minor allelic frequency in beef (Pérez O'Brien *et al.*, 2014b). Our results also confirmed differences between breeds in haplotype block structure (Villa-Angulo *et al.*, 2009; Jasielczuk *et al.*, 2016), but were also strikingly similar for some haplotype blocks across breeds and even breed types, consistent with past research (Pereira *et al.*, 2022).

The unbalanced distribution of alleles in the *cast* gene can however represent a major challenge to accurately estimating LD in haplotype block analyses, while a more even allelic distribution in the three *capn* genes, provided data that would more accurately estimate LD (Lewontin, 1995). Additionally, observations of differences in the regions and length of linkage blocks in Brahman cattle compared to *Bos taurus* cattle, are consistent with previous observations in *Bos indicus* cattle (Villa-Angulo *et al.*, 2009). It is important that genotyping is conducted at a high-enough density to ensure enough animals per genotype group, for statistical analyses and estimation of LD in diverse breed types (Pérez O'Brien *et al.*, 2014a). Limited research is available on haplotype block structure in the calpain-calpastatin system and often included only a few SNPs per gene (Zhang *et al.*, 2012; Quintana, 2017), with few studies using high-density genotyping (Cheong *et al.*, 2008; Braz *et al.*, 2018; Pereira *et al.*, 2022). Our data therefore provide insights into the opportunities for selection in these genes in South African cattle and the value of candidate SNPs across different breeds (De Roos *et al.*, 2008; Van Den Berg *et al.*, 2016).

Haplotype block B4 of the *cast* gene identified across breeds extending 42.1 kb, was very similar in length and position to a linkage block in Angus-Brahman crossbreed beef (Leal-Gutiérrez *et al.*, 2018). These authors also identified a region of high linkage constituting two blocks in a region of the gene very similar to B5 in our

pooled data (Leal-Gutiérrez *et al.*, 2018), although in the breeds other than Brahman, our data suggests these haplotype blocks could extend further downstream. Quintana (2017) identified an 11.2 kb haplotype block in indigenous Spanish cattle in the region of marker *7_760*, which corresponded to more extensive haplotype blocks in our experiment for all breeds except Brahman. The high linkage and low MAF of the *cast* gene suggest that selection has been successful in altering the distribution and recombination of alleles (Walsh & Lynch, 2018) and the high proportion of tender alleles is consistent with previous observations (Morris *et al.*, 2006; Van Eenennaam *et al.*, 2007; Robinson *et al.*, 2012). This was most evident in Angus and Bonsmara bulls that had a favourable allelic frequency of almost 80%, suggesting this tender form of the gene is fixed in the population, at least for the animals sampled here. Being the major gene (and protein) that determines post-mortem tenderization, this could limit the ability to improve tenderness through selection in *cast* in South African Angus and Bonsmara cattle. However, this could explain why Angus is one of the few breeds that have very tender *longissimus thoracis et lumborum* (Warner *et al.*, 2022) and the improved distribution and inheritance of tender alleles, coupled with low recombination in the *cast* gene means that the small progress made from limited genomic variation in these breeds, would not be commercially profitable. Nguni have been selected for traits that are functional and culturally important and, although the proportion of favourable alleles was generally high, still has room for improvement for some markers in the *cast* gene, especially in the 5'-region of the gene, where favourable allelic frequencies were generally lower and haplotype blocks shorter. Many candidate SNPs have been identified in the 3'-UTR of *cast* (Barendse, 2002, 2007) and this could provide the link between functional and structural regions of the gene, through the extended haplotype blocks identified here, except in Brahman. The shorter, more fragmented haplotype blocks indicated the lack of improvement in this gene through selection and highlights another of the many differences of Brahman (*Bos indicus*) cattle compared to taurine, including breed stratification, allele frequencies (distribution of tender alleles), candidate SNPs, calpastatin function and general metabolic responses during the conversion of muscle to meat discussed above. The LD block structure of Charolais was generally similar to Angus for all four genes, although in the *cast* gene, LD blocks were fragmented. Coupled with lower favourable allelic frequency and previous research identifying variability within the breed discussed above, this suggests less selection pressure in the *cast* gene in South African Charolais and could be attributed to a general decline in popularity of the breed, with declining numbers in the South African herd (Ramsay *et al.*, 2006).

The LD of the *capn1* gene was lower than *cast* and contributed to the more variable nature of the haplotype blocks between breeds. The favourable allelic frequency of, for example *capn1-316*, was lower than those reported previously (Page *et al.*, 2004; Morris *et al.*, 2006; Williams *et al.*, 2009), but has been found to be variable between taurine cattle (Williams *et al.*, 2009; Li *et al.*, 2013). The challenge for improving the moderate favourable allelic frequencies for SNPs in *capn1*, lies in the variable nature of the gene between breeds. The first haplotype block (B1) in these pooled data for all five breeds was similar to B1 identified in indigenous taurine and *Bos indicus* cattle using ≃650K SNPs (Braz *et al.*, 2018), but was shorter and started downstream in Angus and Nguni (including *capn1-316*). In Brahman and Charolais, a short upstream block was identified that was similar to B1 in Hanwoo cattle (Cheong *et al.*, 2008). In all breeds except Brahman, B2 (B1 for Bonsmara) extended from approximately intron 14 to intron 19 (Angus and Charolais) or from exon 23 in Bonsmara and

Nguni and is consistent with previous results that identified regions of high linkage near the *capn1-4751* marker (Cheong *et al.*, 2008; Braz *et al.*, 2018; Leal-Gutiérrez *et al.*, 2018; Pereira *et al.*, 2022). The variation in haplotype block structure between breeds in our study was in agreement with other studies and confirms that markers of the *capn1* are breed-specific. However, a general pattern emerged of separate linkage blocks from upstream or near intron 7, often including *capn1-316*, with a more frequently identified block(s) from near or downstream of intron 10 and including *capn1-4751*. The latter linkage block has been found in *Bos indicus* and their crosses (Braz *et al.*, 2018; Leal-Gutiérrez *et al.*, 2018), while South African Brahman cattle did not exhibit high LD in this region. The design of custom SNP arrays for selection should therefore take into account the different locations of linkage regions in the 3' and 5' regions of the gene, using higher density SNPs genotyped over a larger distance for selection across breeds.

The *capn2* gene was characterized by extensive linkage (or low recombination), where both Sanga-types exhibited extensive linkage with a single, large haplotype block. This is consistent with results from Riggs & Vaughn (2015), where strong linkage was continuous, extending upstream and downstream from this gene in crossbred beef. Although more fragmented in the other breeds, a large portion of the gene displayed overlapping haplotype block structures, where candidate SNPs could be applied successfully across breeds (Villa-Angulo *et al.*, 2009). This region extended from intron 2 to exon 5 of the gene for 22 kb including 14 of the 25 SNPs in the coding region of the gene. Although this region has been associated with feed efficiency (Riggs & Vaughn, 2015), growth and body size in some studies (Zhang & Li, 2011; Michenet *et al.*, 2016), it did not generally associate with WBSF. This was likely because many of these studies measured tenderness ≤14 days post-mortem (McClure *et al.*, 2012; Lu *et al.*, 2013; Tizioto *et al.*, 2013), where the association of the gene with the tenderness phenotype (Hulsman Hanna *et al.*, 2014) is more important in the later stages of aging, where genes related to protein accretion become more important genomic effects (De Souza Fonseca *et al.*, 2022). The even distribution of alleles in the *capn2* gene, coupled with extensive linkage presents an opportunity for selection for the later stages of tenderization, although early-ageing is the most advantageous time for large gains to lower costs. Coupled with the pleiotropic effects on growth, body weight and feed efficiency, this could potentially limit the practical application of selection in *capn2* for beef quality traits.

The *capn3* gene has been linked to tenderization of beef (Barendse *et al.*, 2008) and increased sensory scores for quality (Robinson *et al.*, 2012) that could be linked to associations with muscle fat (Royer *et al.*, 2016). The alleles were differentially distributed in *Bos taurus versus Bos indicus* cattle, similar to previous observations where indicine allele distribution deviated from the proportions observed in taurine cattle (Barendse *et al.*, 2008), while linkage in Brahman cattle were not similar to taurine or Sanga-type cattle in the current study. The linkage block structure of taurine cattle and Sanga-types were similar and generally overlapping, but the haplotype blocks were fragmented across the gene, indicative of the moderate linkage observed in the gene. The extent of the blocks throughout the gene (especially in the 3'-region) indicate that the *capn3* gene has been under selection in these breeds and is in agreement with results by Kooverjee *et al*. (2022) that identified signatures of selection in Nguni and Bonsmara cattle. Of the four genes of the calpain-calpastatin system, the *capn3* gene has the lowest tender allelic frequency, presenting an opportunity of selection for beef quality traits

including tenderness, flavour and intramuscular fat, along with diverse non-proteolytic physiological functions of this protein in cellular homeostasis (Ojima *et al.*, 2011; Ono *et al.*, 2016).

Within the limitations of the relatively small sample size, characterization of the genes of the calpain-calpastatin system could only apply to a limited portion of these breeds in South Africa and will need to be confirmed in larger populations (and subpopulations) of the different breeds. The genetic diversity of the genes observed here is however promising and could represent an opportunity to make meaningful progress in selection within these breeds. Similarly, Brahman cattle in this sample were relatively homogenous and should be validated in a broader commercial setting. Although Bonsmara clustered intermediate between Nguni and Angus in the population structure analysis (PCA), characteristics of the alleles and linkage suggest that the Bonsmara is closer to Angus as an improved breed, where admixture and selection has improved the genetic potential for tender beef (at least in the genes of the calpain-calpastatin system). Sanga cattle and their crosses with taurine breeds have adaptive advantages in more extreme climates (leading to thermoregulatory, reproductive and productive advantage over improved taurine breeds), but does not necessitate a compromise in beef quality like Brahman (Warner *et al.*, 2022). An opportunity and a great need exists for more efficient beef production through physiological and production improvements (Dunshea *et al.*, 2016; Terry *et al.*, 2020) and genomic selection (Georges *et al.*, 2019), while the use of smaller-framed, tropically adapted Sanga cattle can lower the carbon-footprint of the beef production chain from growth and finishing (through lower maintenance costs), right through to ageing and storage until marketing (by accelerating tenderization). However, for genomic markers to be effective, the advantages of the favourable genotypes for these traits need to occur in addition to current interventions for improved quality, resulting in a cost benefit (Segers & Lourenco, 2022). Few SNPs have the ability to sustain tenderizing effects on beef throughout extended ageing (Chapter 4) and future research should explore the interactions of these genotypes with the environmental factors that play an important role in determining ultimate tenderness of beef. For breeds such as Angus and Bonsmara, where selection seems to have improved the frequencies and fixed the alleles of the genes in the calpain-calpastatin system, changes in ante– and post-mortem factors that affect tenderization will result in variation in quality between individual carcasses and must be optimized. For these breeds, it could be possible that other causal genes could be more important genetic effectors of tenderization, for example, the genes of intermediary metabolism that are the drivers of the activity of the proteolytic system and many other beef quality traits such as water-holding capacity and colour. In breeds where more genetic variation in the genes of proteolytic tenderization occurred, an opportunity exists to improve the frequency of favourable alleles, while still optimizing the large environmental effects on beef quality. This should however not occur at the expense of the adaptive advantages of indigenous Sanga cattle and it seems that this has been successfully achieved in Bonsmara cattle.

3.5. Conclusion

It seems unlikely that a universal, simple SNP panel or haplotype will be identified for selection for beef quality and with the advances in technology (and reduction in cost), the use of high-density arrays and sequencing will allow the use of more complex genotypes for selection. The Illumina® BovineHD SNP BeadChip

is suitable for genotyping the South African indigenous Sanga-types, Bonsmara and Nguni, allowing for a large enough number of SNPs, spaced at a short enough distance to be informative. The high degree of linkage between SNPs in these regions means that candidate SNPs that affect beef quality could potentially differ between breeds, but could remain useful for selection across breeds when they form part of shared or overlapping haplotype blocks. Between climate change and energy crises and their effect on feed and fuel prices, coupled to the limitations of large-framed, improved taurine cattle to produce beef in challenging environments, Sanga are an attractive alternative to decrease costs, improve the efficiency of production and maintain a high quality beef.

Supplementary Materials (Addendum B): Table S1 (Pooled MAF and genotype frequency of individual SNPs); Table S2 (MAF of individual SNPs stratified by breed); Table S3 (LD as mean r^2 per SNP); Table S4: (LD as D' and r^2 between all SNP); Table S5 (Haplotype blocks stratified by breed)

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Chapter 4: Sustained effects of muscle calpain system genotypes on tenderness phenotypes of South African beef bulls during ageing up to 20 days

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Sustained effects of muscle calpain system genotypes on tenderness phenotypes of South African beef bulls during ageing up to 20 days

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Simple summary

When searching for genetic markers for the selection of more tender beef, it is important to maintain minimal environmental variation from pre-slaughter, right through to the ageing process, to ensure the accuracy of the obtained phenotypes. This is because beef quality traits have a large environmental component that can greatly alter the characteristics of the meat, which would not reflect a true genetic effect. We propose that variable ageing times are especially important in determining whether markers are associated with tenderization or not. Our analyses included candidate genes for the protein degrading enzyme system for calpains, because they contribute the most to tenderization. We were able to validate these markers in South African beef cattle, where they could be useful for selection. The timing of the collection of tenderness data was critical, as only a few (6/134) genetic markers sustained their association with tenderization over ageing to 20 days. A larger tenderization effect earlier in ageing, as shown here for the *capn1_187* and *capn1_4751* markers, would decrease the length of ageing. This would not only increase profits, but also decrease the energy needed during the storage and refrigeration of aged beef, decreasing the carbon footprint of beef production.

Abstract

The most important factor that determines beef tenderness is its proteolytic activity, and the balance between calpain-1 protease activity and calpastatin inhibition is especially important, while contributions can also arise from calpain-2 and, possibly, calpain-3. These processes are, however, affected by the meat ageing process itself. To determine whether genotypes in the calpain–calpastatin system can enhance tenderness through a 20-day ageing period, South African purebred beef bulls (*n* = 166) were genotyped using the Illumina® BovineHD SNP BeadChip through a gene-based association analysis targeting the *cast*, *capn3*, *capn2* and *capn1* genes. The Warner–Bratzler shear force (WBSF) and myofibril fragment length (MFL) of *Longissimus thoracis et lumborum* (LTL) steaks were evaluated between d 3 and d 20 of ageing, with protease enzyme activity in the first 20 h post-mortem. Although several of the 134 SNPs are associated with tenderness, only seven SNPs in the *cast*, *capn2* and *capn1* genes sustained genetic associations, additive to the ageing-associated increases in tenderness for at least three of the four ageing periods. While most genomic associations were relatively stable over time, some genotypes within the SNPs responded differently to ageing, resulting in altered genomic effects over time. The level of ageing at which genomic associations are performed is an important factor that determines whether SNPs affect tenderness phenotypes.

Keywords: SNP; calpain–calpastatin system genes; genomic association; tenderization; ageing

4.1. Introduction

Increased tenderness with beef ageing involves the weakening of the myofibrillar structure of muscle fibres through the degradation of myofibrillar (and other) proteins (Culler *et al.*, 1978; Koohmaraie *et al.*, 1987; Koohmaraie, 1992). The proteolysis that occurs due to the muscle calpain system activity during ageing results in tenderization (Morton *et al.*, 1999) and is mainly attributed to calpain-1 (Koohmaraie, 1992), with prominent modulating effects of calpastatin (Geesink *et al.*, 2006; Koohmaraie & Geesink, 2006). The contribution of calpain-2 protease becomes progressively more important as ageing progresses (Colle & Doumit, 2017), while calpain-3 activity could also potentially contribute to the ultimate tenderness of beef (Lian *et al.*, 2013), although this remains controversial. By extending the ageing period, tougher steaks that age at a slower rate can achieve greater levels of tenderness (Watanabe *et al.*, 1996) and it is important in (for example) cattle with a larger *Bos indicus* genetic background (O'Connor *et al.*, 1997), allowing for an improved quality before going to market. However, this results in logistical challenges of chilled storage and tracking large amounts of beef over extended time periods, whereas the ultimate goal is to accelerate these processes as much as possible (Warner *et al.*, 2017) and shorten the ageing period (Dransfield, 1994).

The aim of selection to improve the tenderness of beef (Taylor *et al.*, 2012) goes hand in hand with the goal to accelerate the ageing process in order to reach the ultimate tenderness as rapidly as possible (Warner *et al.*, 2017). The efficacy of SNPs for selection for tenderness is different between populations (Allais *et al.*, 2011), and there are genetic differences among beef breeds with regard to the calpain system and expression of tenderness. In South Africa, studies based on high-throughput genotyping have been limited and generally focus on genetic characterization and genomic relationships (Makina *et al.*, 2015b; Madilindi *et al.*, 2020). Studies on the genetic diversity and the application of genomic selection use moderate-density SNP arrays such as the 50K (Qwabe *et al.*, 2013; Makina *et al.*, 2014, 2015a; Wang *et al.*, 2015), 80K (Van Der Westhuizen *et al.*, 2014; Zwane *et al.*, 2016; Bosman *et al.*, 2017) and 150K SNPs (Makina *et al.*, 2015b), but these could lack sufficient SNP density for the characterization of indigenous beef breeds (Zwane *et al.*, 2016). Although disease resistance SNPs (Mapholi *et al.*, 2016) and potential beef quality SNPs have been identified (Zwane *et al.*, 2019), association studies using detailed tenderness phenotypes in the calpain–calpastatin system have only been performed with a very limited SNP pool (Frylinck *et al.*, 2009).

Genome-wide association studies (GWAS) are useful for determining novel associations within phenotypes, while SNPs within targeted genes give a higher power of association of analysis for quantitative traits (Williams *et al.*, 2009; Raza *et al.*, 2020). The use of candidate genes or gene-assisted selection is effective (given accurate phenotypic data), because even small gains in quantitative traits are economically valuable (Mullen *et al.*, 2006).

The duration of the ageing period may affect the estimates of heritability, with variations between breeds and ageing time, including interactions between these two variables (Warner *et al.*, 2010). This is likely related to the fact that there are differences in the rate of tenderization between different breeds and individual muscles

(Sierra *et al.*, 2012), even when breeds are subjected to the same production conditions and/or carcasses to the same post-mortem processing procedures.

Accurate phenotypes are a prerequisite for linking genotypic data with traits related to beef quality (Burrow *et al.*, 2001), especially because many of the beef quality phenotypes have a moderate to low heritability (Minick *et al.*, 2004; Smith *et al.*, 2007). Tenderness phenotypes under varying environmental conditions (ante-mortem and post-slaughter practices) are still poorly defined and large datasets with detailed phenotypes and appropriate SNPs in the causative genes are lacking. Studies in controlled environmental and slaughter conditions sometimes only include a small number of animals (Iso-Touru *et al.*, 2018; Guillocheau *et al.*, 2019) and/or a small number of genotypes (Iso-Touru *et al.*, 2018; Bennett *et al.*, 2019) due to the financial costs involved in raising and slaughtering animals. Other genomic association studies of beef quality have pooled data collected from different herds subjected to varying production systems (Morris *et al.*, 2006; Iguácel *et al.*, 2019; Wang *et al.*, 2020), with or without electrical stimulation at varying ageing periods (Morris *et al.*, 2006). Large studies are often constrained by the cost to genotyping (Van Marle-Köster *et al.*, 2015), so that the number of SNPs analysed was sometimes limited to lower-density SNP chips, such as 7K–150K (Grigoletto *et al.*, 2020; Marín-Garzón *et al.*, 2020), inferred through imputation (Bolormaa *et al.*, 2013a; Wang *et al.*, 2020), or only a few individual SNPs from causal genes were used (Morris *et al.*, 2006; Pinto *et al.*, 2010; Castro *et al.*, 2016; Iso-Touru *et al.*, 2018; Bennett *et al.*, 2019; Iguácel *et al.*, 2019). Data from *Bos indicus* using a limited *capn1* and *cast* SNP pool suggested that ageing time (<21 d) may alter genomic associations (Pinto *et al.*, 2010), while it has been suggested that ageing decays genetic associations with tenderness phenotypes over time (Mazzucco *et al.*, 2010; Gruber *et al.*, 2011). Few studies have explored the effect of different post-slaughter practices such as the ageing period on the association between genotypes and tenderness phenotypes (Schenkel *et al.*, 2006; Pinto *et al.*, 2010; Chang *et al.*, 2014; Iguácel *et al.*, 2019), and many studies used a limited number of single nucleotide polymorphisms (SNPs), while no data are available from South African beef cattle.

The augmentation of tenderness through post-mortem practices, such as electrical stimulation, tenderstretch or extended ageing, could eliminate differences in tenderness over time (Watanabe *et al.*, 1996), altering genomic associations depending on the relationship between the sample collection and these factors (Pinto *et al.*, 2010), and making selection less effective. To determine whether extended ageing affects the genomic association between candidate SNPs and tenderness, we performed a regional, gene-based association study using the Illumina® BovineHD (777K) BeadChip focusing on the genes of the calpain–calpastatin system over 20 days of ageing. This is the first study to explore the association between the BovineHD SNP of the *cast* (chromosome 7), *capn-3* (chromosome 10), *capn-2* (chromosome 16) and *capn-1* (chromosome 29) genes with tenderness (WBSF and MFL) up to 20 days of ageing in South African beef cattle. The objective of this study is to determine whether favourable genotypes in causative genes for tenderness could improve the phenotype over extended ageing up to 20 days and whether these associations are similar over ageing periods and between breeds.

4.2. Materials and methods

4.2.1. Animals

Purebred bulls, approximately nine months old, were feedlot finished for approximately 120 days at the Animal Production Unit of the Agricultural Research Council (ARC-AP) unit in Irene, Pretoria, -25°54' +28°12', altitude ≥ 1475 m. Five breeds were included in the study (Table 4.1) as representative of breed types. Angus were representative of British *Bos taurus*, Charolais European *Bos taurus*, Brahman *Bos indicus*, Bonsmara as an indigenous Sanga-type composite breed and Nguni as the indigenous Sanga-type, *Bos taurus africanus*. Bulls were slaughtered to yield A2/A3-class carcasses (Anonymous, 1990, 2015), meaning zero permanent incisors, with lean to medium fatness, and were approximately 12 months old at slaughter. Standard management and slaughter practices were used to minimize the variation in tenderness induced by environmental factors, and the same feedlot, abattoir and similar carcass handling and ageing procedures were used for all bulls.

4.2.2. Slaughter and sampling

Bulls were transported to the ARC-AP abattoir (3.9 km from the feedlot) and held overnight with access to water. Following captive bolt stunning and exsanguination, bulls were slaughtered, carcasses were halved and the right half of each carcass was electrically stimulated for 15 s (500 V peak, 5 ms pulses at 15 pulses/s) and chilled directly (ES). The left half of each carcass served as a control and chilling was delayed for 6 h post-mortem (NS) to allow for accelerated conditioning at 10°C before chilling.

Association analyses were originally performed on the left half and the right half of the carcasses separately, as well as the pooled data. Of all the association analyses performed (NS, ES, NS + ES) for each SNP and phenotype, only 0.3% exhibited genotype × treatment interactions (results not shown) and did not include any of the candidate SNPs identified here. In order to simplify data representation tables, only data from the NS + ES analysis were represented here and any notable differences between association analyses described in the text.

Meat or tail hair samples were collected at slaughter. Meat samples were stored at −20°C until extraction of DNA from a core sample, while tail hair was stored at room temperature until extraction of DNA from hair bulbs. At approximately 20 h post-mortem, the *Longissimus lumborum et thoracis* (LTL; loin) muscle was excised (boned-out). Several 30 mm steaks were collected from predetermined regions of the LTL for each of the laboratory analyses for each ageing period, vacuum-sealed (70 microns) and aged for three, nine, fourteen and twenty days post-mortem at 2 ± 1°C (d 3, d 9, d 14, d 20). The myofibril fragment length (MFL) was determined following ageing, while steaks for the determination of Warner–Bratzler shear force (WBSF) were frozen at −20°C until analysed. Small sub-samples were collected from the lumbar portions of the LTL at 1 h and 20 h post-mortem, snap-frozen to −196°C in liquid nitrogen and immediately stored at −80°C until analyses of muscle calpains and calpastatin activities.

Table 4.1. *Descriptive statistics—means of the pooled data of all breeds and least squares means within breeds of production and tenderness data of South African feedlot-finished beef bulls.*

	All Breeds (*n* = 166)		Angus (*n* = 24)	Bonsmara (*n* = 35)	Brahman (*n* = 35)	Charolais (*n* = 34)	Nguni (*n* = 35)	# *p*-Value
BW (kg)	388 ± 4.26		422 ± 9.65 ^{a}	399 ± 4.26 ^{b}	393 ± 7.44 ^{b}	423 ± 5.39 ^{a}	308 ± 3.39 ^{c}	0.0001
35d ADG (kg/d)	1.54 ± 0.04		1.81 ± 0.08 ^{b}	1.62 ± 0.05 ^{ab}	1.37 ± 0.06 ^{a}	1.88 ± 0.09 ^{c}	1.21 ± 0.07 ^{c}	0.0001
HCW (kg)	219 ± 2.46		234 ± 5.36 ^{ab}	223 ± 2.87 ^{b}	226 ± 4.67 ^{b}	240 ± 3.44 ^{a}	174 ± 2.07 ^{c}	0.0001
CCW (kg)	215 ± 2.42		229 ± 5.29 ^{ab}	218 ± 2.82 ^{b}	221 ± 4.67 ^{b}	234 ± 3.34 ^{a}	170 ± 2.09 ^{c}	0.0001
Dressing%	56.53 ± 0.15		55.62 ± 0.33 ^{b}	55.96 ± 0.33 ^{b}	57.44 ± 0.24 ^{a}	56.64 ± 0.36 ^{ab}	56.44 ± 0.34 ^{ab}	0.0004
Mass Loss (%)	2.18 ± 0.03		2.16 ± 0.07	2.30 ± 0.06	2.22 ± 0.08	2.25 ± 0.07	2.14 ± 0.09	0.0564
EMA (mm^2)	6 084 ± 64		5 853 ± 127 ^{cd}	6 364 ± 134 ^{ab}	5 922 ± 121 ^{bc}	6 647 ± 127 ^{a}	5 444 ± 120 ^{d}	0.0001
WBSF (d 3)	6.15 ± 0.09		5.89 ± 0.20 ^{b}	5.74 ± 0.20 ^{b}	6.63 ± 0.19 ^{a}	6.09 ± 0.21 ^{ab}	6.17 ± 0.24 ^{ab}	0.0122
WBSF (d 9)	4.83 ± 0.08	-21.4%[1]	4.41 ± 0.16 ^{b}	4.53 ± 0.15 ^{b}	5.38 ± 0.21 ^{a}	4.88 ± 0.19 ^{ab}	4.75 ± 0.21 ^{b}	0.0006
WBSF (d 14)	4.17 ± 0.07	-32.3%[1]	3.75 ± 0.11 ^{b}	4.20 ± 0.15 ^{ab}	4.55 ± 0.15 ^{a}	4.08 ± 0.16 ^{ab}	3.99 ± 0.19 ^{b}	0.0028
WBSF (d 20)	3.87 ± 0.07	-37.1%[1]	3.64 ± 0.13 ^{ab}	4.08 ± 0.15 ^{a}	4.09 ± 0.15 ^{a}	3.82 ± 0.14 ^{ab}	3.52 ± 0.15 ^{b}	0.0022
%Δ WBSF [2]			**−38.3%**	**−28.9%**	**−38.3%**	**−37.3%**	**−42.9%**	
MFL (d 3)	35.51 ± 0.48		36.35 ± 1.17 ^{ab}	31.74 ± 0.87 ^{c}	39.65 ± 1.23 ^{a}	35.53 ± 0.95 ^{b}	32.93 ± 0.79 ^{bc}	0.0001
MFL (d 9)	26.21 ± 0.31	-26.2%[1]	25.55 ± 0.66 ^{bc}	23.57 ± 0.47 ^{c}	29.08 ± 0.84 ^{a}	26.65 ± 0.73 ^{b}	25.35 ± 0.49 ^{c}	0.0001
MFL (d 14)	23.92 ± 0.28	-32.6%[1]	22.87 ± 0.57 ^{bc}	22.07 ± 0.44 ^{c}	25.91 ± 0.71 ^{a}	24.67 ± 0.79 ^{ab}	23.72 ± 0.48 ^{bc}	0.0001
MFL (d 20)	21.66 ± 0.23	-39.0%[1]	20.74 ± 0.49 ^{bc}	19.88 ± 0.40 ^{c}	22.93 ± 0.54 ^{a}	22.60 ± 0.57 ^{a}	21.90 ± 0.45 ^{ab}	0.0001
%Δ MFL [2]			**−42.9%**	**−37.4%**	**−42.2%**	**−36.4%**	**−33.5%**	

Least squares means (LSM) ± standard errors (SE) that were significantly different between breeds ($p \leq 0.05$) were indicated with different ascending letter superscripts (a, b, c) in each row.
p-values derived from a GLM; [1] the difference in tenderness at the ageing period, compared to d 3 post-mortem; [2] the difference in tenderness on d 20 compared to d 3 post-mortem within each breed.
35d ADG—average daily gain recorded over the last 35 days before slaughter; BW—live body weight at slaughter; CCW—cold carcass weight; EMA—eye muscle area; HCW—hot carcass weight; MFL—myofibril fragment length (µm); WBSF—Warner–Bratzler shear force (kg).

4.2.3. Tenderness

For WBSF measurements (Wheeler *et al.*, 1997), steaks were thawed overnight, oven-grilled at 200°C to a core temperature of 70°C and cooled to 18°C for core sample collection. A handheld cork borer (12.7 mm diameter) was used to collect six round, uniform cores from each steak, parallel to muscle fibre direction. Core samples were sheared through the centre, perpendicular to the long axis of the muscle fibres with a Universal Instron apparatus (Instron Ltd., Buckinghamshire, England) fitted with a Warner–Bratzler V-notched shear blade (60° angle) (AMSA, 2016). The crosshead speed of the Instron apparatus was set to 200 mm/min and the peak force (kg) for each core was determined.

The MFL was determined following extraction according to Culler *et al*. (1978), as modified by Heinze & Brüggemann (1994). The mean of 100 fragments per steak (or ageing period) was determined using Video Image Analysis (VIA) with an Olympus System microscope, model BX40 (Tokyo, Japan) at a 400× magnification equipped with CC12 video camera.

4.2.4. Muscle calpain protease system

Calpain-1, calpain-2 and calpastatin were extracted from muscle tissue samples and separated by 2-step gradient ion exchange chromatography (Geesink & Koohmaraie, 1999). Using azo-casein substrate (Dransfield, 1996), a unit of calpain protease activity was defined as a 1.0/h increase in absorbance at 366 nm (A_{336}) at 25°C, while protease inhibition by calpastatin was defined as the amount required to inhibit one unit of calpain-2 activity. Enzyme activities and inhibition were expressed as units per grams of meat, while relative units of inhibition by calpastatin per units of calpain-1, or per the combined protease activities of calpain-1 and calpain-2, were also calculated.

4.2.5. DNA extraction and genotyping

DNA was collected from muscle or hair samples and extracted using the NucleoSpin® Tissue kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) with silica membrane columns, including modifications to the basic protocol to enhance DNA yield, as per the user manual. Samples were allowed to stabilize overnight at 4°C and DNA quality and concentration were determined at the Agricultural Research Council, Animal Genetics (Irene, South Africa) or Biotechnology Platform (Pretoria, South Africa) using UV spectrometry (NanoDrop 2000/2000c, Thermo Fisher Scientific, Waltham, MA, USA), fluorometry (Qubit® 3.0, Thermo Fisher Scientific, USA) and ethidium bromide agarose gel electrophoresis.

All the animals were individually genotyped using the BovineHD SNP BeadChip (Illumina®, San Diego, CA, USA) using the HiScanSQ platform and Genome Studio software (Illumina, USA) at the Agricultural Research Council, Biotechnology Platform (Pretoria, South Africa). The National Centre for Biotechnology Information (NCBI) Genome Data Viewer (NCBI, 2021), using bovine genome assembly UMD_3.1.1, was used to determine the extent of the four muscle calpain system gene transcripts (gene ± 8% length). The number of variants that fell within the range for each gene (before quality control) were 12 for the *capn1* gene (chromosome 29, 44,223 bp), 27 for *capn2* (chromosome 16, 70,008 bp), 18 for *capn3* (chromosome 10, 68,507 bp) and 77 for *cast* (163,722 bp). For each SNP, the name was abbreviated as the gene plus the last three digits of the Illumina code

(*gene_123*), except for the SNP *capn1-4751*, which was abbreviated from the Illumina *CAPN1_2* to *capn1_4751*. The allele that favoured tenderness was determined by comparing phenotypic means of the three genotypes, first for the genotype with the lowest WBSF and then MFL (if necessary), where these alleles were indicated with an asterisk and designated the tender allele (A*/C*/G*/T*) that coded for the tender form of the protein. This simplified designation of the alleles was possible because no dominance effects were found in the candidate SNP effects.

Quality control was performed on the SNP using PLINK version 1.9 software (Purcell *et al.*, 2007) to exclude the SNPs with a genotyping (call) rate of <0.95, deviation from Hardy–Weinberg distribution ($P_{HWdev} \leq 1\times10^{-4}$) and minor allelic frequency <5%. Five SNPs were disqualified during quality control, two from the *capn3* gene, two from the *capn2* gene and one from the *cast* gene.

4.2.6. Statistical analyses

A mixed linear model association (mlma) analysis (Yang *et al.*, 2014) was conducted to determine the association of genotypes with the tenderness phenotypes using GCTA 1.92.2 software (Yang *et al.*, 2011) (University of Queensland, Queensland, Australia). The simplified mixed model (α = 0.05) used the genotype of the different SNPs (*bx*) as a fixed effect in a linear model, with a variance-standardized genomic relationship matrix (*g*) as a random accumulated effect of all SNPs and the residual (*e*) or random effect of error. To determine whether these responses were similar between breeds, associations were determined within breeds, with all breeds as covariates or with each individual breed added to the simplified model as covariate fixed effects ($covar_{Br}$):

$$y = a + bx + [covar_{Br}] + g + e \quad (1)$$

We also performed an adjusted association analysis (Benjamini & Hochberg, 1995) on the simplified model. The false discovery rate, as a result of multiple testing using GCTA, was 24% for 134 SNPs in the traits associated with tenderization. When the adjustment in the *p*-value affected the results, these false positive associations were clearly indicated in tables and described in the results.

To determine the least squares means (LSM) of genotypes or genotypes within breeds, Statistical Analysis System (SAS Institute, Cary, NC) software was used (SAS, 2010) to determine the least significant difference (α = 0.05). A generalized linear model procedure was conducted, including the effects of genotype (*Ge*) of the SNP, electrical stimulation treatment (*Tmt*), breed (*Br*), first-order interactions with genotype as well as residual (*e*):

$$y = \mu + Ge + Tmt + Br + GeTmt + GeBr + e \quad (2)$$

Because individual SNPs in a gene are not independent (genotypes of different closely associated SNPs are linked in inheritance or pleiotropy), a linkage disequilibrium (LD) analysis was performed using Haploview 4.2 software (Broad Institute, USA) (Barrett *et al.*, 2005) default analysis (Gabriel *et al.*, 2002). The LD indicates a link between genotypes (alleles at different loci) that was greater than that attributed to chance, i.e., LD (Lewontin, 1964, 1995). This implies that, through this association, selection for one SNP with a high level of LD with another SNP would indirectly result in selection for the other SNP, as they are not genetically independent.

SNPs with Lewontin's D-prime (D') that was greater than the threshold of 0.95 were likely to also be affected when a candidate SNP was included in a selection program. All SNPs that were identified as dependent on a candidate SNP that had additive effects through extended ageing were also subjected to the gene-based association analyses to ensure the direction of change in tenderness phenotypes was also positive and that no (readily apparent) inadvertent negative effects were linked to the SNP that sustained tenderness over extended ageing.

4.3. Results

4.3.1. Tenderness

On d 3 post-mortem, Brahman cattle had the toughest beef based on the highest WBSF values, while Bonsmara was the most tender, but was not different from Angus (Table 4.1; Figure 4.1 a). The WBSF of Charolais and Nguni was intermediate in toughness. All breeds experienced a significant increase in tenderness between d 3 and d 9 of between 19% in Brahman and 25% in Angus. This was followed by another 15–16% decrease in shear force values to d 14, except Bonsmara where the decrease in the WBSF (i.e., improvement in tenderness) was only 7%. Between d 14 and d 20, there was little or no change in the WBSF in Angus and Bonsmara (3% lower), a moderate tenderization in Charolais (6% lower) and continued tenderization in Brahman (10% lower) and Nguni (12% lower WBSF). This meant that Nguni had the most tender beef on d 20 and the WBSF was 13.9% lower than Bonsmara, while Brahman had the highest WBSF scores at this stage of ageing (and Angus and Charolais had intermediate values).

The MFL on d 3 was the shortest in Bonsmara and longest in Brahman (Figure 4.1 b). The MFL in Nguni, Angus and Charolais was similar, where Nguni did not differ from Bonsmara (intermediate–short) and Angus did not differ from Brahman (intermediate–long). All breeds experienced a large decrease in the MFL between d 3 and d 9 post-mortem, which related to 23% in Nguni and 30% in Angus. The reduction in the MFL to d 14 was 10% in Angus and 11% in Brahman, where the decrease in the length of other breeds was 6–7%. On d 20, the MFL was still decreasing from d 14 values by between 8% in Nguni and 10% in Brahman. Although Brahman had the greatest absolute decrease in myofibril lengths (16.72 µm shorter), the long initial MFL was not overcome by ageing and it remained the highest of all breeds (with Charolais) on d 20 of ageing. In Bonsmara, the MFL remained the shortest throughout the entire ageing period (−12.7% of Brahman and Charolais), while Nguni had an intermediate–high MFL (3.8% shorter) and Angus an intermediate–low MFL (8.9% shorter MFL) on d 20.

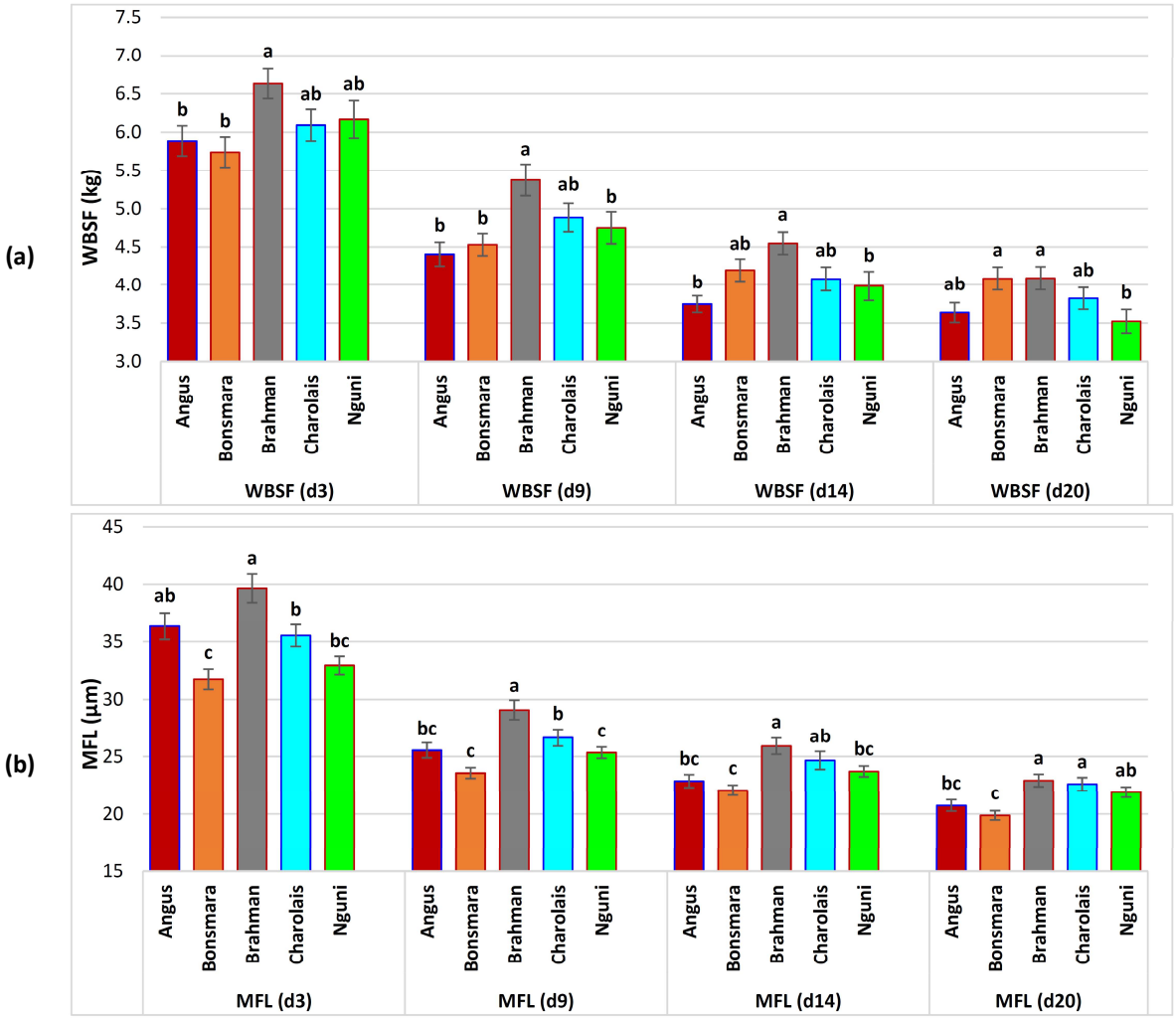

Figure 4.1. *Changes in tenderness in five South African beef breeds over an ageing period of 20 days. Least squares means (LSM) that were statistically significantly different within ageing periods (p ≤ 0.05) are indicated with different ascending letter superscripts (a, b, c). Error bars represent the standard errors of these LSM: (**a**) WBSF—Warner–Bratzler shear force; (**b**) MFL—myofibril fragment length.*

4.3.2. SNP

Ten candidate SNPs were identified in this study that could affect tenderness over extended ageing periods for at least three out of the four ageing periods measured (from a pool of 134 SNPs in the four genes). Of these, one SNP was eliminated in quality control measures due to a very low minor allelic frequency, which could result in false positives. Another two SNPs, one in the *cast* gene and one in the *capn2* gene, were statistically significant but biologically negligible, as the differences in the LSM between genotypes were very small.

4.3.2.1. cast_736

The *cast_736* SNP (rs137217365) was located 28.81 kb from the start of the *cast* gene. The SNP was independent, i.e., it did not exhibit LD (D' > 0.95) with any of the other SNPs of the *cast* gene. It seemed, therefore, that there should not be any unintended effects of non-random inheritance from nearby loci. The genotype that favoured tenderness, or the tender genotype GG*, was relatively rare, and occurred in only 19 of

the 166 bulls (11%). However, data from bulls with the heterozygous genotype showed intermediate tenderizing effects, confirming the effects of the G* allele.

The *cast*_736 genotype (Table 4.2) was associated with biologically small changes in the WBSF in the early ageing periods on d 3 and d 9 post-mortem (≤5.1%), but resulted in a larger decrease in shear force on d 14, while the tenderizing effect on d 20 was not statistically significant ($p \leq 0.1343$). The 0.43 kg decrease in shear force on d 14 in GG* bulls was additive to a tenderizing effect of ageing that resulted in an almost 2 kg decrease in shear force values in all three genotypes. This meant that the tender genotype was still able to induce a 10.3% increase in tenderization in the face of the existing tenderization of ageing at 14 days of 36.0% compared to d 3. The addition of breeds to the simplified model as covariates, each breed as a covariate or within-breed analyses suggested that the effects of this genotype on the WBSF were not breed dependent. However, false discovery rate (FDR) analyses showed that these biologically small effects could be overestimated as a result of multiple testing, rather than a true association. The effects of the *cast_736* SNP on d 3 and d 9 only tended to be significant in the FDR analysis, while the association on d 14 was not significant ($p \leq 0.2340$).

Table 4.2. *The effects of genotype of the* cast_736 *SNP on the tenderness phenotypes, Warner–Bratzler shear force (WBSF) and myofibril fragment length (MFL) of representative purebred South African beef bulls during three to 20 days of ageing and on the muscle protease enzyme system during the first 20 h post-mortem.*

cast_736	* GG (*n* = 19)	GT (*n* = 54)	TT (*n* = 93)	*p*-Value (Simplified)	Genotype Effect
WBSF (d 3)	5.90 ± 0.38 ^{b}	6.05 ± 0.17 ^{ab}	6.22 ± 0.12 ^{a}	0.0130 $^{\$}$	−5.1%
WBSF (d 9)	4.70 ± 0.33 ^{b}	4.77 ± 0.14 ^{ab}	4.92 ± 0.11 ^{a}	0.0111 $^{\$}$	−4.6%
WBSF (d 14)	3.78 ± 0.28 ^{y}	4.17 ± 0.12 ^{z}	4.21 ± 0.09 ^{z}	0.0950 $^{!}$	−10.3%
WBSF (d 20)	3.63 ± 0.27	3.89 ± 0.12	3.90 ± 0.09	0.1343	–
MFL (d 3)	39.29 ± 1.89	34.51 ± 0.81	35.64 ± 0.61	0.4293	–
MFL (d 9)	28.20 ± 1.23 ^{z}	25.36 ± 0.53 ^{y}	26.50 ± 0.40 ^{y}	0.0895	+6.4%
MFL (d 14)	24.42 ± 1.16 ^{a}	23.17 ± 0.50 ^{b}	24.20 ± 0.38 ^{ab}	0.0074	$^{\#}$ −4.2%
MFL (d 20)	20.96 ± 0.94 ^{b}	21.26 ± 0.41 ^{b}	21.97 ± 0.31 ^{a}	0.0448	−4.6%
calpastatin (1 h)	2.15 ± 0.10 ^{z}	2.03 ± 0.04 ^{y}	2.02 ± 0.03 ^{y}	0.0811 $^{!}$	+6.4%
calpastatin (20 h)	1.87 ± 0.12	1.66 ± 0.05	1.72 ± 0.04	0.1819	–
calpain-1 (1 h)	1.53 ± 0.08	1.46 ± 0.04	1.40 ± 0.03	0.8459	–
calpain-1 (20 h)	1.17 ± 0.09	1.06 ± 0.04	1.10 ± 0.03	0.3287	–
calpain-2 (1 h)	1.02 ± 0.04	1.00 ± 0.02	0.98 ± 0.01	0.3944	–
calpain-2 (20 h)	1.03 ± 0.04	1.02 ± 0.02	1.01 ± 0.01	0.4279	–
calpastatin/calpain-1 (1 h)	1.40 ± 0.07 ^{b}	1.44 ± 0.03 ^{ab}	1.49 ± 0.02 ^{a}	0.0398	−5.9%
calpastatin/calpain-1 (20 h)	1.66 ± 0.13	1.80 ± 0.06	1.68 ± 0.04	0.9142	–
calpastatin/calpains(1 h)	0.84 ± 0.03 ^{a}	0.84 ± 0.01 ^{a}	0.86 ± 0.01 ^{a}	0.0105	n/a
calpastatin/calpains (20 h)	0.83 ± 0.04	0.82 ± 0.02	0.81 ± 0.01	0.1441	–

* GG—the genotype that favoured tenderness; least squares means (LSM) ± standard errors (SE) that were significantly different within rows or ageing periods ($p \leq 0.05$) were indicated with different ascending letter superscripts (a, b, c), and those that tended to be significantly different ($p \leq 0.10$) with different descending letter superscripts (z, y, x). n/a—LSM differences were biologically negligible. $^{\#}$ means difference of GG* compared to GT; $^{\$}$ adjusted *p*-values only tended to be significant, $^{!}$ false positive association. MFL—myofibril fragment length; WBSF—Warner–Bratzler shear force.

The *cast_736* SNP was associated with an MFL over the ageing period between d 9 and d 20, with little advantage to adding breed to the model. However, the phenotypic response to genotypes was greatly altered by the time of the ageing period. The MFL on d 9 tended to reach significance, but the response in the GG* genotype was an increase in the myofibril length of 1.70 µm, which would not favour tenderization. At this ageing period, the only significant breed-dependent effect was identified in Charolais, which was the only breed

to exhibit a decrease in the MFL of 6.9% or 1.94 µm (within-breed $p \leq 0.0404$). On d 14 post-mortem, the genotypes failed to result in biologically important changes in the MFL compared to TT (+0.9%), but was 4.2% shorter than GT. The association of the MFL with genotypes on d 20 resulted in a relatively small decrease in myofibril length (1.01 µm shorter) that occurred in addition to the large tenderizing effects induced by ageing. During the period where the *cast_736* SNP affected the MFL (d 9–d 20), ageing resulted in a 4.53 µm decrease in myofibril lengths in TT bulls and 4.10 µm in GT, while, at the same time, GG* bulls experienced a 7.24 µm decrease in MFL.

The lack of a consistent response in the MFL and the small changes in the WBSF could be explained by the differences between genotypes in the muscle protease system. The genotype did not affect calpain protease activities, while small and inconsistent associations were noted for calpastatin inhibition at 1 h post-mortem only (Table 4.2). The LSM of absolute calpastatin inhibition at one hour was not different between genotypes and was identified as a false positive association when *p*-values were adjusted for multiple testing, but tended to associate with inhibition in the mixed linear model. The relative inhibition of calpain-1 by calpastatin at 1 h was decreased by 5.9% in GG* bulls, while the decrease per units of calpain-1 and calpain-2 activity was biologically negligible. The effects on relative calpastatin inhibition remained significant in the adjusted association analysis.

4.3.2.2. cast_763

The *cast_763* SNP (rs135465452) was located 98.91 kb from the start of the gene and was in strong LD (D' > 0.95) with several SNPs associated with tenderness phenotypes (results not shown). When determining whether *cast_763* would be a suitable candidate for selection for tenderness that was sustained in the face of ageing, it was important to note whether the closely-linked SNPs also induced positive tenderizing effects, as selection for the candidate SNP would indirectly result in selection for SNPs in high LD. These SNPs included *cast_741* (−50.0 kb from cast_763), *cast_770* (+17.2 kb from cast_763), *cast_771* (17.9 kb), *cast_772* (18.9 kb) and *cast_779* (36.9 kb). Although none of these SNPs were identified as candidate SNPs for the improvement of tenderness over extended ageing periods, they were found to have associations with some tenderness phenotypes (data not shown). The corresponding alleles for these SNPs were also favourable for tenderness and the general effects of these SNPs were mild reductions in the WBSF between d 3 and d 14 (of between 6.7 and 9.0%), with generally small improvements in the MFL on d 14 (5.0–6.5%).

The *cast_763* SNP did not associate with the WBSF at any of the ageing periods, but was associated with the MFL throughout the entire ageing period between d 3 and d 20 of ageing in the simplified model (Table 4.3). Although the responses in the MFL at these ageing periods were biologically small (4.4–5.5%), the differences between the LSM for the tender CC* genotype reached significance compared to CT. The tenderizing effect of the genotype was additive to an already-tenderizing effect of ageing on the MFL between d 3 and d 20 of 39% for both genotypes. There was no advantage in the addition of breed(s) to the simplified model.

Table 4.3. *The effects of genotype of the* cast_763 *SNP on the tenderness phenotypes, Warner–Bratzler shear force (WBSF) and myofibril fragment length (MFL) of representative purebred South African beef bulls during three to 20 days of ageing and on the muscle protease enzyme system during the first 20 h post-mortem.*

cast_763	* CC (*n* = 113)	CT (*n* = 48)	# TT (*n* = 5)	*p*-Value (Simplified)	Genotype Effect
WBSF (d 3)	5.99 ± 0.11	6.40 ± 0.17	–	0.5672	–
WBSF (d 9)	4.69 ± 0.09	4.97 ± 0.15	–	0.9146	–
WBSF (d 14)	4.09 ± 0.08	4.27 ± 0.13	–	0.7815	–
WBSF (d 20)	3.81 ± 0.08	3.93 ± 0.12	–	0.9412	–
MFL (d 3)	34.77 ± 0.52 ^{y}	36.56 ± 0.85 ^{z}	–	0.0902	-4.9%
MFL (d 9)	25.64 ± 0.35 ^{b}	27.14 ± 0.57 ^{a}	–	0.0289	−5.5%
MFL (d 14)	23.38 ± 0.33 ^{b}	24.45 ± 0.54 ^{a}	–	0.0005	−4.4%
MFL (d 20)	21.28 ± 0.27 ^{b}	22.30 ± 0.43 ^{a}	–	0.0081	−4.6%
calpastatin (1 h)	1.96 ± 0.03 ^{y}	2.07 ± 0.04 ^{z}	–	0.0828 $^{!}$	−5.5%
calpastatin (20 h)	1.63 ± 0.03	1.76 ± 0.05	–	0.1042	–
calpain-1 (1 h)	1.40 ± 0.02 ^{z}	1.42 ± 0.04 ^{z}	–	0.0848 $^{!}$	n/a
calpain-1 (20 h)	1.04 ± 0.03	1.09 ± 0.04	–	0.3695	–
calpain-2 (1 h)	0.98 ± 0.01	1.00 ± 0.02	–	0.4187	–
calpain-2 (20 h)	1.01 ± 0.01	1.03 ± 0.02	–	0.5901	–
calpastatin/calpain-1 (1 h)	1.44 ± 0.02	1.50 ± 0.03	–	0.9886	–
calpastatin/calpain-1 (20 h)	1.69 ± 0.04	1.77 ± 0.06	–	0.4553	–
calpastatin/calpains(1 h)	0.83 ± 0.01	0.87 ± 0.02	–	0.5752	–
calpastatin/calpains (20 h)	0.80 ± 0.01 ^{y}	0.85 ± 0.02 ^{z}	–	0.0876	−5.6%

* CC—the genotype that favoured tenderness; least squares means (LSM) ± standard errors (SE) that were significantly different within rows or ageing periods ($p \leq 0.05$) were indicated with different ascending letter superscripts (a, b, c), and those that tended to be significantly different ($p \leq 0.10$) with different descending letter superscripts (z, y, x). # Tough homozygotes were not available for comparison; ! adjusted *p*-values identified a false positive association; MFL—myofibril fragment length; WBSF—Warner–Bratzler shear force.

The small differences in the myofibril length could be ascribed to small changes in protease enzyme inhibition that tended to occur. These differences were not consistently present and could be false positive associations (Table 4.3). The absolute calpastatin inhibition at 1 h post-mortem associated with the genotype was 5.5% lower, while relative calpastatin per combined calpain-1 and calpain-2 proteolysis at 20 h was 5.6% lower in the *CC genotype compared to CT. Although a tendency towards a decrease in calpain-1 at 1 h was observed, the difference was biologically negligible (a 1.4% shorter MFL) and could be considered unaffected by the genotype.

4.3.2.3. capn2_780

The *capn2_780* SNP (rs135646764) was located 55.07 kb from the start of the gene. The SNP was in LD (D' > 0.95) with other SNPs that affected tenderness in the gene-based association study, *capn2_760* (−46.93 kb), *capn2_763* (−35.72 kb), *capn2_766* (−26.53 kb) and *capn2_772* (−21.40 kb). Selection for the tender allele of the *capn2_780* SNP would include indirect selection for these SNPs, all of which were linked to the corresponding tender alleles. These SNPs were found to have no association with the WBSF or MFL, but did affect changes in enzyme function, which was a general increase in calpastatin and calpains, where calpain proteolysis increased to a greater extent than calpastatin inhibition. This resulted in a decrease in the relative calpastatin inhibition per units of calpain proteolysis, which would favour tenderization (results not shown). The tough GG genotype was relatively rare (*n* = 15), where 10 of these bulls were Brahman, while also not being sufficient for determining the LSM. The G allele was rare in Angus, with only three heterozygotes and no GG bulls. Because few "tough homozygotes" were available for comparison, genotype differences were compared

between AA* (*n* = 112) and AG (*n* = 39), with the exception of the Angus breed, where the association could not be tested (minor allelic frequency = 0.06).

There were no associations between genotypes and the WBSF at any of the ageing periods (Table 4.4), while the *capn2_780* genotype tended to affect the MFL throughout the ageing period (Figure 4.2 a). The tenderizing effect of the SNP on the MFL on d 14 was only apparent when breed(s) were added to the simplified model as covariate(s). The tender, or AA* genotype resulted in a >6% reduction in the MFL compared to AG on d 3 and d 9, which was sustained as a small 5.4% decrease on d 20. Although these tenderizing effects were biologically small, they were additive to a 39% decrease in the MFL already induced by ageing in both genotypes (Figure 4.2 a).

The covariate effect of breed(s) was only present on d 14 post-mortem (Figure 4.2 b), where the Bonsmara MFL remained unchanged, while tenderizing effects of the genotype were moderate in Brahman (8.1%) and large in Charolais (15.6%). The AA* genotype in Nguni bulls resulted in a 6.9% increase in myofibril lengths, or 1.54 µm, which was not a tenderizing response.

The fact that the consistent decrease in the MFL over the ageing period was not accompanied by a concomitant decrease in shear force values at any of the ageing periods was most likely due to a lack of changes in the proteolytic enzymes in the different genotypes (Table 4.4). The adjusted analyses (FDR) did not change the results of the multiple associations.

Table 4.4. *The effects of genotype of the* capn2_780 *SNP on the tenderness phenotype, Warner–Bratzler shear force (WBSF) of representative purebred South African beef bulls during three to 20 days of ageing and on the muscle protease enzyme system during the first 20 h post-mortem.*

capn2_780	*** AA(*n* = 112)**	**AG(*n* = 39)**	**# GG(*n* = 15)**	***p*-Value (Simplified)**	**Genotype Effect**
WBSF (d 3)	6.05 ± 0.11	6.54 ± 0.20	–	0.3279	–
WBSF (d 9)	4.77 ± 0.09	4.83 ± 0.17	–	0.1467	–
WBSF (d 14)	4.10 ± 0.08	4.30 ± 0.15	–	0.2274	–
WBSF (d 20)	3.84 ± 0.08	3.93 ± 0.14	–	0.3831	–
calpastatin (1 h)	2.06 ± 0.03	1.87 ± 0.05	–	0.9969	–
calpastatin (20 h)	1.75 ± 0.03	1.48 ± 0.06	–	0.7869	–
calpain-1 (1 h)	1.45 ± 0.02	1.35 ± 0.04	–	0.3737	–
calpain-1 (20 h)	1.10 ± 0.03	0.99 ± 0.05	–	0.3225	–
calpain-2 (1 h)	1.00 ± 0.01	0.95 ± 0.02	–	0.9139	–
calpain-2 (20 h)	1.03 ± 0.01	0.96 ± 0.02	–	0.6730	–
calpastatin/calpain-1 (1 h)	1.47 ± 0.02	1.42 ± 0.04	–	0.7586	–
calpastatin/calpain-1 (20 h)	1.74 ± 0.04	1.62 ± 0.07	–	0.9579	–
calpastatin/calpains(1 h)	0.85 ± 0.01	0.82 ± 0.02	–	0.7954	–
calpastatin/calpains (20 h)	0.83 ± 0.01	0.77 ± 0.02	–	0.7862	–

* AA—the genotype that favoured tenderness; least squares means (LSM) ± standard errors (SE) were not significantly different ($p > 0.10$).
# Tough homozygotes were not available for comparison; WBSF—Warner–Bratzler shear force.

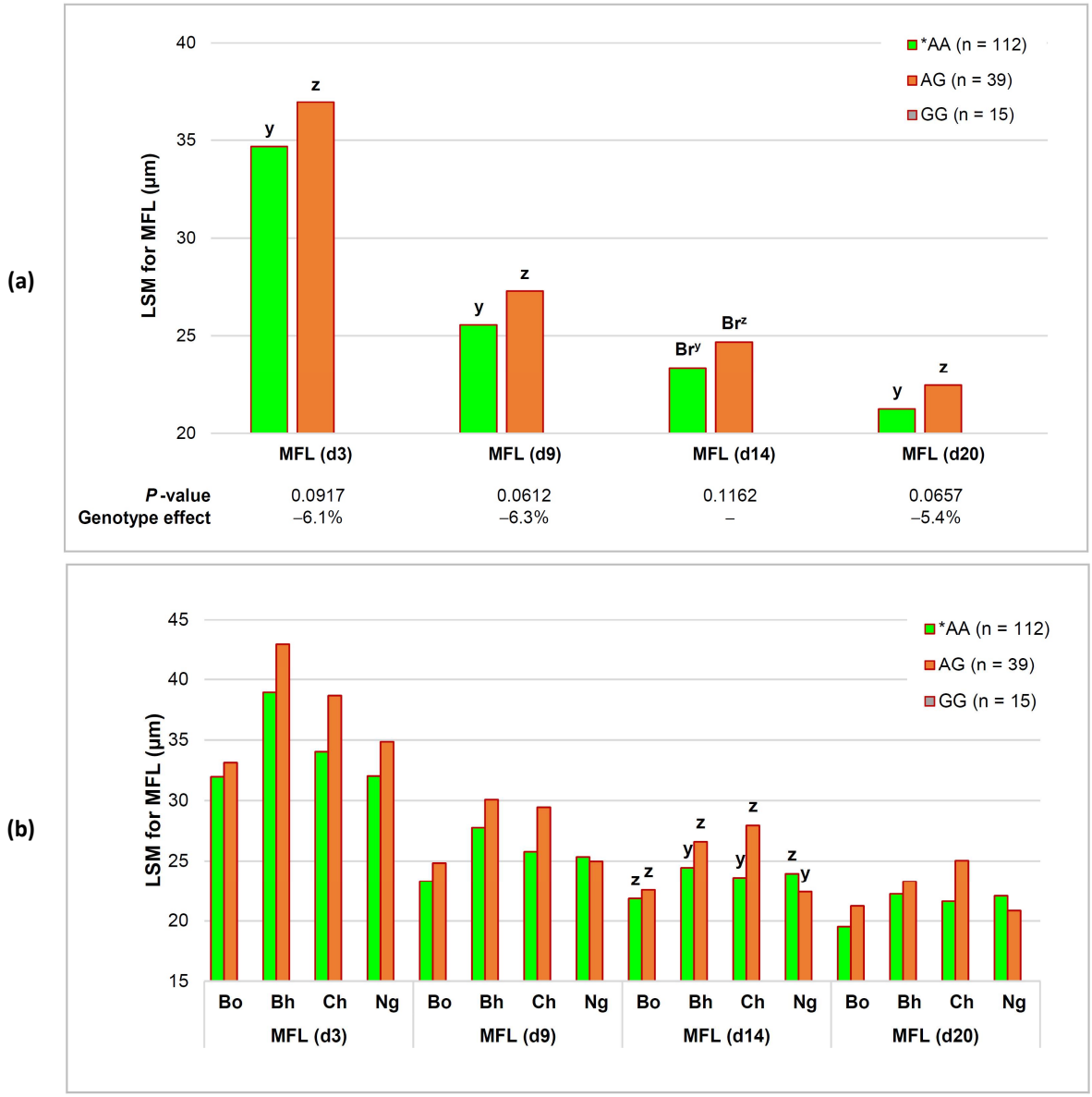

Figure 4.2. *Additive effects of the* capn2_780 *genotype and ageing on myofibril fragment length (MFL). AA*—the genotype that favoured tenderness. Within each ageing period, different descending letter superscripts (z, y, x) tended to be significantly different (*p ≤ *0.10): (**a**) genotype and ageing effects data pooled for all breeds; Br—different only when breeds were added to the simplified model; (**b**) genotype and ageing effects on MFL for individual South African beef breeds. Bo—Bonsmara; Bh—Brahman; Ch—Charolais; Ng—Nguni.*

4.3.2.4. capn1_184

The *capn1_184* SNP (rs17871986) was located 4.51 kb from the start of the gene and exhibited a high level of LD (D' > 0.95) with three other SNPs associated with tenderness within close proximity of the SNP. The selection for the tender G* allele of *capn1_184* favoured selection for the "tender alleles" for these SNPs that were closely linked to it (data not shown). The *capn1_183* (-0.76 kb), *capn1_316* SNP (+1.10 kb) and *capn1_185* (+2.96 kb) SNPs were all associated with increased tenderization, with similar or smaller means differences than those discussed below. There was a shortage of tough homozygous bulls (AA) in the *Bos taurus* and Sanga-types and a lack of tender homozygotes (GG*) in Brahman. However, the addition of breed(s) as covariate(s) did not

improve the simplified mixed model in any of the phenotypes tested. A greater pool of animals would be required to fully elucidate the breed differences, if any exist.

The *capn1_184* SNP exhibited a strong, stable association with the WBSF over the entire ageing period (Figure 4.3 a) and during intermediate ageing (d 9–d 14) for the MFL (Table 4.5), resulting in large phenotypic responses ≥ 19%). The differences between means observed for the different genotypes sustained levels greater than 1 kg shear force throughout the different ageing periods, where a 20.3% decrease in the WBSF of d 3 was maintained throughout, and up to 22.8% on d 20. The effect on the WBSF on d 20 was likely ascribed to multiple testing (adjusted $p \leq 0.1250$) and this effect would need to be confirmed in a larger dataset. These genotype associations were additive to decreases in the WBSF of between 34.2 and 37.1% in all three genotypes over the ageing period (Figure 4.3 a). Similar results were observed for the MFL at d 9 and d 14 of ageing, where myofibril lengths in GG* bulls were 7.31 µm and 8.04 µm shorter than AA bulls, respectively.

Table 4.5. *The effects of genotype of the* capn1_184 *SNP on the tenderness phenotype, myofibril fragment length (MFL) of representative purebred South African beef bulls during three to 20 days of ageing and on the muscle protease enzyme system during the first 20 h post-mortem.*

capn1_184	*** GG (*n* = 77)**	**AG (*n* = 66)**	**AA (*n* = 23)**	***p*-Value (Simplified)**	**Genotype Effect**
MFL (d 3)	33.47 ± 0.65	35.48 ± 0.72	41.57 ± 1.74	0.1810	–
MFL (d 9)	24.67 ± 0.42 ^{c}	26.20 ± 0.47 ^{b}	31.98 ± 1.12 ^{a}	0.0242	−22.9%
MFL (d 14)	22.50 ± 0.38 ^{y}	24.05 ± 0.42 ^{y}	30.54 ± 1.02 ^{z}	0.0771	−26.3%
MFL (d 20)	20.92 ± 0.31	21.54 ± 0.35	25.70 ± 0.84	0.1557	–
calpastatin (1 h)	1.99 ± 0.04	1.99 ± 0.04	2.23 ± 0.09	0.4096	–
calpastatin (20 h)	1.69 ± 0.04	1.64 ± 0.05	1.81 ± 0.11	0.6957	–
calpain-1 (1 h)	1.47 ± 0.03	1.41 ± 0.03	1.32 ± 0.08	0.2095	–
calpain-1 (20 h)	1.13 ± 0.03	1.05 ± 0.04	0.97 ± 0.09	0.7597	–
calpain-2 (1 h)	1.00 ± 0.01	1.00 ± 0.01	0.98 ± 0.03	0.7125	–
calpain-2 (20 h)	1.04 ± 0.01	1.02 ± 0.01	0.98 ± 0.04	0.5729	–

* GG—the genotype that favoured tenderness; least squares means (LSM) ± standard errors (SE) that were significantly different within rows or ageing periods ($p \leq 0.05$) were indicated with different ascending letter superscripts (a, b, c), and those that tended to be significantly different ($p \leq 0.10$) with different descending letter superscripts (z, y, x). MFL—myofibril fragment length.

Large, consistent decreases in the WBSF and large, but less consistent, shorter MFLs were likely the result of the associations of the *capn1_184* SNP with the inhibition of calpastatin relative to protease activities (Figure 4.3 b). The calpastatin per calpain-1 at 1 h and 20 h decreased by ≥ 19%, while calpastatin per combined calpain-1 and calpain-2 activities at 1 h was 17% lower. The LSM for heterozygous bulls was intermediate for calpastatin per unit of calpain-1 proteolysis, but not different from the tender genotype for inhibition relative to the combined proteolysis of calpain-1 and calpain-2. This would allow for an increased degradation of muscle proteins, greatly favouring the tenderization of beef.

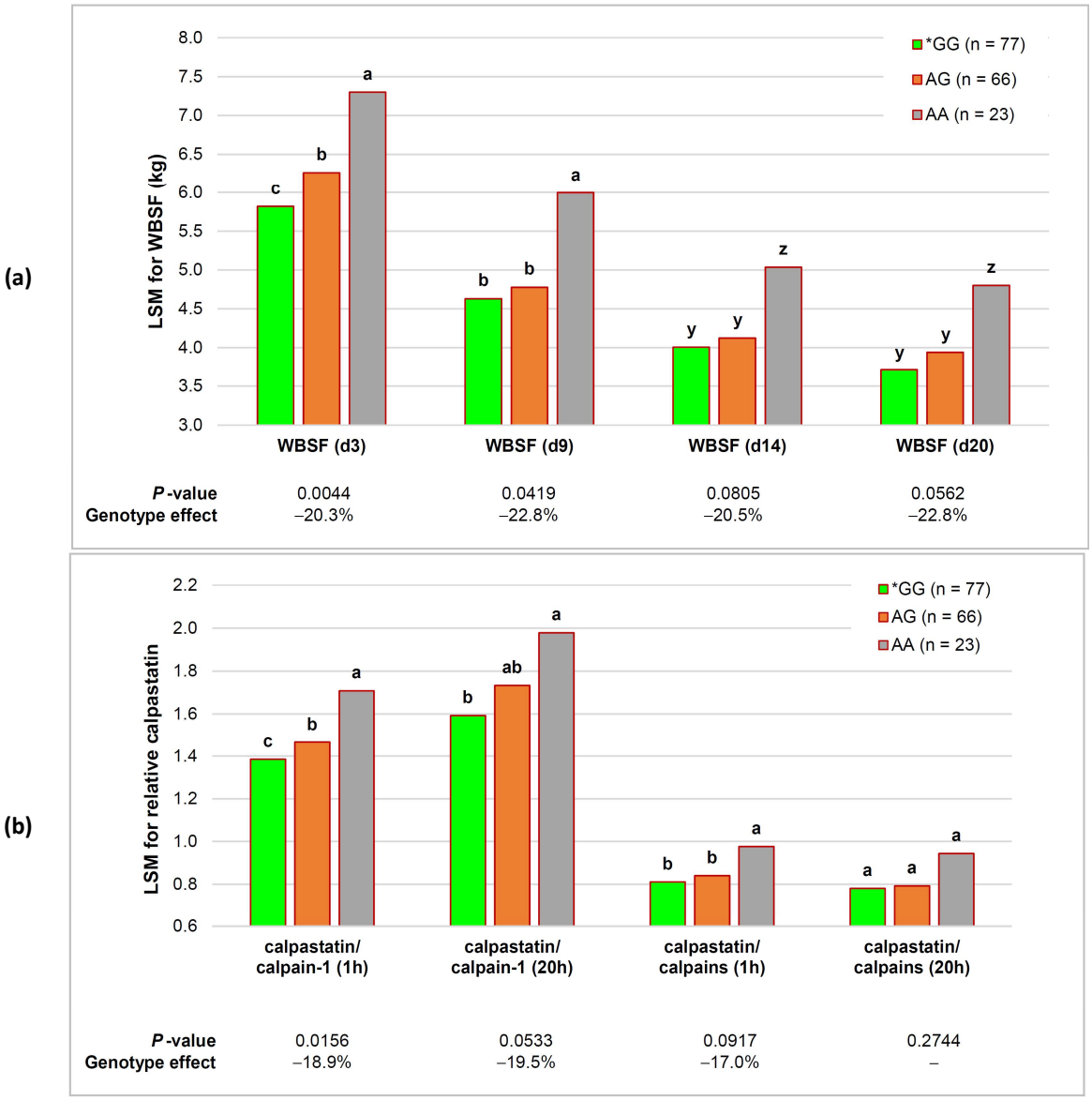

Figure 4.3. *Additive effects of the* capn1_184 *genotype and ageing on tenderness in representative purebred South African beef bulls. *GG—the genotype that favoured tenderness. Within each ageing period, different ascending letter superscripts (a, b, c) were significantly different (*p ≤ *0.05), and those that tended to be different (*p ≤ *0.10) were indicated with different descending letter superscripts (z, y, x): (**a**) Warner–Bratzler shear force (WBSF); the effect on d 20 was not significant after FDR adjustment (adjusted* p ≤ *0.1250); (**b**) relative calpastatin inhibition of protease(s).*

4.3.2.5. capn1_187

The *capn1_187* SNP (rs135658374) was located 17.59 kb from the start of the gene and was in strong LD (D' > 0.95) with the *capn1_4751* SNP (+6.57 kb) discussed below. The distribution of alleles for Brahman was skewed toward the tough allele, while other breeds generally experienced a shortage of CC genotypes. The discussion, therefore, focused on the differences between the tender homozygous bulls (TT*) and heterozygotes (CT) for breed dependence, although there was little or no advantage to adding breeds to the simplified model as covariates.

The SNP was associated with the WBSF over the entire ageing period. The least squares means differences between homozygous bulls were very close to 1 kg on d 3 and d 9 post-mortem (0.43–0.49 kg for heterozygous

bulls), and the large gain in the tenderness of early ageing was decreased somewhat by d 20, where the shear force improved by 0.77 kg in TT* and 0.34 kg in CT. These genotype effects on shear force occurred additive to the 37% reduction in the WBSF that occurred in all genotypes by d 20 post-mortem (Figure 4.4 a). Adjusting for multiple testing during association, the adjusted *p*-value for the WBSF on d 20 decreased from a significant $p \leq 0.0302$ to a tendency $p \leq 0.0558$.

The association of the *capn1_187* genotype with the MFL decayed over the time of ageing, with the most prominent effect on d 3, where an 11.78 µm decrease in length was observed in TT* bulls (26.6% shorter). As the ageing period progressed, the improvement in the MFL gradually decreased and was 5.42 µm (18.2%) on d 9 and non-significant by d 14.

There was a tendency toward a breed-dependent association of the *capn1_187* SNP and absolute calpastatin at 20 h (Table 4.6). A large increase in the absolute inhibition (toughening) was observed in Angus, Brahman and Charolais (+13–15%), with no effect in Bonsmara and a moderate tenderizing response (11% lower calpastatin) in Nguni. Although this apparent toughening effect was concerning, the adjustment of the *p*-values for multiple testing identified it as a potential false positive association (adjusted $p \leq 0.1607$). The means differences for relative calpastatin activity per calpain(s) were, generally, unaffected, while the relative calpastatin inhibition per calpain-1 at 1 h was tenderizing (15.5% lower).

Table 4.6. *The effects of genotype of the* capn1_187 *SNP on the tenderness phenotype, myofibril fragment length (MFL) of representative purebred South African beef bulls during three to 20 days of ageing and on the muscle protease enzyme system during the first 20 h post-mortem.*

capn1_187	*** TT (*n* = 76)**	**CT (*n* = 57)**	**CC (*n* = 33)**	***p*-Value (Simplified)**	**Genotype Effect**
MFL (d 3)	32.54 ± 0.64 ^{c}	35.33 ± 0.76 ^{b}	44.32 ± 2.10 ^{a}	0.0090	−26.6%
MFL (d 9)	24.30 ± 0.43 ^{c}	26.23 ± 0.51 ^{b}	29.71 ± 1.40 ^{a}	0.0047	−18.2%
MFL (d 14)	22.25 ± 0.40	24.38 ± 0.47	26.65 ± 1.30	0.1270	–
MFL (d 20)	20.61 ± 0.32	21.80 ± 0.38	24.02 ± 1.06	0.1885	–
calpastatin (1 h)	2.00 ± 0.04	2.04 ± 0.04	1.96 ± 0.12	0.2181	
calpastatin (20 h)	1.73 ± 0.04 ^{z}	1.69 ± 0.05 ^{z}	1.61 ± 0.14 ^{z}	0.0640 $^{!}$	$^{\#}$ variable
calpastatin/calpain-1 (1 h)	1.38 ± 0.02 ^{z}	1.44 ± 0.03 ^{z}	1.63 ± 0.08 ^{y}	0.0909	−15.5%
calpastatin/calpain-1 (20 h)	1.63 ± 0.05	1.69 ± 0.06	1.84 ± 0.16	0.1571	
calpastatin/calpains(1 h)	0.81 ± 0.01	0.83 ± 0.01	0.90 ± 0.04	0.1989	
calpastatin/calpains (20 h)	0.79 ± 0.01	0.80 ± 0.02	0.85 ± 0.04	0.5493	

* TT—the genotype that favoured tenderness; least squares means (LSM) ± standard errors (SE) that were significantly different within rows or ageing periods ($p \leq 0.05$) were indicated with different ascending letter superscripts (a, b, c), and those that tended to be significantly different ($p \leq 0.10$) with different descending letter superscripts (z, y, x). $^{\#}$ Responses of the *TT genotype were variable between breeds and not always tenderizing, $^{!}$ false positive association; MFL—myofibril fragment length.

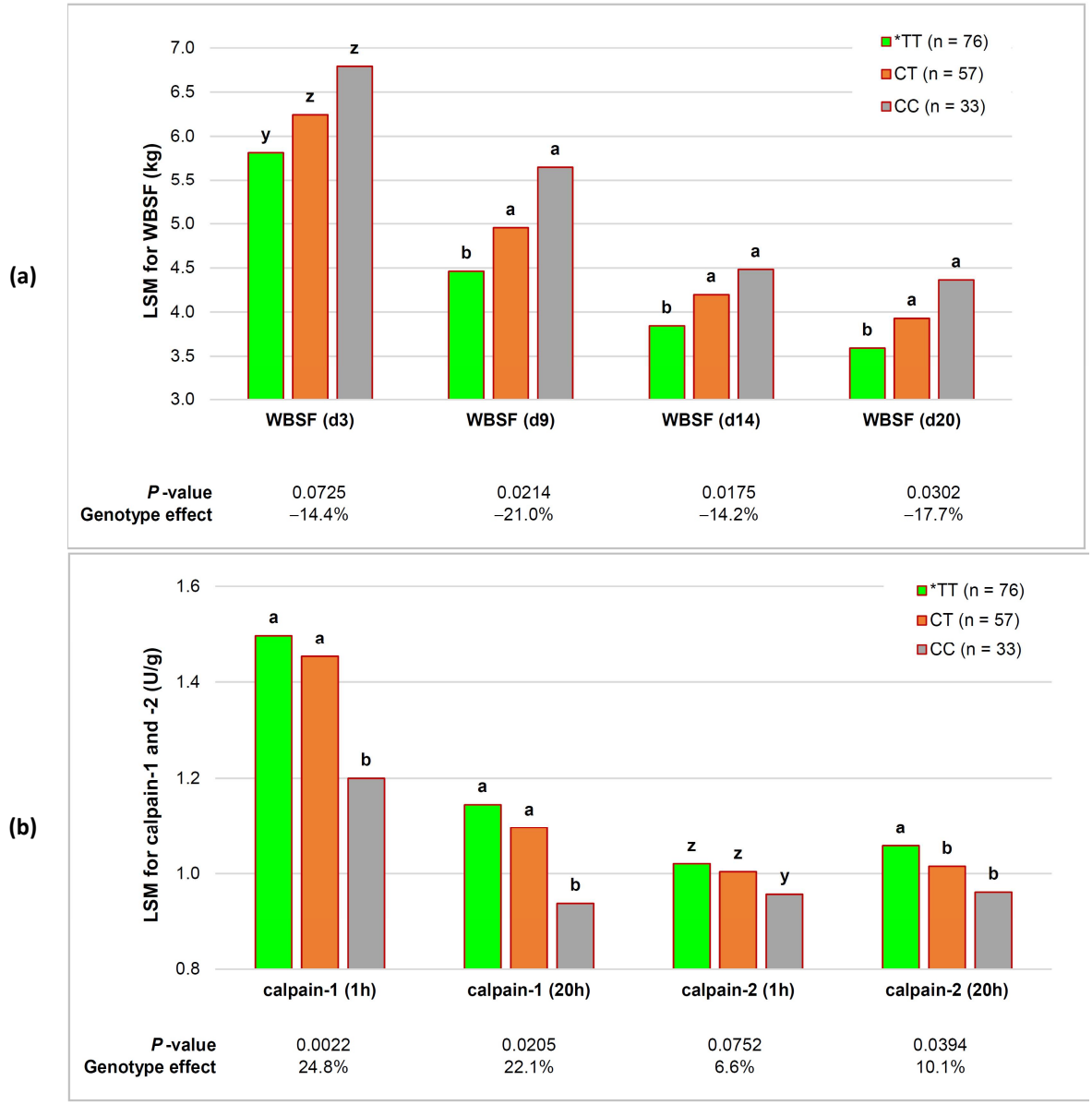

Figure 4.4. *Additive effects of the* capn1_187 *genotype and ageing on tenderness in representative purebred South African beef bulls. *TT—the genotype that favoured tenderness. Within each ageing period, different ascending letter superscripts (a, b, c) were significantly different* (p ≤ *0.05), and those that tended to be significantly different* p ≤ *0.10) were indicated with different descending letter superscripts (z, y, x): (**a**) Warner–Bratzler shear force (WBSF); the effect on d 20 only tended to be significant after FDR adjustment (adjusted* p ≤ *0.0558); (**b**) calpain protease activities.*

The large consistent responses in tenderization (especially the WBSF) were, therefore, not in response to the decreased calpastatin inhibition of protease activities, but rather the large and consistent responses of the proteases themselves to genotypes (Figure 4.4 b). Increased protease activity in genotypes that contained the T* allele were large for muscle calpain-1 activities (17—25%), with moderate tenderizing effects in calpain-2 (≤10%). These increased rates of proteolysis would favour myofibril fragmentation, decreasing the length of the fibrils and favouring a decrease in shear force values.

4.3.2.6. capn1_4751

The *capn1-4751* SNP (rs17872050) was located 24.17 kb from the start of the *capn1* gene. It exhibited strong LD (D' > 0.95) with the *capn1_187* SNP discussed above (−1.86 kb) and some downstream SNPs, with the tender allele for *capn1_4751* corresponding to the tender alleles of these SNPs (results not shown). The *capn1_189* SNP (+6.04 kb), *capn1_190* (+10.34 kb) and *capn1_191* (+13.79 kb) decreased the WBSF in late ageing by between 9.0% on d 14 and up to 16.2% on d 20, and selection for the *capn1_4751* SNP would result in favourable responses in these tenderness phenotypes.

The SNP associated with the WBSF only between d 14 and d 20, resulting in a moderate tenderization of 0.47—0.50 kg shear force (Table 4.7), additive to the existing ageing tenderizing effects. The association between the *capn1-4751* SNP and MFL was, however, sustained from d 3 to d 14 post-mortem, although the effects on myofibril lengths were moderate. The MFL was decreased by 10.4% on d 3, 9.3% on d 9 and 8.1% on d 14 in CC* bulls compared to TT. These moderate gains were still important, as they occurred additive to the 32–33% decrease in the MFL that occurred between d 9 and d 20 of ageing, and the genotype effect deteriorated but did not disappear over time in late ageing as the myofibril lengths decreased. There was no advantage to adding breeds to the simplified mixed model as covariates.

Table 4.7. *The effects of genotype of the* capn1_4751 *SNP on the tenderness phenotypes, Warner–Bratzler shear force (WBSF) and myofibril fragment length (MFL) of representative purebred South African beef bulls during 3 to 20 days of ageing and on the muscle protease enzyme system during the first 20 h post-mortem.*

capn1_4751	*** CC (*n* = 53)**	**CT (*n* = 63)**	**TT (*n* = 50)**	***p*-Value (Simplified)**	**Genotype Effect**
WBSF (d 3)	5.89 ± 0.16	6.17 ± 0.15	6.26 ± 0.22	0.3758	–
WBSF (d 9)	4.47 ± 0.14	4.94 ± 0.13	4.85 ± 0.19	0.3035	–
WBSF (d 14)	3.84 ± 0.12 ^{b}	4.14 ± 0.11 ^{ab}	4.31 ± 0.17 ^{a}	0.0357	−10.8%
WBSF (d 20)	3.58 ± 0.11 ^{b}	3.88 ± 0.11 ^{ab}	4.07 ± 0.16 ^{a}	0.0226	−12.0%
MFL (d 3)	32.43 ± 0.79 ^{y}	35.72 ± 0.73 ^{z}	36.17 ± 1.09 ^{z}	0.0790	−10.4%
MFL (d 9)	24.20 ± 0.52 ^{b}	26.14 ± 0.48 ^{a}	26.69 ± 0.72 ^{a}	0.0474	−9.3%
MFL (d 14)	22.11 ± 0.49 ^{y}	24.36 ± 0.45 ^{z}	24.07 ± 0.67 ^{z}	0.0762	−8.1%
MFL (d 20)	20.32 ± 0.40	21.90 ± 0.37	21.94 ± 0.55	0.1056	–
calpastatin (1 h)	2.02 ± 0.04	2.03 ± 0.04	2.08 ± 0.06	0.1216	–
calpastatin (20 h)	1.73 ± 0.05	1.73 ± 0.05	1.74 ± 0.07	0.1424	–
calpain-1 (1 h)	1.54 ± 0.03 ^{a}	1.43 ± 0.03 ^{b}	1.48 ± 0.05 ^{ab}	0.0340 $^{\$}$	+4.1%
calpain-1 (20 h)	1.14 ± 0.04	1.13 ± 0.04	1.08 ± 0.05	0.1847	–
calpain-2 (1 h)	1.03 ± 0.01	1.00 ± 0.01	1.03 ± 0.02	0.2252	–
calpain-2 (20 h)	1.05 ± 0.02	1.04 ± 0.01	1.03 ± 0.02	0.3517	–
calpastatin/calpain-1 (1 h)	1.35 ± 0.03	1.45 ± 0.03	1.46 ± 0.04	0.3813	–
calpastatin/calpain-1 (20 h)	1.64 ± 0.06	1.69 ± 0.05	1.77 ± 0.08	0.7056	–
calpastatin/calpains(1 h)	0.79 ± 0.01	0.84 ± 0.01	0.84 ± 0.02	0.8265	–
calpastatin/calpains (20 h)	0.79 ± 0.02	0.80 ± 0.02	0.83 ± 0.02	0.3613	–

*CC—the genotype that favoured tenderness; least squares means (LSM) ± standard errors (SE) that were significantly different within rows or ageing periods ($p \leq 0.05$) were indicated with different ascending letter superscripts (a, b, c), and those that tended to be significantly different ($p \leq 0.10$) with different descending letter superscripts (z, y, x). $^{\$}$ adjusted *p*-values only tended to be significant; MFL—myofibril fragment length; WBSF—Warner–Bratzler shear force.

4.4. Discussion

Of the total 134 SNPs tested in the gene-based association analyses, 62 SNPs showed some association with tenderness phenotypes in the WBSF or MFL in at least one of the ageing periods ($p \leq 0.10$), while 38 SNPs associated significantly ($p \leq 0.05$) with tenderness (results not shown). Only 12 of these SNPs, or less than 20%, associated with tenderness over extended periods of ageing, before quality control or biological importance of differences were considered. For these SNPs, it was more likely to identify associations from data collected at a single ageing period or from animals that showed variation in ageing, where 92% of the associations would be identified if any one ageing period was used to determine both the WBSF and MFL. However, more than 80% of the SNPs that could affect tenderness would likely fail to associate with tenderness from a single measurement, because they only associated with tenderness phenotypes for a short period of ageing. The least likely ageing period to identify an association in these data was d 9, where only 40% of all the possible SNPs (24/62) showed an association with the WBSF or MFL, while at the other ageing periods (d 3, d 14 and d 20), approximately 50–55% of all possible associations were identified.

Although the effects of genotypes in the calpain–calpastatin system are well-established, few studies have formally investigated the changes induced by ageing over time, especially when it came to using high-density (777K) genotyping in South African beef cattle. Here, the SNPs in the *cast* and *capn2* gene that could sustain tenderizing effects for extended ageing periods were rare and did not have large effects on a variety of phenotypes of physical tenderness or protease activity. Previous studies similarly identified a limited number of 777K SNPs in the *capn1* and *cast* genes associated with tenderness, but these associations were limited to earlier ageing periods (≤d 7) and did not extend into the d 14 intermediate ageing period (Tizioto *et al.*, 2013). In our data, there were, however, a few SNPs in the *capn1* gene with larger sustained phenotypic responses in the tender genotypes (genotypes containing tender alleles) and could be linked physiologically to responses in the muscle calpain–calpastatin system.

Linkage disequilibrium (LD) is a requirement for effective genomic selection (Meuwissen *et al.*, 2001; Wientjes *et al.*, 2013), but can also cause inadvertent negative effects from SNPs that are not targeted through selection (Wright, 1980), but linked in inheritance or pleiotropy (Williams, 2005). Additionally, genotyping needs to be conducted at a sufficiently high density to identify LD between SNP markers (McKay *et al.*, 2007; Pérez O'Brien *et al.*, 2014; Makina *et al.*, 2015b) or the correlation in inheritance between alleles from different loci. The mean LD (D') between the SNPs in the *cast* and *capn2* genes was high, 0.84 and 0.92, respectively, while greater levels of diversity (moderate D') were present in the *capn1* (0.69) and the *capn3* (0.63) genes. All the SNPs in LD with the candidate SNPs for extended ageing were also linked to tender alleles that would result in responses that favoured tenderness, with no apparent unintended negative effects on tenderization (Wright, 1980). In addition to tenderness, other phenotypes such as the colour, energy supply and water-holding capacity were recorded, but no clear adverse effects were identified through LD (results not shown).

It must be considered that the SNPs in this study were specifically chosen to sustain an effect on tenderness (WBSF or MFL) over time. If the three genotypes of an SNP responded to ageing in the same way, the association with the phenotype should remain relatively unaffected over the ageing period. One could assume that this should be true for the SNPs identified in this study, and this was generally true, with little

difference in the absolute size of the tenderizing response (kg or µm) between d 3 and d 20, within each of the genotypes (bulls homozygous for the tender alleles, heterozygotes and bulls homozygous for the tough alleles).

If there was an interaction between the individual genotypes and tenderization over the ageing period, one genotype would tenderize more (or less) than the other genotypes over time. Longer ageing periods are generally employed to allow beef that tenderizes at a slower rate, to reach a greater tenderness before going to market. Over time, the differences between tough steaks in early ageing and those that tenderize rapidly by early ageing can decrease and even become negligible (Watanabe *et al.*, 1996). One could expect that it would become more difficult to identify genomic effects after longer periods of ageing (e.g., d 20), because the differences between the means of the toughest vs. most tender steaks decreased over time. An example of such an effect was shown in the *capn1_187* SNP, where the change in the MFL of the TT* and CT genotypes (containing the tender allele) was 11.93 µm and 13.53 µm, respectively (a 37–38% decrease in myofibril length to d 20). However, the change in the CC genotype was 20.3 µm or 45.8% between d 3 and d 20, and because the bulls with the "tough genotype" exhibited a greater response to ageing, the beneficial effect of the T* allele also gradually disappeared over time.

For this same SNP, however, (*capn1_187*), the effect on the WBSF was, generally, very stable and there was little difference between the responses of bulls with different genotypes and ageing for 20 days. Tender homozygous bulls (TT*) experienced a 2.22 kg reduction in the WBSF (38.2%), heterozygous bulls 2.32 kg (37.1%) and tough homozygous (CC) bulls 2.43 kg (35.7%). The differences between means of the genotypes, therefore, remained very stable over the different ageing periods, with little difference between d 3 and d 20 in the percentage difference of TT* compared to CC. This large tenderizing response, from very early ageing would be the ideal in a candidate SNP, because it would eliminate the financial and logistical costs associated with extended ageing; therefore, increasing profitability (Dransfield, 1994; Warner *et al.*, 2017).

Conversely, the association of the *cast_736* SNP on the MFL changed dramatically over time from an effect of attenuated tenderization of GG* bulls on d 9, to a tenderizing effect of the same genotype on d 20. This dramatic change in the association between d 9 and d 20 was the result of an enhanced response to ageing in the GG* genotype, where the change in myofibril lengths of these bulls was 7.24 µm between d 9 and d 20, but only 4.1 µm in GT and 4.53 µm in TT.

The effects of the *cast_736* SNP on the WBSF that increased during ageing to a 10% decrease in shear force by d 14 in GG* bulls was promising. It was previously identified as a candidate SNP for selection for tenderness on approximately d 3 and d 14 of ageing (White, 2020). The *cast_736* SNP fell within the range of a quantitative trait locus (QTL) for shear force identified previously, but was not one of the candidate SNPs tested by the 50K array used (McClure *et al.*, 2012) and was also included in the GeneSeek HD Genomic Profiler (150K). Previous studies failed to associate the genotype of this SNP with tenderizing effects (Bolormaa *et al.*, 2013b; Tizioto *et al.*, 2013). The moderate tenderizing response that was retained well into ageing could represent a meaningful gain in tenderness above either GT or TT bulls on d 20, if the proportion of GG* bulls could be increased through selection for this SNP. It would also have to be targeted specifically for selection, as it did not show LD with any other candidate SNPs in this experiment. To our knowledge, this is the first association of this SNP with tenderness phenotypes and confirmed a role for the SNP in the QTL that could be independent of the

other SNPs in the QTL. However, the effects on the MFL and calpastatin that accompanied this improvement in the WBSF precluded the use of the SNP, as an unintended negative response in calpastatin inhibition (an increase) could result in a decreased proteolysis and increased toughening. The counterintuitive, toughening effect of the SNP on calpastatin in the "tender" genotype could explain the inconsistent effects in the MFL.

Although the present results showed small but persistent effects of the *cast_763* SNP on the WBSF, previous studies failed to identify an association between the SNP and tenderization (White, 2020). Although the SNP fell within the location of a shear force QTL in the gene, the *cast_763* SNP was not included in the these analyses as a potential candidate SNP (Barendse, 2011; McClure *et al.*, 2012; Lee *et al.*, 2014) or did not associate with tenderness (Bolormaa *et al.*, 2013b; Tizioto *et al.*, 2013). Even though these effects were small, they proved statistically significant results in both models used to analyse these data. It follows that a small potential exists for an improvement in the marker through selection that would be linked to improvements in early and intermediate ageing through LD with neighbouring SNPs, including measures of the WBSF, which were not significant for the SNP itself, as part of the QTL in this region of the gene (McClure *et al.*, 2012). This SNP was, however, already distributed toward the favourable allele, with a large proportion of CC* bulls (n = 113). This is characteristic of many SNPs in the *cast* gene (Curi *et al.*, 2010; Johnston & Graser, 2010; King *et al.*, 2012), where the average tender allele frequency from all 77 SNPs genotyped in the *cast* gene averaged 71.3%, being the minor allele in only 9 (12%) of these SNPs. This could be the result of selection practices, as confirmed by the relatively high LD of the gene (D' = 0.84).

The *capn2_780* SNP generally only tended to have a small effect on the MFL, but was persistent through most of the ageing periods tested. It was linked to SNPs that could favour responses in the muscle calpain system in this study that could indirectly enhance tenderization through proteolysis in selection. Although SNPs in the *capn2* gene were tested as possible candidates for selection for tenderness (Costello *et al.*, 2007), this SNP has not yet been causally linked to tenderness (McClure *et al.*, 2012; Bolormaa *et al.*, 2013b; Tizioto *et al.*, 2013).

The *capn1_184* SNP was located within QTL for tenderness phenotypes (shear force and panel score), where it was linked to the WBSF (Tizioto *et al.*, 2013), while other studies found no association of the SNP with tenderness (McClure *et al.*, 2012; Lee *et al.*, 2014; White, 2020). It was an excellent candidate for selection to improve tenderness over the entire ageing period for representative South African beef breeds, while positive gains would also be determined in the SNP through LD. Not only did the association of this SNP with the WBSF persist over the entire ageing period, but there were large gains during the intermediate ageing period (d 9 and d 14) and the 21–26% improvement in tenderness (≤d 14) was much greater than the 7% improvement in the WBSF or 9% in the MFL between d 14 and d 20. This implied that beef could go to market sooner and this 1.1 kg difference in shear values between genotypes was large enough to make a considerable (perceptible) difference to the consumer. These results were in stark contrast with a previous study that failed to show an association of this SNP with direct or indirect measures of tenderness on d 3 or d 14 of ageing (White, 2020).

The *capn1_187* and *capn1_4751* SNPs were candidates for extended effects on tenderness throughout ageing in this study. They were slightly less effective than *capn1_184* in determining the WBSF, at least as effective for the MFL and had more pronounced effects on calpain protease activities. The *capn1_4751* SNP was also linked to downstream SNPs that caused at least similar responses in tenderization. The *capn1-4751* SNP was

the only one of the SNPs studied here that was identified as a causal SNP in QTL for shear force (Barendse, 2011; McClure *et al.*, 2012), and has been consistently included in research papers since its discovery in 2005 (White *et al.*, 2005) and is included in most of the high-throughput arrays and commercial chips.

Although *capn1_187* was not identified as a candidate for selection for tenderness of beef (Bolormaa *et al.*, 2013b; Tizioto *et al.*, 2013), *capn1_4751* associated with several measures of tenderness in many studies. The *capn1-187* SNP was located within the range of QTL for shear force, but not included as a genotype in these research articles (Barendse, 2011; McClure *et al.*, 2012; Lee *et al.*, 2014). The large response in tenderization during early ageing of these SNPs was the ideal pattern of association, increasing tenderness rapidly within the first days of ageing, even though a diminishing effect of the genotype on the tenderness was noted in later ageing (Mazzucco *et al.*, 2010; Gruber *et al.*, 2011). The association of the *capn1-187* SNP with tenderness phenotypes could arise from its strong LD with the causal SNP *capn1-4751*, through association in inheritance rather than an effect on the calpain-1 large subunit protein itself. The *capn1_4751* SNP has been shown to exhibit very low tender allelic frequencies in some Brahman (Smith *et al.*, 2009), and both *capn1_4751* and *capn1_187* showed only 10% tender alleles in the Brahman breed, where the effect of the SNP remained tenderizing.

The present results agreed with that of previous studies (White, 2020), namely, that there were no significant interactions between breeds and genotype effects, although the phenotypes themselves were subject to breed effects. This meant that although there were differences between breeds for allelic frequencies, the associations of these SNPs with phenotypes remained intact within the different breeds and the SNPs could be used across different populations (White, 2020), as long as they remained polymorphic. This absence of breed effects could be the result of the conservation of these critical genes in evolution, because they perform a fundamental function as modulator proteins maintaining normal cellular function (Ono & Sorimachi, 2012; Spinozzi *et al.*, 2021). Because the six SNPs identified here could potentially increase tenderness in beef that was aged for as little as three days and up to three weeks, they could be effective for selection across different supply chains. Not only would the tenderness of rapidly tenderizing steaks be improved, but selection for these SNPs can improve the rate of tenderization of slow-ageing beef and across a variety of breed types. Although a larger dataset would be needed to confirm the results of these associations with tenderness, the increase in myofibril fragmentation and enhanced protease enzyme degradation associated with these SNPs made them good candidates for inclusion in custom selection arrays, in order to improve tenderization. It is, however, difficult to draw any firm conclusions from the relatively small number of animals genotyped here and detailed phenotypic data from a large number of animals subjected to high-density genotyping or sequencing (Ono & Sorimachi, 2012; Spinozzi *et al.*, 2021) would be required to confirm these observations. Brahman-favourable allelic frequency was consistently below the average for all four genes, but particularly in *capn1,* where it was only 15.8% (vs. the average 50.8% tender alleles in *capn1*).

4.5. Conclusion

One possible way of decreasing the effective ageing period is through genomic selection for SNPs that (ideally) accelerate ageing over extended periods of time. Because the SNPs of the calpain system are responsible for coding the most important proteases of tenderization and their inhibitor, these genes are likely targets for selection. However, the duration of ageing can alter genomic associations over time. Some of the SNPs in the muscle calpain system sustained genomic associations with tenderness throughout extended ageing periods, while some individual genotypes also responded differently to ageing, altering these associations over time. We found that ageing was accelerated in some tough genotypes in the *cast* and *capn1* genes, compared to genotypes that contained the tender alleles, progressively eroding genotype differences as the ageing was extended.

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Chapter 5: Quantitative trait nucleotides of the muscle calpain system genes for myofibril fragmentation, calpastatin inhibition of proteases and energy metabolites in South African beef bulls

This thesis chapter will be prepared for publication in a peer-reviewed scientific journal.

Quantitative trait nucleotides of the muscle calpain system genes for myofibril fragmentation, calpastatin inhibition of proteases and energy metabolites in South African beef bulls

Abstract

Very few genomic association analyses are available for South African beef cattle, especially for genes of the proteins of the calpain-calpastatin system that are the most important physiological determinants of beef tenderization. Electrical stimulation is a common post-mortem intervention to accelerate the progression of rigor and advance the onset of tenderization. We therefore determined the association of genetic markers in the *capn1*, *capn2*, *capn3* and *cast* genes with several growth, carcass and beef quality traits during meat ageing for up to 20 days, in five South African beef breeds, using 134 variants. Electrical stimulation failed to negate the genetic effects on beef traits and many important candidate single nucleotide polymorphisms (SNPs) maintained their high ranking among markers that determine these traits. These genes were associated with physiological processes that affect tenderization; intermediary metabolic (energy) metabolites, calpastatin inhibition of proteases and myofibril fragment length (MFL), rather than Warner-Bratzler shear force. Some quantitative trait nucleotides (QTNs) in the *cast* gene are associated with MFL at different ageing periods (*7_761*-rs136056291 and *7_780*-rs135279064), while pleiotropic effects of other QTNs included association with calpastatin protease inhibition over ageing periods (*7_670*-rs109677393 and *7_764*-rs109102936), plus water-holding capacity (*10_727*-rs109474612). The QTN *7_350*-rs41255587 for MFL, exhibited associations with energy metabolites, with a novel QTN for multiple energy metabolic traits in the *capn2* gene (*16_763*-rs137662975). Associations of SNPs with glycogen content, creatine phosphate and ATP concentrations were linked to QTNs for tenderness in the *cast* and *capn2* genes. This suggests that the glycolytic processes that drive proteolytic degradation of myofibrils, are under control or closely linked to genetic markers of these genes.

Keywords: calpain-calpastatin system genes; quantitative trait nucleotides; regional genomic association; South African beef; tenderness; energy metabolism

5.1. Introduction

Electrical stimulation of beef carcasses is commonly applied in South African abattoirs as a tenderizing intervention to accelerate the depletion of creatine phosphate (CrP) and adenosine triphosphate (ATP) in muscle, in order to advance rigor (Carse, 1973; Chrystall & Hagyard, 1976; Hopkins & Thompson, 2001a). It was initially used to prevent the shortening of sarcomeres during rapid chilling of small sheep carcasses (Davey *et al.*, 1976). The rapid depletion of ATP accelerates the glycolytic processes and pH decline, as well as the release of Ca^{2+} into the sarcoplasm (Hopkins & Thompson, 2001b), which accelerates the proteolytic processes of tenderization (Strydom *et al.*, 2005; Simmons *et al.*, 2008). Calpain activity is sustained during optimal chilling conditions, although excessive chilling can attenuate glycolytic and proteolytic activities preventing the development of the mechanisms of pH decline and Ca^{2+} release, resulting in poor proteolytic tenderization (Dransfield, 1994a). Conversely, elevated carcass temperatures can lead to excessive pH decline, protein

denaturation and premature deactivation of calpain protease activity, resulting in tougher beef (Kim *et al.*, 2012; Strydom & Rosenvold, 2014). This tenderizing effect of the proteases is established by the balance between the enzymatic degradation of myofibrillar proteins by calpain-1 and calpain-2 and the protease inhibitory actions of calpastatin (Koohmaraie *et al.*, 1987; Koohmaraie, 1992), which is improved by the application of electrical stimulation (Rhee & Kim, 2001). Effective application of electrical stimulation (Frylinck *et al.*, 2015) can improve the tenderness of carcasses that are compromised by older age (Pike *et al.*, 1993), indicine breed type (Dransfield, 1994b; Ferguson *et al.*, 2000; Gursansky *et al.*, 2010) and β-adrenergic agonist supplementation in the feed (Hope-Jones *et al.*, 2010) and decreases the variation in tenderness between carcasses (Geesink *et al.*, 2001; Barbut, 2014). These effects are however transient (Dransfield *et al.*, 1992; Strydom *et al.*, 2005), but the early acceleration of ageing can increase the turnover of beef through cold-storage facilities, decreasing costs and lowering the environmental impact of beef production (Warner *et al.*, 2010).

The procedures of post-mortem carcass handling need to be controlled carefully to balance the timing, level and duration of electrical stimulation with the carcass temperature and pH decline (Takahashi *et al.*, 1984). This will ensure the best outcome for facilitating enzymatic proteolysis (Ducastaing *et al.*, 1985) and maintain the fluid balance (Hwang & Thompson, 2001a) of the muscle fibres in the processes that control the conversion of muscle to meat. Environmental factors such as nutritional status and stress (susceptibility) of cattle and carcass handling (electrical stimulation and chilling protocols) may have profound effects on intermediary energy metabolism in the sarcoplasm (Rhee & Kim, 2001), affecting the pH, tenderness, water-holding capacity (WHC) and colour of beef (Hwang & Thompson, 2001a; Savell *et al.*, 2005). The temperature of the carcass should not exceed 35°C at the onset of rigor (at pH 6.0) to prevent heat-toughening (Dransfield, 1993; Simmons *et al.*, 1996; Strydom & Rosenvold, 2014), or decrease below 10°C at full rigor, to prevent cold-shortening (Davey *et al.*, 1976; Thompson, 2002). For individual carcasses of similar size, the thermal inertia resulting from the insulatory properties of intramuscular and subcutaneous fat can modify the response of carcass temperature to chilling, where the larger, often marbled carcasses of improved breeds of cattle are more susceptible to high rigor temperatures (Hope-Jones & Strydom, 2021), while the smaller leaner carcasses of tropically adapted breeds (Shackelford *et al.*, 1991; Wright *et al.*, 2018) are more prone to cold-shortening (Ferguson *et al.*, 2000; Gursansky *et al.*, 2010). Electrical stimulation may increase carcass temperature and can sometimes result in structural disruption in muscle fibres (Uytterhaegen *et al.*, 1992) that further accelerate the release of Ca^{2+}, protease activities (Hwang & Thompson, 2001b; Hwang *et al.*, 2003) and tenderization (Judge *et al.*, 1980; Mikołajczak *et al.*, 2019) and decrease differences between carcasses from different breeds (Ferguson *et al.*, 2000). However, improper control of electrical stimulation risks accelerating the autolytic inactivation of enzymatic proteolysis through pH and temperature effects and can compromise tenderization during ageing by decreasing the degradation of myofibrillar proteins (Dransfield, 1993; Geesink *et al.*, 1994). The disruption of the structural elements of muscle fibres compromises the intracellular fluid spaces that increase carcass fluid loss (Savell *et al.*, 1978a), decrease the WHC of meat (Strydom *et al.*, 2000; Frylinck & Heinze, 2003) and may increase cooking losses (Uytterhaegen *et al.*, 1992). These carcass mass and purge losses constitute a loss of meat yield (lower profit), while increased cooking losses (Savell *et al.*, 1978b; Eikelenboom *et al.*, 1985) can

compromise the juiciness of beef (Bouton *et al.*, 1980), delivering a poor quality product to the consumer (poor eating experience).

Tenderness is a quantitative trait with several quantitative trait loci (QTL) across several genes (McClure *et al.*, 2012), while individual single nucleotide polymorphisms (SNPs) in the *capn1* or *cast* genes can explain up to 20% of the genetic variation in tenderness (Johnston & Graser, 2010; Giusti *et al.*, 2013; Calvo *et al.*, 2014). Haplotypes can be more effective to identify associations with beef traits in comparison to individual SNPs (McClure *et al.*, 2012; Dunner *et al.*, 2013; Tait Jr. *et al.*, 2014), explaining more of the variation in quantitative traits (Stone *et al.*, 2005) such as tenderness (Barendse, 2011). The strong linkage disequilibrium in the *cast* and *capn2* gene of these bulls has previously been found to result in overlapping haplotype block structures, allowing for selection of candidate SNPs across different breeds (Chapter 3). Selection for SNPs in these genes would not only improve beef quality traits such as tenderness, but have been found to decrease the variation in tenderness between carcasses (Eggen & Hocquette, 2003; Schenkel *et al.*, 2006; Gruber *et al.*, 2011; Picard *et al.*, 2015) and accelerate the rate of ageing (Chang *et al.*, 2014), which are important challenges in beef production (Koohmaraie *et al.*, 2003). However, genomic selection has found limited application in the beef industry compared to dairy (Taylor *et al.*, 2012; Georges *et al.*, 2019), likely due to the practise of terminal cross-breeding in beef cattle and the focus on cost-effective commercial interventions that establish large improvements in beef quality. As environmental factors contribute so much to beef quality traits, accurate phenomic data is an important requirement for accurate association analyses (Burrow *et al.*, 2001). The heritability of meat quality traits is generally moderate to low (Minick *et al.*, 2004; Smith *et al.*, 2007), and it is important to minimize the effects of environmental variation on beef phenotypes (Warner *et al.*, 2010).

The variants included in the commercial Illumina® BovineHD (777K) SNP BeadChip were initially selected using a diverse group of *Bovidae*, including crossbred cattle breeds, indicine and African taurine cattle (Illumina® Data Sheet, 2015). A shorter distance between adjacent SNPs compared to other commercial arrays could improve the accuracy of multi-breed association analyses (Van Raden *et al.*, 2009; Lund *et al.*, 2014; Pérez O'Brien *et al.*, 2014; Van Den Berg *et al.*, 2016) and estimates of linkage disequilibrium (De Roos *et al.*, 2008; Daetwyler *et al.*, 2014). Limited research has been published on genetic associations between calpain system genes and beef quality traits in South African beef cattle (see Chapter 4) (Frylinck *et al.*, 2009), with no assessment of accurate, detailed beef quality traits in electrically stimulated *vs*. non-stimulated beef carcasses. The aim of this study was to identify the most important SNPs that determine detailed growth, carcass and beef quality traits in the *capn1*, *capn2*, *capn3* and *cast* genes in five South African beef cattle breeds and whether the effects of genotypes were altered by the application of controlled electrical stimulation, compared to non-electrically stimulated beef (control).

5.2. Materials and methods

5.2.1. Animals

All bulls (n = 166) were progeny of registered, purebred sires (sourced from several reputable breeders) and finished in intensive, feedlot conditions at the Agricultural Research Council (ARC), Animal Production Unit. Three international breeds and two local breeds that are commonly used for terminal crossbreeding in

commercial beef production in South Africa were chosen to represent *Bos taurus* (Angus, n = 27; Charolais, n = 34), *Bos indicus* (Brahman, n = 35), indigenous composite (Bonsmara, n = 35) and indigenous Sanga cattle (Nguni, n = 35). Bulls were finished on a typical high grain diet for approximately 120 days in the feedlot facilities of the ARC. At approximately 12 months of age, bulls were transported 4 km to the ARC abattoir holding pens, with free access to water overnight. Bulls were slaughtered using captive bolt stunning and exsanguination, producing carcasses that were classified as A2 or A3 (zero incisors, lean to medium fatness) (Anonymous, 1990, 2015). These procedures and protocols were approved by the Ethics Committee of the Animal Production Institute of the Agricultural Research Council (ARC AEC-I 2010 001) and the Animal Ethics Committee of the University of Pretoria (EC171114-161).

5.2.2. Phenotypes

Phenotypes for growth and carcass traits were collected, with determination of detailed beef quality traits over four meat ageing periods up to 20 days post-mortem, while minimizing environmental variation (using the same feedlot facilities, abattoir and laboratories) for all the data points. Phenotypes that estimated growth traits were calculated from body weights recorded in the feedlot for the average daily gains (ADG) for the last 63 days or 35 days and body weight gain during finishing. Bulls were weighed on the day prior to slaughter, to determine the final liveweight measurement (BW). Carcass trait measurements included the hot carcass weight (HCW), cold carcass weight (CCW) and eye muscle area (EMA) from tracings of the cut surface of the loin at the tenth rib (T10), while carcass mass loss percentage was calculated from the difference between the HCW and CCW (Addendum C, Table S1).

Electrical stimulation of the right half of each carcass (ES) occurred within 30 minutes of stunning, for 15 seconds at 500 V peak, using 5 ms pulses at 15 pulses per second, after which carcass halves were moved into a chiller at 4 ± 2°C. The left half of the carcasses was not electrical stimulated (NS) and accelerated conditioning was applied for 6 hours at 10°C, to reach carcass temperatures of 20°C, before overnight chilling at 4 ± 2°C. Concurrently, data was collected from the *Longissimus lumborum* muscle of the carcass halves, including pH and temperature, using a digital handheld meat pH meter (CyberScan pH II pH/mV/°C meter) fitted with a polypropylene spear-type gel electrode (Thermo Fisher Scientific, Waltham, Ma, USA). Small meat samples were collected from the region of the sixth lumbar vertebra (L6) and snap-frozen in liquid nitrogen at −196°C, followed by immediate storage at −80°C until assay, for the determination of energy metabolites (1 h, 3 h, 6 h post-mortem) and muscle calpain system proteases (calpain-1 and calpain-2) and their inhibitor calpastatin (1 h post-mortem).

At 20 h post-mortem, chilled *Longissimus lumborum et thoracis* muscles were boned-out from each of the carcass halves, from the lumbar region (L6) up to the thoracic region (T10) for the final collection of samples for analyses of intermediary metabolites (20 h), calpain system proteins (20 h), muscle composition (proximate analyses) and collagen content and solubility. Four 30 mm steaks were collected from predetermined sections of the *Longissimus* muscle for each of the ageing periods up to 20 days post-mortem (d 3, d 9, d 14 and d 20). These steaks were aged in vacuum bags (70 microns, 2 ± 1°C) for the determination of meat drip loss (purge),

cooking loss, water-holding capacity (WHC), Warner-Bratzler shear force (WBSF) and myofibril fragment length (MFL) at all ageing periods and sarcomere lengths (SL) on d 3 post-mortem.

Steaks for the determination of WBSF were frozen at –20°C until laboratory cooking and shear force analysis and were thawed at 4 ± 1°C for 24 h before being cooked by oven-broiling (Mielé, model H217, Mielé & Cie, Gütersloh, Germany) using direct radiant heat (AMSA, 1995, 2016). Ovens were pre-set to 200°C and steaks were cooked to an internal temperature of 71 ± 1°C at the geometric centre of each steak, determined with a thermocouple device (Comark C9003, Comark Instruments, Northampton, UK). Steaks were cooled to an ambient core temperature (18°C) before six round cores of 12.7 mm diameter were collected from each steak with a handheld cork borer, parallel to the muscle fibre direction (at an angle of approximately 45° in these loin steaks). Cores were sheared through the centre, perpendicular to the long axis of the muscle fibres with a Warner-Bratzler shear device (AMSA, 2016), with a V-notched blade (60° angle). Crosshead speed was 200 mm/min and the peak force (kg) for each core was determined using a Universal Instron apparatus (Instron Ltd, Buckinghamshire, England). The mean value of the load at maximum (kg) was used in statistical analysis, after elimination of outliers.

Video Image Analysis (VIA) using an Olympus System microscope, model BX40 (Tokyo, Japan) equipped with CC12 video camera was used to determine MFL, SL, EMA and WHC, where imaging data was processed with the AnalySIS Life Science software package (Soft Imaging Systems Gmbh, Münster, Germany). Myofibrils were extracted according to Culler *et al*. (1978), as modified by Heinze & Brüggemann (1994) and the mean of 100 fragments per sample was used in data analyses. The SL were determined according to the method of Dreyer *et al*. (1979), using a final value that was calculated as the mean of 50 lengths. The WHC or the amount of water expressible by applying an external pressure force (Jauregui *et al.*, 1981), was determined by calculating the ratio of meat area and liquid area after applying constant pressure of 300 psi for 60 seconds to a 400 – 600 mg fresh meat sample on filter paper (Irie *et al.*, 1996).

The calpains (calpain-1 and calpain-2 proteases) and calpastatin (protease inhibitor) were extracted from 5 g samples (Dransfield, 1996) and separated by two-step gradient ion exchange chromatography (Geesink & Koohmaraie, 1999). The calpain assay used azo-casein as substrate to eliminate background absorbance of non-specific proteins in the extracts (Dransfield, 1996). Activity of the proteins per grams of muscle tissue was defined as one unit of calpain activity, being equivalent to an increase in absorbance of 1.0 per hour at 366 nm (A_{366}) at 25°C. One unit of calpastatin activity was equivalent to the amount of calpastatin that inhibited one unit of calpain-2 activity. The total enzyme or inhibitor activity was expressed as units (U) per grams of meat, while relative inhibition of calpastatin per units of calpain-1, or per the combined protease activities of calpain-1 and calpain-2 was also calculated.

Muscle intermediary metabolite concentration was determined enzymatically by amyloglucosidase (AGS) hydrolysis of glycogen (Dalrymple & Hamm, 1973) and spectrophotometric analysis. Muscle energy parameters included glycogen content (glycosyl units/g), glucose, glucose 6-phosphate (G6P), lactate, creatine phosphate (CrP) and adenosine triphosphate (ATP) concentrations (µmol/g). Glycolytic potential was calculated based on the observations that C-6 substrates or intermediates can yield two rounds of glycolysis (2×pyruvate) and C-3

intermediates one round. Glycogen, G6P and glucose concentrations were multiplied by two and added to the lactate concentration, to give the glycolytic potential (µmol/g).

Collagen content and solubility were determined according to the method originally described by Neuman & Milan (1950) and adapted by Bergman & Loxley (1963), Hill (1966) and Weber (1973), based on the oxidation of hydroxyproline. The composition of muscle tissue was analysed using the standard methods of the Association of Official Analytical Chemists International (AOAC, Rockville, USA) (AOAC, 2019). Freeze-dried samples were used for the determination of the percentages of dry matter (DM), moisture, ash, crude protein (CP) and intramuscular fat (IMF).

5.2.3. Genotypes

Genomic DNA was extracted from hair and muscle tissue samples collected at slaughter using the NucleoSpin® Tissue kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). All bulls were individually genotyped using the BovineHD SNP BeadChip (777K variants) and analysed using Genome Studio Software Version 2.0.4 (Illumina®, San Diego, CA, USA). Illumina SNP names were shortened by using the chromosome number, followed by an underscore and the last three digits of the name (Addendum C, Table S2), for example BovineHD0700028721 = *7_721*. The *CAPN1_1* and *CAPN1_2* SNPs were coded for their positions, *29_316* and *29_4751* respectively. The alleles or genotypes that were favourable for tenderness are indicated with an asterisk (*) and determined from phenotypic means for WBSF, MFL or calpain system proteins.

Haplotype analyses and favourable allelic frequencies were reported previously (Chapter 3). Haplotypes were numbered from H1, the haplotype with the largest number of tender alleles to haplotypes with increasing number of "tough" alleles (Addendum C, Table S3). Due to the relatively small sample group that was used to accommodate the cost of detailed phenotypic analyses and higher definition genotyping, many of the haplotypes including homozygotes (especially for the unfavourable alleles in *capn3* and *cast*) included too few bulls (≤10%) for meaningful discussion of haplotype effects. The effects of the individual genetic markers (SNPs) will therefore be discussed within these linkage blocks.

5.2.4. Data analyses

The PLINK version 1.9 software program (Purcell *et al.*, 2007) was used to perform quality control of SNPs of the *capn1*, *capn2*, *capn3* and *cast* genes (134 SNPs), where four SNPs failed to reach a minor allelic frequency of 5% and one SNP failed Hardy-Weinberg exact test ($P_{HWdev} \leq 1\times10^{-4}$). The PLINK software was used to perform simple least squares associations, within breeds and within treatment groups (NS and ES), to provisionally validate the use of the Illumina® BovineHD SNP BeadChip for use in South African beef. Analysis of the data using the mixed linear model-based association or MLMA function (Yang *et al.*, 2014) of the Genome-wide Complex Trait Analysis (GCTA) 1.92.2 software (Yang *et al.*, 2011) was conducted in the pooled data, within breed and within treatment groups and used to test the interaction of genotypes with the ES treatment (G×E). This model (Chapter 4, Section 4.2.6) incorporated random effects of all SNPs from a variance-standardised genomic relationship matrix that prevents false positive associations. Within the treatment groups, breed was incorporated as a covariate in the association analyses, but similar to the analyses in PLINK, failed to provide

accurate power of association (Müller *et al.*, 2017) or correction for false negative associations. More detail on these preliminary association analyses, as well as the more complex associations described below, is supplied in Addendum A, Section 6.

The challenge of eliminating false positive and identifying false negative associations (Kaler *et al.*, 2020) was addressed by Fixed and random model Circulating Probability Unification (FarmCPU) software that fits fixed and random models to the data, iteratively, to determine associations (Liu *et al.*, 2016). These analyses generate an optimised *p*-value by permuting each phenotype to break its associations with genotypes. These optimised *p*-values (α=0.01) were used to adjust *p*-values for all the data models, with these *p*-values considered a significant association (candidate SNPs), while putative effects were considered at α=0.05 (probative SNP). The mean optimal *p*-value for all phenotypes was determined as $p \leq 0.0013$, which was similar to the level of adjustment for putative effects in adjusted Bonferroni correction (α=0.05, *p*-value = 0.10 ÷ 83 or the number of independent SNPs tested, *p*-threshold = 0.0012). However, this adjustment would only identify 53% of the optimised *p*-values for individual phenotypes and blanket adjustments for all phenotypes were abandoned in favour of the individual optimised *p*-values for each trait (for all software and models used). Association analyses were performed on the data for the NS (control) and the ES treatment groups separately, using the FarmCPU software, while incorporating the first four principal components from the genomic relationship matrix of the 777K genotypes, as covariates in the model.

In addition to these analyses, the Genome Association and Prediction Integrated Tools (GAPIT) software package using R software (Wang & Zhang, 2021) was used to analyse the data, incorporating the best-fit for the first four principal components of the genomic relationship matrix (777K), in Mixed Linear Model (MLM) (Yu *et al.*, 2006), the Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway (BLINK) (Huang *et al.*, 2018) and Multi-Locus Mixed Model (MLMM) (Segura *et al.*, 2012) association analyses. The quantile-quantile (QQ) plots were used to determine the model that best fit the association of individual phenotypes with SNPs, between FarmCPU, MLM, BLINK and MLMM, while estimated allele effects were recorded from FarmCPU or MLM, when these models included the SNP as an important genetic marker affecting the trait. At the time of analyses BLINK and MLMM models in GAPIT did not estimate allele effects, but supplied quantitative trait nucleotides (QTNs) and associated *p*-values. For all the models, QTNs were identified and the most notable associations of nearby SNPs that fell within the putative range of the optimized *p*-value were discussed. Several allele effects on metabolites were identified, which could be confounded by an interaction between calpain system function and pH changes. These associations were repeated with ultimate pH (pH_u) as an additional covariate, to determine whether the associations were confounded by pH_u. The addition of pH_u to the models had little or no effect on the *p*-values or the size of allele effects on metabolites.

5.3. Results and discussion

*5.3.1. The calpastatin gene (*cast*)*

These data are supplemented by the characterization of the muscle calpain system genes that described minor– and "tender" alleles, linkage and linkage disequilibrium block structure of the pooled data and of

individual cattle breeds (see Chapter 3). The *cast* gene exhibited strong linkage between loci (D' = 0.84; r^2 = 0.48) and extensive linkage blocks with a great degree of overlap between different breeds (Addendum B, Table S5)

5.3.1.1. Single nucleotide polymorphisms in haplotype block 1 (SNPs in cast*-B1,* 7_721 – 7_731*)*

The *cast* SNP, *7_729* in the upstream region of the gene was identified as a QTN for body weight gain during finishing (Table 5.1). This region of the gene was found to contain a high level of linkage disequilibrium (LD), with a haplotype block in the 5'-UTR extending between SNPs *7_721* to *7_731* (Addendum B, Table S5). The tender genotypes of the calpain-calpastatin system have sometimes been linked to negative associations with growth traits (King *et al.*, 2012; Gomes *et al.*, 2013) and it is often assumed that the greater level of proteolysis induced by tender alleles that favour beef quality, can have a negative effect on protein synthesis *in situ* and therefore also carcass size and meat yield. This region of the gene (H1, *7_721 – 7_731*) was previously found to associate with growth phenotypes such as eye muscle area (EMA) (Tizioto *et al.*, 2013; Royer *et al.*, 2016), BW and weaning weight (Royer *et al.*, 2016; Ardicli *et al.*, 2017, 2019), while other researchers have failed to identify a link with growth or meat yield (Xia *et al.*, 2016), even using higher density arrays (Crispim *et al.*, 2015; Espigolan *et al.*, 2015; Fernandes Jr. *et al.*, 2016; Seabury *et al.*, 2017). The positive association of weight gain with more tender genotypes found here, confirms an increase in body weight and feed conversion ratio that was observed for SNPs in this region previously (Ardicli *et al.*, 2017, 2019), although these authors did not include tenderness phenotypes to determine a possible negative interaction with beef quality traits. However, increased growth has been found to have a positive association with tenderization in pork and increased calpain-1 protease activity that suggests selection for growth does not decrease tenderization (Kristensen *et al.*, 2002).

5.3.1.2. SNPs in cast*-B2 (*7_732 – 7_738*)*

The second haplotype block (B2; seven SNPs) was part of overlapping blocks in all breeds, except Charolais, where the last three SNPs were not included in the block (Addendum B, Table S5). Although no QTNs were identified in this haplotype block (Table 5.1), the SNP *7_736* was identified as a candidate for WBSF on d 9 post-mortem in the ES group. This SNP was previously identified as a candidate for selection for tenderness at different ageing periods (Chapter 4) (White, 2020). The SNP was also identified as the most likely candidate for WBSF d 9 in the NS group, but failed to reach statistical significance ($p \leq 0.0031$). Differences in post-mortem practices (ageing period and electrical stimulation) could explain why the association with tenderness was not consistently observed (Tizioto *et al.*, 2013; Ramayo-Caldas *et al.*, 2016). The effect of the *7_736* SNP on WBSF can be explained by a putative association with G6P concentration at 20 h post-mortem, where the 1.48 µmol/g increase represents a 23.4% increase of the mean G6P of 6.32 µmol/g. The concentration of G6P was demonstrated to be a biomarker of tenderness (King *et al.*, 2019) and increased glucose metabolites such as G6P have been linked to tenderization, through enhanced glycogenolysis, glycolysis and lactate build-up, accelerated pH decline and cytosolic Ca^{2+} release (Ouali *et al.*, 2013; Matarneh *et al.*, 2017, 2021). Furthermore, decreased *cast* gene expression in pork was linked to greater glycolytic potential (Sieczkowska *et al.*, 2010) that will facilitate tenderization of meat, while *cast* SNPs affected energy metabolic pathways of *Bos indicus* cattle in proteomic research (Rosa *et al.*, 2018).

Table 5.1. *Quantitative trait nucleotides and candidate SNPs in the* cast *gene for growth, carcass and beef quality traits in control (NS) and electrically stimulated beef (ES) of bulls from five South African breeds, genotyped using the Illumina® BovineHD SNP BeadChip (777K).*

cast	Trait	Tmt	Model	*p*-value	Effect of tender genotype
***cast*-B1 (32.1 kb, *7_721 – 7_731*)**					
7_729	BW gain	NS	BLINK	**9.43×10^{-5}**	**+13.85 kg#**
***cast*-B2 (9.8 kb, *7_732 – 7_738*)**					
7_736	WBSF (d 9)	ES	MLMM	0.0024	–0.41 kg#
	glucose 6-phosphate (20 h)	ES	FarmCPU	(0.0028)	+1.48 µmol/g
***cast*-B3 (7.7 kb, *7_739 – 7_742*)**					
7_740	carcass mass loss percentage	ES	FarmCPU	**1.28×10^{-5}**	**–0.24%**
7_349	BW (kg)	NS	FarmCPU	0.0013	18.0 kg
	HCW (kg)	NS	FarmCPU	**1.62×10^{-4}**	**12.6 kg**
	CCW (kg)	NS	FarmCPU	**1.81×10^{-4}**	**12.2 kg**
***cast*-B4 (42.1 kb, *7_743 – 7_670*)**					
7_747*	calpastatin per calpain-1 (20 h)	NS	FarmCPU	0.0030	–0.13
	MFL (d 14)	ES	BLINK	(0.0026)	–3.29 µm#
7_748	calpastatin (20 h)	ES	MLMM	**5.66×0^{-6}**	**–0.60 U/g#**
7_754	MFL (d 14)	ES	BLINK	0.0009	–2.58 µm#
7_757	MFL (d 14)	ES	BLINK	0.0026	–3.29 µm#
7_759	MFL (d 14)	ES	MLMM	0.0026	–3.72 µm#
7_761	MFL (d 9)	NS	FarmCPU	**1.51×10^{-5}**	**–13.04 µm**
	MFL (d 14)	NS	FarmCPU	**3.63×10^{-11}**	**–19.05 µm**
	MFL (d 14)	ES	FarmCPU	**3.62×10^{-7}**	**–16.14 µm**
7_670	calpastatin per calpains (1 h)	NS	FarmCPU	0.0003	–0.20
	MFL (d 3)	NS	FarmCPU	**6.69×10^{-6}**	**–7.69 µm**
	MFL (d 3)	ES	FarmCPU	**7.60×10^{-6}**	**–6.75 µm**
	MFL (d 9)	ES	FarmCPU	**1.18×10^{-6}**	**–7.74 µm**
	MFL (d 20)	NS	FarmCPU	**2.93×10^{-6}**	**–3.37 µm**
Independent SNPs (*7_763*, *7_764* and *7_765*)					
7_764	calpastatin (1 h)	NS	MLMM	**2.51×10^{-6}**	**–0.32 U/g**
	MFL (d 9)	ES	MLMM	**9.42×10^{-5}**	**–5.81 µm**
	MFL (d 14)	NS	MLMM	**4.05×10^{-5}**	**–2.91 µm**
	MFL (d 20)	NS	MLMM	**5.69×10^{-5}**	**–2.74 µm**
7_765	calpastatin per calpains (1 h)	NS	MLMM	**3.71×10^{-4}**	**–0.13**
	MFL (d 14)	ES	BLINK	**8.57×10^{-6}**	**–5.00 µm**

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cast	Trait	Tmt	Model	*p*-value	Effect of tender genotype
***cast*-B5 (53.0 kb, *7_766*– *7_794*)**					
7_766*	MFL (d 14)	NS	MLM	0.0013	–7.69 µm
7_769	MFL (d 14)	ES	MLMM	0.0006	–2.83 µm#
7_773	ATP (1 h)	NS	MLMM	**3.49×10^{-4}**	**–0.10 µmol/g#**
7_774	calpastatin (20 h)	ES	MLMM	**6.70×10^{-5}**	**–0.31 U/g#**
7_777	creatine phosphate (6 h)	NS	FarmCPU	**9.84×10^{-5}**	**–0.76 µmol/g**
7_116	lactate (3 h)	ES	FarmCPU	(0.0023)	+7.42 µmol
7_778	MFL (d 14)	ES	MLMM	**1.63×10^{-4}**	**–1.33 µm#**
7_780	calpastatin (20 h)	ES	MLMM	(0.0012)	–0.27 U/g#
	MFL (d 14)	ES	MLMM	**1.77×10^{-8}**	**–5.35 µm#**
	MFL (d 20)	ES	MLMM	**3.01×10^{-5}**	**–3.06 µm#**
7_350	MFL (d 14)	NS	BLINK	0.0004	–2.07 µm#
	MFL (d 20)	ES	BLINK	**5.52×10^{-7}**	**–3.17 µm#**
	glucose (6 h)	NS	MLMM	0.0022	+0.19 µmol/g#
	lactate (6 h)	NS	BLINK	0.0008	+2.12 µmol/g#
7_793	glucose 6-phosphate (20 h)	ES	FarmCPU	(0.0030)	+1.31 µmol/g
7_794	calpastatin (1 h)	NS	MLMM	0.0007	–0.11 U/g#

cast-B1 – Haplotype block 1 of the *cast* gene; Tmt – treatment group non-electrically stimulated (NS) or electrically stimulated (ES); QTNs are indicated in bold text, while putative SNP effects are indicated in parenthesis.
– allele effects were estimated from MLM in GAPIT; BW – final live body weight; CCW – cold carcass weight; HCW – hot carcass weight; MFL – myofibril fragment length; WBSF – Warner-Bratzler shear force.
*Within B4, SNPs *7_747*, *7_751*, *7_752*, *7_756* and *7_758* were in perfect disequilibrium, while within B5, *7_766* and *7_768* were in perfect disequilibrium.

5.3.1.3. SNPs in cast*-B3* (7_739 – 7_742*)*

The third haplotype block (7.7 kb) included five SNPs that were part of overlapping haplotype blocks in all breeds except Brahman, where one SNP (*7_349*) was an independent SNP. Analysis in FarmCPU identified an association between the marker *7_740* and carcass mass loss percentage in the ES group, where the effect was estimated to be a reduction of 0.24 percentage points (Table 5.1). This is equivalent to an 11.3% reduction from the mean carcass loss of 2.16%, for all bulls. Since this is equivalent to only 25 g per carcass (as HCW), it would not be cost-effective to select for the SNP based on carcass mass loss alone, even though cumulatively small losses in commercial abattoirs can account for a significant loss of profit. However, a SNP 4 kb downstream from *7_740* affected juiciness of beef (Lee *et al.*, 2014) and the role of this region in other fluid losses that could affect consumer eating quality, such as purge or cooking loss, should be investigated further. The biologically small positive effect for carcass mass loss, is however in linkage with more important QTNs that could prove to be cost-effective for selection for meat yield and affect profit margins, through marker *7_349* discussed below.

The SNP *7_349* did not seem to affect quality traits in any of the models, but was linked to body and carcass weights of the control group (NS) in the FarmCPU analyses (Table 5.1). The SNP was identified as a QTN for HCW (12.6 kg) and CCW (12.2 kg) and associated with BW (18.0 kg), as a candidate SNP below the optimised threshold *p*-value. Similarly large weight differences have been observed previously in Brahman-type cattle for birth and weaning weight for the nearby SNP *7_741* (+2.6 kb), but not for carcass weights (Howard *et al.*, 2015). Several studies have failed to identify an association with these SNPs with growth (Crispim *et al.*, 2015; Weng *et al.*, 2016; Bordbar *et al.*, 2019) or carcass traits (Espigolan *et al.*, 2015; Xia *et al.*, 2016; Lopes *et al.*, 2021). Research that identified associations with carcass yield traits and liveweight from nearby SNPs (3.0 – 4.7 kb downstream of *7_349*), were dedicated to growth phenotypes (Royer *et al.*, 2016) and it is unlikely that this SNP is associated with any tenderness phenotypes, as found in our study and others (Bolormaa *et al.*, 2013; Tizioto *et al.*, 2013; Xia *et al.*, 2016), or a range of other beef quality traits for this QTN in our data.

However, this region of the gene has been linked to adaptive traits, such as thermotolerance (Dikmen *et al.*, 2015), feed efficiency (Abo-Ismail *et al.*, 2018) and reproductive traits in dairy cattle (Garcia *et al.*, 2006; Obando, 2017). Beef breeds that are adapted to tropical environments often produce smaller carcasses (Shackelford *et al.*, 1991; Bester *et al.*, 2001; Riley *et al.*, 2003) and a potential link to adaptation rather than the unlikely link to tenderness could explain the associations observed here with weight parameters. This presents an opportunity for future research of adaptive traits in beef.

5.3.1.4. SNPs in cast*-B4 (*7_743 – 7_670*)*

Haplotype block four (*cast*-B4) of the *cast* gene extended 42.1 kb between SNPs *7_743* and *7_670* including 20 SNPs and was overlapping in Angus, Bonsmara and Nguni, but fragmented in Brahman and Charolais (Addendum B, Table S5). This region of the gene extends from 3 kb downstream of exon 3 up to the region of exon 8 and was associated with MFL phenotypes, particularly in the ES treatment group and mainly for MFL on d 14 post-mortem (Table 5.1). This confirms the results of previous studies that found effects on shear force on d 14 of ageing (King *et al.*, 2012; McClure *et al.*, 2012), where tenderization during the first two weeks post-mortem has been found to be dependent mainly on protease activities (De Souza Fonseca *et al.*, 2022). These SNPs also exhibited associations with the absolute inhibition of calpastatin, or the relative inhibition of calpain proteases by calpastatin, and a reduction in inhibition of calpain protease activity, will result in greater myofibril fragmentation (Whipple & Koohmaraie, 1993) that results in more tender beef. This link to proteases explain the more consistent effect on myofibril fragmentation, as the protease activity is allowed a greater opportunity to degrade structural proteins (King *et al.*, 2012) and for ageing to progress effectively, making beef more tender.

The SNP *7_748* was identified as a QTN for an almost 35% reduction in calpastatin inhibition at 20 h post-mortem in the ES treatment group. FarmCPU identified two candidate SNPs for relative calpastatin inhibition in the NS group; *7_747* (2.9 kb upstream of the QTN) as a candidate SNP for calpastatin protease inhibition per calpain-1 protease activity at 20 h and *7_670* (exon 8) for inhibition per combined calpain activity at 1 h post-mortem. This reduction in calpastatin inhibition or increased, sustained calpain protease

degradation, drives the multiple associations with (and QTNs for) myofibril fragmentation identified in this haplotype block (Whipple & Koohmaraie, 1993).

The most likely candidates for selection for sustained MFL of this haplotype block, were the markers *7_761* (just upstream of exon 6) and *7_670* (described above) located 2.4 kb apart. The combined effects of these two QTNs were linked to almost all the MFL measurements (treatment groups and ageing periods), except MFL on d 20 in the ES group where the associations failed to reach statistical significance (*7_761*, $p \leq 0.0031$; *7_670*, $p \leq 0.0107$). Further investigation into a previously identified, possible reduction in ADG as a result of selection for *7_670* for tenderization (King *et al.*, 2012) should be investigated, although the combined effect of anabolic steroid treatment by these researchers could confound these results. Similar to other studies (Crispim *et al.*, 2015; Mateescu *et al.*, 2017; Savoia *et al.*, 2019), we found no evidence of a negative effect of greater tenderization on growth traits in these South African beef bulls ($p \leq 0.4236 - 0.9970$). The ability of these SNPs to sustain relatively large, economically viable effects on MFL (i.e. tenderization), during extended ageing periods up to 20 days post-mortem, regardless of ES, in a region of strong linkage in all five breeds (Addendum B, Table S5), make them ideal candidates for inclusion in arrays for across-breed selection. These data should be confirmed in a larger sample group using sequencing of the surrounding region (Braz *et al.*, 2018), combined with detailed, carefully controlled phenotypic data.

5.3.1.5. Independent cast *SNPs (*7_763*;* 7_764*;* 7_765*)*

The *cast* SNPs *7_764* and *7_765* were not part of haplotype blocks for the pooled data, because they were not informative in Nguni or Bonsmara bulls and were independent SNPs in Brahman bulls. They were however in strong LD in the two *Bos taurus* breeds, where they were also in strong linkage with other SNPs including *7_761* and *7_670* (Addendum B, Table S5) and a similar result between associations of these SNPs was expected. The markers *7_764* and *7_765* demonstrated strong associations with MFL, with QTNs in (especially) the intermediate ageing period (d 9 and d 14) and with calpastatin protease inhibition in the first hour post-mortem (Table 5.1). These QTNs are however limited in their application in South African beef, by being uninformative in Nguni and Bonsmara cattle that compose a large proportion of the South African herd (Ramsay *et al.*, 2006).

5.3.1.6. SNPs in cast*-B5 (*7_766 – 7_794*)*

Haplotype block five constituted a large region of the gene (53 kb), including SNPs downstream of the gene (30 total SNPs) that were continuous in most breeds, except Brahman where a short (five SNPs), separate block was identified in the downstream portion of the sequence transcript (Addendum B, Table S5). The SNPs of the 3'-UTR were some of the first SNPs investigated for their effects on tenderization (Barendse, 2002; Casas *et al.*, 2006).

Three QTNs were identified for MFL on d 14 (*7_778*, *7_780*) and d 20 (*7_780* and *7_350*) and marker *7_780* can be linked to a putative reduction in calpastatin inhibition at 20 h (Table 5.1), facilitating myofibril fragmentation. The QTNs for MFL were all identified in the ES treatment group, while in the NS group, these SNPs all remained within the top ten SNPs that determined myofibril fragmentation (results not shown),

although calpastatin inhibition at 20 h was unaffected in the control ($p \leq 0.2973$). Candidate SNPs for MFL were also identified for MFL on d 14, of both the NS and ES treatment groups for the effects of *7_766* that was in perfect LD with *7_768*. The increase in the power of associations of the ES treatment group compared to control, directly contradicted the common perception that tenderizing interventions such as electrical stimulation and ageing decrease the genetic effect on phenotypes (Ferguson *et al.*, 2000; Gruber *et al.*, 2011).

Two QTNs for instant energy supply in muscle (ATP 1 h and CrP 6 h) of the control group were identified for *7_773* and *7_777* respectively. Rapid depletion of ATP is characteristic of accelerated rigor (Newbold & Harris, 1972), where CrP is normally responsible for replenishing ATP supply through phosphorylation. The continued depletion of CrP observed here, could indicate that the effect was sustained, at least up to 6 h post-mortem. This is further supported by putative associations identified by FarmCPU for elevated lactate at 3 h for *7_116* and continued supply of glucose 6-phosphate at 20 h post-mortem for *7_793*, even in the ES treatment group. This was confirmed by association of *cast* SNPs in pork that correlated with lactate concentration, WHC and drip loss (Krzęcio *et al.*, 2005), establishing the link between greater pH decline or lactate build-up and *cast* SNPs (Reardon *et al.*, 2010; Ropka-Molik *et al.*, 2014; Ardicli *et al.*, 2017). In the control, *7_350* was associated with both glucose and lactate concentrations at 6 h as a candidate marker for selection. Both glucose and glucose 6-phosphate are molecular markers for increased tenderness (King *et al.*, 2012) and can indicate that glycogenolysis is increased, with more (but not excessive) lactate production, accelerated pH fall and Ca^{2+} release, favouring the degradation of myofibrils by calpains (Ouali *et al.*, 2013; Matarneh *et al.*, 2017, 2021).

*5.3.2. The calpain-3 gene (*capn3*)*

The linkage disequilibrium of the *capn3* gene was moderate (D'=0.63; allele association = 0.20) (Chapter 3, Table 3.4) and it had short haplotype block structures (two to four SNPs in length), while almost half of the SNPs were not informative in Brahman (Addendum B, Table S2). Signatures of selection have been identified for Nguni and Bonsmara cattle in all the regions of the haplotype blocks in *capn3* (Kooverjee *et al.*, 2022). The selective changes in the characteristics of the *capn3* gene in Sanga and Sanga-type cattle warrants further investigation, even though no link to temperament (Hulsman Hanna *et al.*, 2014), feed efficiency (Seabury *et al.*, 2017; Martin *et al.*, 2019) or reproductive phenotypes (Hawken *et al.*, 2012; Saatchi *et al.*, 2014; Elzo *et al.*, 2016; Galliou *et al.*, 2020) have been identified in international breeds. However, *capn3* is known for pleotropic effects that extend beyond its protease activity, including energy metabolism (Ono *et al.*, 2016; Jahnke *et al.*, 2020), the activity of the rate-limiting enzyme of glycolysis, phosphofructokinase and Ca^{2+} efflux from the sarcoplasmic reticulum (Ojima *et al.*, 2011). Only a few QTNs were identified with any confidence and SNP *10_727* was a candidate for tenderness traits in these beef cattle (Table 5.2).

5.3.2.1. SNPs in capn3-*B4 (*10_725 – 10_728*)*

Linkage block (B4) exhibited more overlap between breeds than the other haplotype blocks of *capn3* and was the region where QTNs were identified. It could potentially be linked to the tenderness marker *capn3*-T4 that is only 1.5 kb upstream of the linkage block and was used in GeneSTAR grading of beef (Barendse *et al.*, 2008). The genetic marker *10_725* was a QTN for carcass mass loss (Table 5.2) in the ES treatment group

($p \leq 0.0002$), but not in the control ($p \leq 0.8054$). Carcass mass loss was decreased by 0.30 percentage points and, similar to results in the *cast* gene, this constitutes a significant prevention of total mass loss from the carcass during chilling (13.9% lower carcass mass loss), but only a 31 g gain in carcass weight. This SNP was in linkage with another genetic marker, *10_727* that was identified as a pleiotropic QTN, affecting both tenderization (discussed below) and WHC. This SNP was associated with an 11.1% increase in WHC in these data, a trait that has previously been associated with proteolytic activity (Tizioto *et al.*, 2013), but not in *capn3*. This involves the osmotic effects of charged proteins and changes in the cellular fluid spaces that normally hold water in the intact muscle fibre (Huff-Lonergan & Lonergan, 2005). This constitutes a large reduction in fluid loss from carcasses, which is also a significant reduction in the potential loss of profit as meat yield. Electrical stimulation can sometimes increase fluid losses from beef throughout the production chain (Savell *et al.*, 1978a, 1978b; Strydom *et al.*, 2000; Frylinck & Heinze, 2003) and by decreasing these losses, selection for this SNP could provide positive effects through to the end of the beef supply chain, by improving the juiciness of cooked steaks (or eating experience of the consumer). This is consistent with results that linked the GeneSTAR-T4 marker to juiciness and overall liking of streaks by consumer panels at d 7 post-mortem (Robinson *et al.*, 2012). Although our data precludes any definitive link of haplotype block four with this effect, more detailed genotyping in the region upstream of this linkage block should be conducted to confirm whether the region affects the water-holding capacity of beef.

Table 5.2. *Quantitative trait nucleotides and candidate SNPs in the* capn3 *gene for growth, carcass and beef quality traits in control (NS) and electrically stimulated beef (ES) of bulls from five South African breeds, genotyped using the Illumina® BovineHD SNP BeadChip (777K).*

capn3	**Trait**	**Tmt**	**Model**	***p*-value**	**Effect of tender genotype**
***capn3*-B4 (10.8 kb, *10_725 – 10_728*)**					
10_725	carcass mass loss percentage	ES	BLINK	**2.16×10^{-4}**	**–0.30%[#]**
10_727	calpastatin per calpain-1 (20 h)	NS	FarmCPU	**2.45×10^{-4}**	**–0.17**
	MFL (d 9)	NS	FarmCPU	0.0010	–1.98 µm
	MFL (d 14)	ES	FarmCPU	**3.01×10^{-5}**	**–2.34 µm**
	water-holding capacity (d 20)	NS	FarmCPU	**2.05×10^{-4}**	**+0.03**

QTNs are indicated in bold text, while putative SNP effects are indicated in parenthesis; *capn3*-B4 – Haplotype block 4 of the *capn3* gene; Tmt – treatment group non-electrically stimulated (NS) or electrically stimulated (ES).
[#] – allele effects were estimated from MLM in GAPIT; MFL – myofibril fragment length.

This region of the gene has most often been associated with tenderness phenotypes (Barendse *et al.*, 2008; Robinson *et al.*, 2012), although results were not always consistent (Cafe *et al.*, 2010a; Johnston & Graser, 2010). We identified *10_727* as a QTN for MFL on d 14 in the ES treatment group, where association in the NS group failed to reach statistical significance, but was still one of the important SNP affecting the trait ($p \leq 0.0035$). It was also a candidate SNP for MFL on d 9 in the control that remained important, but not significant in the ES group ($p \leq 0.0066$). These effects on myofibril fragmentation can be explained by an association with relative calpastatin inhibition per calpain-1 proteolysis, although this QTN was only important

in the NS treatment group ($p \leq 0.7977$). This confirms the results of previous research that found tenderness on d 14 post-mortem was especially affected by genetic markers in this region (Gruber *et al.*, 2011).

*5.3.3. The calpain-2 gene (*capn2*)*

A high linkage was identified in the *capn2* gene (Chapter 3, Table 3.4), where all loci were associated in Nguni and Bonsmara (one large haplotype block), while linkage blocks were fragmented, but contained almost all SNPs in other breeds (Addendum B, Table S5). This confirms the results of Riggs & Vaughn (2015) that identified an area of extensive linkage of chromosome 16, including the entire *capn2* gene. These authors associated the haplotype block with feed efficiency (Riggs & Vaughn, 2015) and investigation of possible adaptive responses should be investigated. This association of SNPs in the *capn2* gene with feed efficiency was however not confirmed by subsequent research (Seabury *et al.*, 2017; Martin *et al.*, 2019).

5.3.3.1. SNPs in capn2*-B1 (*16_758 – 16_760*)*

Although the SNP *16_758* was identified as a QTN in the BLINK analysis (Table 5.3), this 5% reduction in calpastatin inhibition of proteases is unlikely to affect beef quality traits. A putative effect of the SNP on calpastatin at 20 h was equivalent to an 8.7% reduction in inhibition of proteolysis, being biologically more relevant, but statistically less likely. A small decrease in WBSF on d 20 was putatively identified for the SNP *16_760*, the most important SNP for WBSF on d 20 in the treatment group, but was ranked 73rd in the control ($p \leq 0.5484$). The SNPs of this region were however previously associated with WBSF on d 7 and an association of *16_759* with WHC was identified, linked to processes that involve proteases (Tizioto *et al.*, 2013) that can disrupt the fluid spaces of the myofibrils (Offer & Trinick, 1983; Ertbjerg & Puolanne, 2017). We found no evidence of an association with WHC in any of the treatment groups or ageing periods ($p \geq 0.0660$).

5.3.3.2. SNPs in capn2*-B2 (*16_761 – 16_772*)*

The SNP *16_763* was associated with four measurements of anaerobic energy metabolism in the ES treatment group (Table 5.3). Novel QTNs of the SNP with glycogen concentrations at 1 h and 3 h post-mortem were identified and it was a probative SNP for glycogen concentration at 6 h and glycolytic potential at 1 h post-mortem. The effect of the *16_763* SNP on glycosyl units was a 9.2% increase at 1 h and 7.9% at 6 h, but only 3.1% of the mean concentration at 6 h, while glycolytic potential was 4.4% higher at 1 h. These effects could drive a more rapid fall in sarcoplasmic pH, already accelerated by electrical stimulation, to favour greater myofibril fragmentation (Matarneh *et al.*, 2017), as evidenced by downstream QTNs in the haplotype block. However, these effects of the genetic marker were not limited to the ES treatment group and in the NS group, the marker was consistently ranked first or second most important for glycogen concentrations ($p \leq 0.0129$) and fourth most important SNP for glycolytic potential ($p \leq 0.0187$). Although the physiological link between calpain protease activity and the post-mortem anaerobic metabolic pathway is well-established (Ferguson & Gerrard, 2014), association of SNPs with glycolytic processes have not been established. However, increasing evidence is accumulating about the poorly-understood control mechanisms that connect the genes to the proteins they transcribe and their function at a cellular level.

Table 5.3. *Quantitative trait nucleotides and candidate SNPs in the* capn2 *gene for growth, carcass and beef quality traits in control (NS) and electrically stimulated beef (ES) of bulls from five South African breeds, genotyped using the Illumina® BovineHD SNP BeadChip (777K).*

capn2	**Trait**	**Tmt**	**Model**	***p*-value**	**Effect of tender genotype**
***capn2*-B1 (8.6 kb, *16_758* – *16_760*)**					
16_758	calpastatin per calpains (20 h)	NS	BLINK	**3.00×10^{-4}**	**–0.04#**
	calpastatin (20 h)	NS	BLINK	(0.0034)	–0.14 U/g#
16_760	WBSF (d 20)	ES	BLINK	(0.0024)	–0.30 kg#
***capn2*-B2 (21.9 kb, *16_761* – *16_772*)**					
16_763	glycogen (1 h)	ES	BLINK	**9.32×10^{-5}**	**+2.66 glycosyl U/g#**
	glycogen (3 h)	ES	BLINK	**5.36×10^{-5}**	**+1.98 glycosyl U/g#**
	glycogen (6 h)	ES	MLMM	(0.0024)	+0.53 glycosyl U/g#
	glycolytic potential (1 h)	ES	BLINK	(0.0014)	+4.66 µmol/g#
16_765	MFL (d 14)	ES	FarmCPU	**5.71×10^{-7}**	**–3.41 µm#**
16_769	calpastatin (1 h)	ES	FarmCPU	**3.47×10^{-4}**	**–0.32 U/g#**
	MFL (d 14)	NS	FarmCPU	**9.28×10^{-7}**	**–3.99 µm#**
***capn2*-B3 (11.6 kb, *16_773* and *16_778*)**					
16_776	calpastatin per calpains (1 h)	NS	BLINK	1.47×10^{-4}	–0.05#
	ATP (3 h)	ES	BLINK	3.53×10^{-4}	0.16 µmol/g#
Independent SNPs (*16_780* and *16_781*)					
16_780	carcass mass loss percentage	ES	BLINK	**8.38×10^{-5}**	**0.40%#**
	ATP (1 h)	NS	MLMM	**2.20×10^{-4}**	**–0.34 µmol/g#**
16_781	calpastatin (1 h)	NS	MLMM	0.0011	–0.14 U/g#
	calpastatin per calpains (20 h)	NS	MLMM	(0.0022)	–0.03#
	glucose 6-phosphate (20 h)	ES	FarmCPU	0.0021	+0.90 µmol/g

QTNs are indicated in bold text, while putative SNP effects are indicated in parenthesis; *capn2*-B1 – Haplotype block 1 of the *capn2* gene; Tmt – treatment group non-electrically stimulated (NS) or electrically stimulated (ES).
– allele effects were estimated from MLM in GAPIT; MFL – myofibril fragment length.

These include SNP effects on binding of transcription factors and the covalent modification of proteins in signal-transduction pathways that affect multiple cellular functions and are shared between seemingly unrelated cellular processes to SNPs (Mackay *et al.*, 2009; Rodrigues *et al.*, 2017; Leal-Gutiérrez *et al.*, 2020b; Zhu *et al.*, 2021). Stress associated with the transport, holding and slaughter of cattle normally activates β-adrenergic receptor signalling through G-proteins and protein kinase C (Ca^{2+}-dependent protein kinase), resulting in a sarcoplasmic signal transduction cascade (phosphorylation/dephosphorylation or covalent modification of enzymes) and greater glycogenolysis, depleting anaerobic glycolytic substrate (Ferguson & Gerrard, 2014). However, *in situ*, cellular stress responses can also lead to activation of calpain protease activity (Kemp & Parr, 2012), where all isoforms of protein kinase C are substrates for calpain-1 proteolysis (Goll *et al.*, 2003), decreasing β-adrenergic signalling through protein kinase C and conserving glycolytic substrate. Although

the characterisation and validation of SNP effects (including the calpain-calpastatin system) remains an active area of research, this is increasingly being combined with transcriptomics, proteomics and metabolomics in a systems biology or gene network approach to elucidate these complex interactions that fall between DNA and cellular function (Gagaoua *et al.*, 2022; Warner *et al.*, 2022). Other important substrates for calpain-1 that could have important homeostatic effects in cells include membrane Ca^{2+} ATPase, ryanodine receptor, kinases, phosphatases and transcription factors (Goll *et al.*, 2003; Place *et al.*, 2015; Ono *et al.*, 2016; Spinozzi *et al.*, 2021) and an important question is whether the different calpain-calpastatin system genotypes can differentially alter these cellular functions *in situ*, during the ante-mortem period.

Two QTNs were identified in B2 for MFL in d 14 (*16_765* and *16_769*) and absolute calpastatin inhibition (*16_769*). Marker *16_765* effect on MFL (14.3% shorter lengths) was only present in the ES treatment group and the SNP failed to affect MFL on d 14 in the control ($p \leq 0.5713$). However, the nearby linked SNP, *16_769* was associated with MFL on d 14 in the control and an important SNP in the ES group ($p \leq 0.0601$), with a 16.7% decrease in fibril lengths, that can be explained by an association with a 15.7% decrease in absolute calpastatin inhibition at 1 h in the ES group ($p \leq 0.0062$ in NS). The effects of the SNP in this region on tenderness traits were inconsistently identified (Hulsman Hanna *et al.*, 2014), where generally no association with shear force was identified (Bolormaa *et al.*, 2011; McClure *et al.*, 2012; Xia *et al.*, 2016; Mateescu *et al.*, 2017; De Souza Fonseca *et al.*, 2022).

5.3.3.3. SNPs in capn2*-B3 and downstream independent SNPs (*16_773 – 16_778*;* 16_780 *and* 16_781*)*

Although QTNs were identified for *16_776* and *16_780* for small, favourable effects on carcass and beef quality traits (Table 5.3), the potential gains that could be made would not be financially viable for selection purposes. Similarly, the effects of marker *16_781* on calpastatin was biologically small, while the increase in glucose 6-phosphate for this genetic marker represented a 14.2% increase in glycolytic substrate.

*5.3.4. The calpain-1 gene (*capn1*)*

A moderate association was identified between loci in the *capn1* gene (D' = 0.69, $r^2 = 0.26$) (Chapter 3, Table 3.4), with three relatively short haplotype blocks identified in the gene. Haplotype blocks were more fragmented within breeds, although the region encompassing B2 and B3 (≥44,085,769 on chromosome 29) of the gene, exhibited overlapping haplotype blocks for most breeds (except Brahman). Extended regions of high linkage in the 3'-region of the gene are consistent with reports by other authors (Cheong *et al.*, 2008; Braz *et al.*, 2018; Leal-Gutiérrez *et al.*, 2018).

5.3.4.1. Independent SNPs of capn1 *(*29_183*,* 29_186*,* 29_187*)*

The SNP *29_186* was a candidate SNP for a small (5.3%) decrease in MFL on d 14 (Table 5.4), while *29_187* was a candidate for selection for a 0.41 kg decrease in WBSF on d 9 in the ES treatment, but these effects were not important in the control ($p \leq 0.4466$). Although the SNP *29_186* was not identified as a candidate for selection in Angus, *29_187* and surrounding, sequenced SNPs associated with WBSF on d 14 in a region 4.6 kb up– and downstream of this SNP, including *29_370* (Leal-Gutiérrez *et al.*, 2020b). Similarly, *29_186* failed to

associate with WBSF and estimates of WBSF in analyses using 777K genotypes, while *29_187* did, although neither of these experiments determined measures of myofibril fragmentation (Leal-Gutiérrez *et al.*, 2020b; White, 2020).

Table 5.4. *Quantitative trait nucleotides and candidate SNPs in the* capn1 *gene for growth, carcass and beef quality traits in control (NS) and electrically stimulated beef (ES) of bulls from five South African breeds, genotyped using the Illumina® BovineHD SNP BeadChip (777K).*

capn1	**Trait**	**Tmt**	**Model**	***p*-value**	**Effect of tender genotype**
independent SNPs (*29_183*, *29_186*, *29_187*)					
29_186	MFL (d 14)	ES	MLMM	0.0034	–1.25 µm[#]
29_187	WBSF (d 9)	ES	MLMM	0.0013	–0.41 kg[#]
***capn1*-B1 (3.0 kb, *29_184* – *29_185*)**					
29_184	calpastatin per calpain-1 (1 h)	ES	BLINK	**2.56×10^{-4}**	**–0.14[#]**
	calpastatin per calpain-1 (20 h)	NS	BLINK	0.0022	–0.21[#]
	MFL (d 14)	ES	MLMM	**4.99×10^{-5}**	**2.97 µm[#]**
29_316	MFL (d 14)	NS	FarmCPU	0.0011	–1.32 µm
	calpastatin per calpains (20 h)	NS	MLM	0.0016	+0.08
***capn1*-B3 (7.7 kb, *29_189* – *29_191*)**					
29_189	WBSF (d 9)	ES	FarmCPU	0.0004	–0.45 kg

QTNs are indicated in bold text, while putative SNP effects are indicated in parenthesis; *capn1*-B1 – Haplotype block 1 of the *capn1* gene; Tmt – treatment group non-electrically stimulated (NS) or electrically stimulated (ES).
[#] – allele effects were estimated from MLM in GAPIT; MFL – myofibril fragment length; WBSF – Warner-Bratzler shear force.

5.3.4.2. SNPs of capn1*-B1 (*29_184 – 29_185*)*

The only QTN of the *capn1* gene was *29_184* (Table 5.4) that decreased MFL on d 14 by 12.4% and was the result of a 9.6% reduction in the relative calpastatin per calpain-1 protease activity at 1 h post-mortem in the ES treatment group. This marker was also an important SNP affecting these traits in the NS group, but failed to reach statistical significance ($p \leq 0.0732$). It was a candidate SNP for decreasing calpastatin per calpain-1 at 20 h in the control, but not the ES treatment group ($p \leq 0.9711$). These data are consistent with previous research that identified associations of the *29_184* SNP with WBSF on d 7 and WBSF and sensory tenderness on d 14 post-mortem (Tizioto *et al.*, 2013; Leal-Gutiérrez *et al.*, 2020b), although some research has failed to identify an effect on tenderness traits (Xia *et al.*, 2016; White, 2020). The *29_184* marker was previously identified as a SNP that could sustain effects on tenderization through extended ageing from these data, where less stringent control of false positive or false negative effects were applied (Chapter 4). Although we failed to identify the sustained effects here, using optimised *p*-values for each phenotype, the SNP remained one of the most important SNPs affecting tenderization in the different ageing periods (Addendum C, Table S4). Using MFL as an example, more consistent rankings within the ES treatment group were identified with the GAPIT-run models (BLINK, MLM and MLMM) than within NS, where it failed to affect MFL on d 3 or d 20. However, it remained

within the top ten markers affecting MFL at all ageing periods in all three models of the ES treatment group and was in the top five for 10 out of 12 results. Results for ranking as important SNPs affecting WBSF was far more variable, but it was an important SNPs for WBSF on d 3 in NS and ES, while in the MLM model, remained in the top ten SNPs for all ageing periods in the control.

Marker *29_316* was associated with a small (5.5%) decrease in MFL on d 14 in the control but not the ES group ($p \leq 0.6326$), that was accompanied by an increase in calpastatin inhibition per combined protease activity of calpain-1 and calpain-2 at 20 h post-mortem. The SNP *29_316* has been extensively researched and correlated to diverse traits, especially in taurine cattle (White *et al.*, 2005). These included growth related traits such as weaning weight (Saatchi *et al.*, 2014), ADG (Cafe *et al.*, 2010a), body measurements (Casas *et al.*, 2005), carcass weights (Gill *et al.*, 2009; Espigolan *et al.*, 2015), EMA (Espigolan *et al.*, 2015) and carcass yield grade (King *et al.*, 2012). It is generally investigated for associations with beef quality traits such as WBSF (Page *et al.*, 2002), myofibril fragmentation (Curi *et al.*, 2010; King *et al.*, 2012), sensory panel tenderness (Casas *et al.*, 2005), ageing rate (Chang *et al.*, 2014) and beef colour (Cafe *et al.*, 2010a). Associations with fatness have been identified for IMF (Barendse, 2011), rib fat measurements (Cafe *et al.*, 2010a) and marbling (Li *et al.*, 2013), as well as cooking loss (Cafe *et al.*, 2010b) and reproductive traits (Collis *et al.*, 2012).

5.3.4.3. SNPs of capn1-*B3 (*29_189 – 29_191*)*

The marker *29_189* was associated with a 0.45 kg reduction in WBSF on d 9 post-mortem in the ES treatment group, but not in the NS group ($p \leq 0.3616$) and confirms the inconsistent associations of the region with tenderness traits (Tizioto *et al.*, 2013; Leal-Gutiérrez *et al.*, 2018, 2020a). These associations of *capn* and *cast* genetic markers with "other" physiological processes can be linked to a diverse range of cellular substrates for calpains including haem (Casas *et al.*, 2014) and proteins such as those affecting platelet function (Randriamboavonjy & Fleming, 2012; Jung, 2013).

Evidence suggests an interaction between the calpain-calpastatin system and intermediary metabolism that is a facilitating role for glucose metabolism in post-mortem protein degradation, but could be ascribed to direct physiological functions of the calpains. The physiological role of calpains in metabolic processes of the living cell can determine the post-mortem metabolic status of muscle fibres directly, in a feedback loop that affects their own post-mortem activity, through transcription or translation factors (Ono *et al.*, 2016; Rosa *et al.*, 2018; Guillocheau *et al.*, 2019), Ca^{2+} transporters (Singh *et al.*, 2004; Place *et al.*, 2015; Bruce, 2018), metabolic hormones and receptors (Sreenan *et al.*, 2001; Ghosh *et al.*, 2005; Yuasa *et al.*, 2016) and proteins of signal transduction and covalent modification (Park *et al.*, 1993; Goll *et al.*, 2003). In addition to these substrates that could alter cellular homeostasis, calpains have also been found to interact with intermediary metabolic enzymes and pathways (Purintrapiban *et al.*, 2001; Paul *et al.*, 2003; Ojima *et al.*, 2011; Kemp *et al.*, 2013; Ono *et al.*, 2016; Rosa *et al.*, 2018; Jahnke *et al.*, 2020) and together these effects suggest that the interaction between calpains and nutrient metabolism could go beyond the coincidental.

5.4. Conclusion

We identified QTNs for fluid balance in the genes of the muscle calpain system that can be used to select for lower beef yield losses, but potentially also improve beef eating quality. Four QTNs for energy and glycolytic substrates or intermediates were linked to neighbouring SNP effects on tenderness traits. Although no QTNs for WBSF were identified, several QTNs for MFL were identified and many of these included effects on calpastatin inhibition of calpain protease activity. While electrical stimulation altered SNP marker effects on traits in some instances, there was no clear distinction of attenuation or augmentation of associations by treatment, which remains to be explained. Energy metabolism is central to the development of several beef quality traits, by facilitating cellular conditions that drive the proteolytic degradation of myofibrils, which in turn can affect, not only tenderization, but also water-holding capacity. This causal relationship between lactate production (pH decline), protease activity and water-holding capacity should be investigated further. Future research should focus on sequencing regions surrounding the QTNs that have the largest biological importance, especially those with pleiotropic effects on beef quality traits.

Supplementary Materials (Addendum C): Table S1 (Phenotypic means); Table S2 (Description of SNP); Table S3 (Number of bulls per haplotype); Table S4 (SNP 29_184 ranking for tenderness between treatments).

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Chapter 6: Summary and critical evaluation

Although South African beef cattle breeds have been extensively characterized, little research is available on the association of genomic markers with beef quality traits (Frylinck *et al.*, 2009). Our research is the first association analysis of the calpain-calpastatin system genes in South African beef cattle, using 777K genotyping and detailed, accurate phenotypes that were determined under rearing, slaughter and post-mortem conditions that were controlled to minimise environmental variation. Environmental effects interact with breed types (genetics) and production system (Wheeler *et al.*, 1990; Frylinck *et al.*, 2013) and is therefore expected to affect the outcome of association analyses (Goddard & Hayes, 2007). Few studies have addressed the relationship between extended beef ageing (Castro *et al.*, 2016; White, 2020) or the effects of electrical stimulation (Riley *et al.*, 2019, 2020a, 2020b) on the association of SNPs with beef quality traits. The aim of this research was to investigate whether these tenderizing interventions, extended ageing and electrical stimulation, could attenuate or even eliminate the genomic associations between causal genes and beef quality traits. The candidate gene approach was chosen to increase the power of association of analysis for these quantitative traits (Williams *et al.*, 2009; Raza *et al.*, 2020) and a large number of accurate, diverse phenotypes were collected in order to determine a detailed representation of the conversion of muscle to meat.

Coupled to the cost of the relatively high-resolution genotyping required for the multibreed analyses, the cost of the bulls, finishing, sampling and laboratory analyses precluded the use of a large number of animals. These data could therefore only capture a small fraction of the genetic variation within the different breeds and would not be suitable to determine accurate genomic-enabled breeding values or accurate predictions for selection, particularly when complex models are applied in association analyses, but provide ample data to test the principle that environmental influences can eliminate the advantage of favourable genotypes in the calpain system. There is an opportunity to conduct genomic association analyses in South African indigenous cattle like the Nguni breed that displays unique genomic variation (Makua *et al.*, 2019; Zwane *et al.*, 2022), but this should be conducted using accurate phenotypes, detailed environmental information and using a sufficiently large number of animals to capture the genomic variation (ideally through high-coverage sequencing) to accurately predict important phenotypes like growth, beef quality and adaptive traits.

This research demonstrated that genotyping with the Illumina® BovineHD SNP (777K) BeadChip, with lower ascertainment bias toward Sanga-type and generally toward Brahman cattle (except in *capn3*), provided sufficient density to determine linkage block structural differences between breeds, although the limited number of bulls in our study could only represent a partial estimate of linkage and should be confirmed in a larger study. With the moderate (*capn3* and *capn1*) to high (*capn2* and *cast*) LD observed within these genes, overlapping haplotype blocks offer an opportunity for genetic marker identification and selection across breeds for pleiotropic effects in the calpain-calpastatin system. These results confirmed the close relationship of the Sanga composite breed, Bonsmara, with improved taurine breeds such as Angus cattle, through principal components analysis, favourable allelic distribution and linkage block structure of the calpain system genes. These similarities are likely the result of increasing production pressure on carcass yield and consequent admixture of the breed with British and European taurine cattle. These data confirmed that Nguni cattle have

the genetic potential to produce tender beef and identified opportunities for improvement of favourable allele frequency in some genes. Association studies and predictive models that are used to improve the quality of Nguni beef in future, should however incorporate phenotypes of the adaptive advantages and genetic variation that are unique to the breed (Zwane *et al.*, 2022), to ensure the conservation of these traits that could be of great value in a changing environment. The physical proximity and moderate to high linkage disequilibrium of regions of these genes, intimately linked to tenderization and traits linked to adaptive responses like immunity, metabolism and heat tolerance, suggest that directional selection for beef quality traits could result in unintended changes in adaptive physiology.

The principal limitation of these data (sample size) illustrates the challenges faced in genomic studies that lack high-throughput phenotypes. Determining comprehensive, multi-dimensional, accurate phenotypes on each individual bull, through traditional methods that require technical and interpretive skill that are time-consuming and expensive to perform, coupled to the cost of higher density genotyping, prohibited the use of a larger sample size. There is still great reliance on traditional measurement of phenotypes, similar to the procedures in this research and on quantitative selection in South Africa, with only limited application genomic predictions and automated technologies like real-time ultrasonography. The challenge of closing the phenomic gap on the rapidly advancing genomic technologies was identified as an important target more than a decade ago (Houle, 2010; Hocquette *et al.*, 2012a). However, is seems that genetic research in beef cattle still face these same challenges (Rexroad *et al.*, 2019), as high-throughput genomic tools have continue to progress and become increasingly affordable in the last decade, while phenotyping remains "the rate-limiting step in genomic selection" (Hocquette *et al.*, 2012b). One of the major concerns is the development of automated tools that are able to determine accurate, repeatable measures of traits, rapidly recording a large amount of data points in a cost-effective manner, allowing financial gains from their application. The most promising technologies for the immediate future involve visual measurements and monitoring in the infrared spectrum to determine traits, including adaptive traits, behaviour, body condition, temperament, reproductive and some beef quality traits. Alternatively, automation of phenotype recording in abattoirs present an opportunity for the advancement of phenomics, provided these technologies deliver good prediction of traits and a financial incentives for the infrastructure development (Ventura *et al.*, 2020). Advanced phenomic applications depend heavily on the type of production system and can most readily be applied within intensive systems, especially where individual identification, pedigree information, animal recording and infrastructure are already in place. Automated feeding systems that aim to record and improve feed efficiency provide an incentive through increased profit (Silva *et al.*, 2016) and the infrastructure is readily-adaptable to multi-dimensional phenotypic observations. Similarly, intensive dairy production has adopted precision farming technologies to collect a large amount of data for genomic predictions and precision farming (Wu *et al.*, 2023). These data would however represent only a small proportion of the South African beef herd (Visser *et al.*, 2020) and the challenge of technological infrastructure development, automation, the maintenance and interpretation of large-data, coupled to genomic evaluation of individuals, remain a significant obstacle for small-scale and informal farming systems (Ventura *et al.*, 2020; Houaga *et al.*, 2023). Large extensive systems, informal small-holder and subsistence farming present unique challenges and the application of technologies and goals for genomic advancement from developed

countries based on scientific research principles are unlikely to find direct application or acceptance, where animals are spread over wide areas and used for cultural or religious purposes (Mrode *et al.*, 2020). It is therefore important that animal recording of a greater complexity of phenotypes (in accordance with the complexity of the physiological processes) is more widely incentivised, especially in rural communities, in order to collect more data for genomic predictions across a wider range of production types, environments and slaughter procedures. A major challenge worldwide however, remains finding a high-throughput solution for difficult-to-measure traits, like muscle metabolic parameters, myofibril degradation and enzyme control and activities.

The results of association analyses in this research, over extended ageing up to 20 d post-mortem, provided evidence that there was an interaction between the effects of SNPs on beef tenderization and extended beef ageing, where most SNP effects were limited to a defined period of meat ageing. Only a few SNPs were able to sustain tenderizing effects in beef over several beef ageing periods and these data are consistent with the altered processes of beef tenderization over the ageing period (De Souza Fonseca *et al.*, 2022). Several QTNs and candidate associations were identified in the genes of the calpain system following electrical stimulation treatments. The effect of treatment on associations was generally a difference in the strength of the associations (size of the effects), while several SNP maintained their ranking among genetic effects on a wide variety of traits, with no evidence that electrical stimulation attenuated genomic effects. This is consistent with electrical stimulation as a tool to accelerate the cellular processes that drive the conversion of muscle to meat. However, the effect of electrical stimulation on association analyses was not simple in all instances and genomic effects could depend on additional factors that are related to the complex development of beef quality traits post-mortem. These differences in some SNP-associations between treatment groups could depend on the specific SNP or trait, or more likely, depend on physiological changes in cellular function that would require determination of traits of greater complexity. Whether these cellular conditions are affected by the functions of these proteins *in situ*, unrelated to the direct actions of the calpain system, or are the result of the complex processes of the conversion of muscle to meat facilitating allele effects, remains to be established.

The muscle calpain enzyme system proteins are all found in the sarcoplasm and are best-known to determine post-mortem proteolysis of muscle proteins, determining protein flux within the fibres and structural organisation of proteins (Spinozzi *et al.*, 2021). Differences between breeds or individuals for calpain system genotypes could therefore alter the development and growth of muscle fibres (Nonneman & Koohmaraie, 1999; Kemp *et al.*, 2013), resulting in myofibrillar structural differences. This in turn could affect the ability of proteases to degrade sarcoplasmic proteins, the ability of these altered myofibrillar structures to retain water and to refract light from the surface of meat. The link between the genotypes of calpastatin and calpains and the different measures of water holding of meat (Ribeca *et al.*, 2013; Tizioto *et al.*, 2013; Iso-Touru *et al.*, 2018) should be investigated in greater detail, particularly the increased fluid losses from carcass weight during chilling and ageing observed in our data that, cumulatively, could represent a substantial loss of profit to abattoirs. Other measures of fluid balance like drip or purge, as well as juiciness can also have a large impact on eating quality for the consumer and should be investigated further. Associations between calpain system SNPs and colour traits like L* and a* have been identified in past research (Saucedo Uriarte *et al.*, 2021) and the link between the possible structural influence of different calpains, as well as the effects of these genotypes on

intermediary metabolic responses identified in this research, represents an opportunity to further elucidate the physiological mechanisms that connect these complex traits.

Future research should determine whether the calpain-calpastatin system and the genotypes for markers within these genes, could alter muscle cellular conditions to such an extent that it alters the post-mortem processes of the conversion of muscle to meat, beyond the enzymatic degradation of proteins. Since their substrates include proteins of gene transcription and translation (Ono *et al.*, 2016; Leal-Gutiérrez *et al.*, 2018; Guillocheau *et al.*, 2019) and proteins of post-translational, signal transduction pathways (Park *et al.*, 1993; Goll *et al.*, 2003; Yamaguchi *et al.*, 2012; Yuasa *et al.*, 2016) they could affect a range of cellular functions directly by altered control, including intermediary metabolism. The calpains interact with enzymes of intermediary metabolic pathways directly (Purintrapiban *et al.*, 2001; Ojima *et al.*, 2011; Ono *et al.*, 2016), including oxidative metabolism and mitochondrial function (Rosa *et al.*, 2018; Jahnke *et al.*, 2020), where glycolytic activity and pH decline of meat (and the oxidative state of the sarcoplasm) play a central role in determining the development of several beef quality traits that are not simply coincidental to an effect of the rate of pH-temperature decline.

Novel QTNs in the *cast* and *capn2* genes for increased glycolytic metabolism and water holding in *capn3* exhibited positive allele effects on beef quality phenotypes that were independent of protease activity of the enzyme system. The associations of calpain-calpastatin system SNPs with metabolic substrates, intermediates and products (independent of pH_u), raises the important question of whether calpain genotypes can affect the storage and mobilization of glycogen, or glycolytic enzyme activities through *in situ* effects on intermediary metabolic control at the level of transcription, translation or covalent modification. Although these associations could indicate that the complex interactions of intermediary metabolic pathways are required to facilitate the genomic effects on tenderization, the weight of evidence relating calpains to pH change and metabolic biomarkers for tenderization, suggest a more direct role for calpains.

Investigation of genomic associations in beef cattle is increasingly becoming more integrated with other research areas in order to incorporate a systems biology approach to analyse data. The complex interactions of several cellular processes in controlling and modifying beef quality attributes, means that complex predictive models that integrate all these data will be required to accurately predict phenotypes. In future, these data should combine genomic data with determination of mRNA concentration (gene expression), post-translational modification, signal-transduction pathways, enzyme activities of proteases and metabolic enzymes, proteomics, metabolic profiles and determination of cellular conditions, in order to determine the complex interactions that establish the transition from muscle to meat. It is important that the ante– and post-mortem conditions are recorded and tenderising interventions specified, for example the time of ageing and the use of electrical stimulation. These diverse environmental effects, production types, carcass conditions and ageing procedures are not additive to genetic effects on phenotypes (Rexroad *et al.*, 2019) and genomic prediction should incorporate the physiological principle; that environmental effects can have overreaching effects on many beef quality phenotypes. These complex data, in a multi-omics approach, would more accurately determine the effects of QTNs on various beef quality traits. Studies of this nature, particularly using large sampling groups, would require collaboration of scientists from a variety of disciplines, as well as a substantial financial input, which remains an important constraint to research and publication for South African researchers. The next few

years will be an exciting period for farm animal genomics, as gene editing could become the future of bovine genomics (Van Eenennaam & Mueller, 2022), while the recent advances in these technologies, will allow the insertions of entire genes with desirable genotypes in future, that could completely alter the way we improve cattle (Tou *et al.*, 2023).

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Addendum A: Detailed materials and methods

1. Animals

Purebred South African bulls of 5 breeds were finished and slaughtered in 3 separate years, 50 animals (10 × per breed) in 2011, 50 animals (≃10 × per breed) in 2012 and 75 animals (15 × per breed) in 2014 and early 2015. Bulls were progeny of registered pure breed bulls and cows and sourced from several reputable breeders that were registered with the appropriate breeders' associations. Breeds were chosen to be representative of specific types used in South Africa as purebred animals or for crossbreeding (Plate 1). These breeds were Angus (British *Bos taurus*), Brahman (Zebu type *Bos indicus*), Bonsmara (South African composite breed with a large Sanga proportion), Charolais (European *Bos taurus*) and Nguni (Sanga-type *Bos taurus africanus*). Bulls were approximately 9 months old when entering the feedlot at the Agricultural Research Council, Animal Production Unit (ARC-AP; -25°54' 28°12', altitude ≥1475 m). Weight and feed intake data were collected for the 120 days they were reared in the feedlot and they were slaughtered at approximately 12 months of age to yield A2/A3 carcasses, which are cattle with zero permanent incisors and with lean to medium fatness (Anonymous, 1990, 2015). All treatments and procedures were approved by the Ethics Committee of the Animal Production Institute of the Agricultural Research Council (ARC AEC-I 2010 001) and the Animal Ethics Committee of the University of Pretoria (EC171114-161).

2. Slaughter, carcass measurements, electrical stimulation and chilling

After weighing to determine final liveweight one day before slaughter (BW), animals were transported (3.9 km) to the abattoir of the ARC-AP. Animals were penned overnight (with access to water) before slaughter using captive bolt stunning and exsanguination (Plate 2). Hair samples for genomic DNA extraction were collected from hides directly after exsanguination for 2014 samples, whereas DNA was extracted from muscle tissue in the other slaughter years.

Carcasses were submitted to general inspection, inspection for disease, aged according to dentition, classed and halved. The two halves of the carcass were weighed to record hot carcass weight (HCW). Electrical stimulation occurred within 30 minutes post-mortem. Stimulation was applied for 15 seconds at 500 V peak, using 5 ms pulses at 15 pulses per second. The right side of carcasses was electrically stimulated and chilled directly at 4°C (ES), while the left side of the carcasses served as control and was not electrically stimulated and subjected to delayed chilling (NS). The left carcass halves were kept at 10°C for 6 hours when carcass temperatures reached 20°C ± 0.13, to allow enhanced development of metabolic changes in muscle (accelerated conditioning), before being moved into the chiller (4°C) overnight.

Plate 1. *South African purebred beef breeds used in the experiment.*

An - Angus; Bo - Bonsmara; Bh - Brahman; Ch - Charolais; Ng - Nguni

Plate 2. *Slaughter procedures of cattle at the Agricultural Research Council abattoir.*

3. Sampling

3.1. 1 h, 3 h and 6 h post-mortem

Musculus longissimus thoracis et lumborum (LTL) loin muscle were used for all samples. Carcasses were sampled from caudally at 1 h, 3 h and 6 h post-mortem, approximately at the level of the sixth lumbar vertebra (L6). Carcass measurements for pH and temperature were determined before sample collection directly on the carcass at 1 h, 3 h, 6 h post-mortem (ES and NS) in all slaughter groups (years). The pH and temperature was determined with a digital handheld meat pH meter (CyberScan pH II pH/mV/°C meter), fitted with a polypropylene spear type gel electrode (Thermo Fisher Scientific, Waltham, MA, USA). All pH calibrations (pH 4 and pH 7) were conducted at 10°C.

At the same time, small muscle samples were collected and snap-frozen in liquid nitrogen ($N_{2\ell}$) at −196°C, from ≥1 h post-mortem for the analysis of muscle energy (ES and NS). In the 2011 slaughter group, no samples were collected for energy at 3 h and 6 h post-mortem. At 1 h post-mortem, small samples were collected and snap-frozen for the determination of calpain system enzyme/inhibitor activity (ES and NS). These samples were immediately frozen at −80°C until laboratory analyses.

3.2. 20 h post-mortem

Cooled carcass measurements were taken at approximately 20 h post-mortem. Cold carcass weight (CCW) was recorded by weighing each half of the carcass and tracings were made of the LTL eye muscle (between the ribs T9 – T10). Both left and right LTL were boned-out from carcasses from the level of L6 caudally, up to T9/10 cranially and post-rigor pH and temperature were recorded from excised loins.

Small samples were collected and snap-frozen from lumbar regions of the excised LTL for the determination of sarcomere length (SL, NS), calpain system enzymes/inhibitor (ES and NS) and muscle energy (ES and NS). Samples for the determination of muscle collagen and proximate analysis (NS) and meat samples for DNA extraction were also collected. Larger samples and steaks (≃30 mm) were collected and vacuum-sealed from remaining, more cranial portions of the muscle.

The LTL steaks were collected from both halves of the carcass, from predetermined portions of the muscle, with 4 steaks each for laboratory analysis and Warner-Bratzler shear force (WBSF) tenderness evaluation following ageing for 3, 9, 14 and 20 days post-mortem (see below for details). Laboratory analyses included sarcomere length (day 3 only), drip loss, water-holding capacity (WHC), Commission Internationale de l'Éclairage (CIE) colour measurements (lightness, redness, yellowness, chroma and hue angle) and myofibril fragmentation length (MFL), for all ageing periods.

Steaks collected for 3 d ageing were over-wrapped before ageing, while steaks collected for the longer ageing periods were vacuum-sealed before ageing. Steaks were vacuum-sealed for 9 d, 14 d and 20 d ageing in 70 micron, food-grade bags with a Boss twin-chamber vacuum machine, type Titan-X 630 (Meistertech Z3000, Germany) with a 100 m^3/h vacuum pump vacuumed at 99% at 1°C. Vacuum-packed (or over-wrapped) steaks were aged at chiller temperatures of 2 ± 1°C and used for the determination of colour, drip loss and WHC on 3 d, 9 d, 14 d and 20 d post-mortem using fresh samples. Samples collected for tenderness analysis by WBSF were aged in vacuum bags and then frozen at −20°C until cooking and shearing.

4. Laboratory methods

4.1. Tenderness (Warner-Bratzler shear force)

As the most widely used standard instrumental (objective) measure of tenderness (Wheeler *et al.*, 1997), WBSF determinations were taken for LTL from both halves of all carcasses for all ageing periods (Bratzler, 1949; Warner, 1952). The 30 mm frozen steaks collected at 20 h post-mortem (–20°C), were thawed at 4 ± 1°C for 24 h before being cooked by oven-broiling (Mielé, model H217, Mielé & Cie, Gütersloh, Germany) using direct radiant heat (AMSA, 1995, 2016). Ovens were pre-set to 200°C and steaks were cooked to an internal temperature of 71 ± 1°C, determined with a thermocouple device (Comark C9003, Comark Instruments, Northampton, UK) at the geometric centre of each steak. Cooked steaks were cooled to ambient core temperature of 18°C. Six round, "good" cores (free of visible fat and connective tissue) of 12.7 mm diameter were collected from each steak with a handheld cork borer, parallel to the muscle fibre direction (at an angle of approximately 45° in these steaks). Cores were sheared through the centre with a Warner-Bratzler shear device (AMSA, 2016), with a V-notched blade (60° angle), perpendicular to the long axis of the muscle fibres. Crosshead speed was 200 mm/min and the peak force (kg) for each core was recorded using a Universal Instron apparatus (Model 4301, using Series IX Merlin Automated Materials Testing System software, Instron Ltd, Buckinghamshire, England).

The mean value of the load at maximum (kg) of 6 cores was used in statistical analysis, after elimination of outliers with the formulas:

$$\boldsymbol{upper\ limit = \ mean\ shear\ force\ (kg)\ + \frac{SD \times t}{\sqrt{n}}}$$

$$\boldsymbol{lower\ limit = \ mean\ shear\ force\ (kg) - \frac{SD \times t}{\sqrt{n}}}$$

where;

$$\boldsymbol{t\ (5df;\ 0.01) = 4.032}$$

and n = 6;

$$\boldsymbol{\sqrt{6}\ =\ 2.4495}$$

These values can either be expressed as kg (as shown here), or often as the SI unit, Newtons (N) of peak force using *g* as a conversion factor (kg ÷ 9.81), depending on convention.

4.2. Myofibril fragmentation

Myofibril fragment lengths were determined for 100 fragments per sample by means of Video Image Analysis (VIA) using an Olympus System microscope, model BX40 (Tokyo, Japan) at a 400× magnification equipped with CC12 video camera. Myofibrils were extracted according to Culler *et al*. (1978), as modified by Heinze & Brüggemann (1994).

4.3. Eye muscle area (EMA)

The outline of the LTL eye muscle was traced onto transparent film directly from the carcass at the cranial excision point of the loin (T9 - T10). The area within the tracing was analysed using video image analysis (VIA), using AnalySIS Life Science software package (Soft Imaging Systems Gmbh, Münster, Germany).

4.4. Colour

Instrumental meat colour was determined on fresh samples (3 d post-mortem) or vacuum-aged samples (9, 14 and 20 days). The 30 mm steaks collected at 20 h post-mortem were divided in two steaks of 15 mm thickness. One of these steaks was allowed to bloom in darkness for 60 minutes (freshly cut surface facing upwards), at chiller temperatures of (2 ± 1°C), before colour was recorded in triplicate at three different sites. For the 2011 slaughter group, colour was measured using a Minolta spectrophotometer (Model CR200, Osaka, Japan) with an 8 mm diameter measuring area, diffuse 228 illumination and 0° viewing angle. For the 2012 and 2014 slaughter groups, a Konica-Minolta 600d spectrophotometer, with the SpectraMagic NX Pro software package, was used with illuminant D65, geometry di 8° de 8°, observer angle 10° and measurement aperture 8 mm. Measurements were taken, excluding the spectral component (SCE), after calibration using a white reference.

Three fundamental outputs were used, following the Commission Internationale de l'Éclairage's (CIE) three-dimensional space for L*, a* and b* determinations in the CIELAB colour convention (AMSA, 2012), where means of the three measurements were used for statistical analysis. Lightness (L*), redness (a*), yellowness (b*), chroma (C*) and hue angle (h_{ab}) were determined by the spectrophotometer for all ageing periods (MacDougall, 1977, 1982, 1986, 1994, 2002) and used in statistical analyses.

4.5. Calpain system assay

The calpains (calpain-1 and calpain-2 proteases) and calpastatin (protease inhibitor) were extracted from 5 g samples (Dransfield, 1996) and separated by 2-step gradient ion exchange chromatography (Geesink & Koohmaraie, 1999). The calpain assay used azo-casein as substrate to eliminate background absorbance of non-specific proteins in the extracts (Dransfield, 1996). Activity of the proteins per grams of muscle tissue was defined as one unit of calpain activity, being equivalent to an increase in absorbance of 1.0 per hour at 366 nm (A_{366}) at 25°C. One unit of calpastatin activity was equivalent to the amount of calpastatin that inhibited one unit of calpain-2 activity. Total enzyme / inhibitor activity was expressed as units (U) per grams of meat, while relative inhibition of calpastatin per units of calpain-1, or per the combined protease activities of calpain-1 and calpain-2 was also calculated.

4.6. Muscle energy

Samples collected at 1 h, 3 h, 6 h and 20 h post-mortem, snap-frozen and stored at −80°C were used for the enzymatic determination of metabolites of muscle energy supply. The method used amyloglucosidase (AGS) hydrolysis of glycogen (Dalrymple & Hamm, 1973) and spectrophotometric analysis. For the 2011 slaughter group, only samples from 1 h and 20 h post-mortem were collected (n = 116). Muscle energy parameters

included glycogen content (glycosyl units/g), glucose, glucose 6-phosphate (G6P), lactate, creatine phosphate (CrP) and adenosine triphosphate (ATP) concentration (µmol/g).

Glycolytic potential was calculated using a formula that incorporates muscle metabolites for glycolysis, where C-6 substrates can yield two rounds of glycolysis (or 2 × C-3 intermediates, pyruvate) and C-3 substrates one round of glycolysis. The formula for calculating glycolytic potential is:

$$\underset{(\boldsymbol{\mu mol/g\ muscle})}{\boldsymbol{Glycolytic\ Potential}} = \mathbf{2} \times (\,[\,\boldsymbol{glycogen}\,] + [\,\boldsymbol{glucose\ 6\text{-}phosphate}\,] + [\,\boldsymbol{free\ glucose}\,]\,) + \boldsymbol{lactate}$$

4.7. Drip loss

While preparing the 30 mm steaks for colour measurement, the purge remaining in the vacuum bag was calculated by weighing the sealed vacuum bag containing the steak, the steak after lightly drying with tissue paper and the empty vacuum bag. Drip was expressed as a percentage of the combined mass of the steak and drip:

$$\boldsymbol{purge} = \boldsymbol{sealed\ bag} - (\boldsymbol{dry\ steak} + \boldsymbol{empty\ bag})$$

$$\boldsymbol{drip\ loss}\ (\%) = \frac{\boldsymbol{purge}}{\boldsymbol{steak} + \boldsymbol{purge}}$$

4.8. Collagen content and solubility

Collagen content and solubility were measured according to the method originally described by Neuman & Milan (1950) and adapted by Bergman & Loxley (1963), Hill (1966) and Weber (1973). The method is based on the oxidation of hydroxyproline and condensation of the product with para-dimethylaminobenzaldehyde, followed by spectrophotometric determination between A_{480} nm and A_{620} nm (A_{560} nm) of the red (pink) solution. Total collagen was calculated with the formula:

$$\boldsymbol{collagen} = \frac{\boldsymbol{hydroxyproline}}{\boldsymbol{total\ nitrogen}\ (\boldsymbol{wet})}$$

Soluble collagen was calculated as the difference between total collagen and insoluble collagen:

$$\boldsymbol{Soluble\ Collagen} = total\ collagen - insoluable\ collagen$$

Collagen solubility was calculated with the formula:

$$\boldsymbol{Collagen\ Solubility}\ (\%) = \frac{\boldsymbol{soluable\ collagen}}{\boldsymbol{total\ collagen}}\ \%$$

4.9. Proximate analysis

The composition of muscle tissue was analysed using the standard methods of the Association of Official Analytical Chemists (AOAC, Rockville, USA) International (AOAC, 2019). Freeze-dried samples were used for the determination of the percentages of dry matter (DM), moisture (ASM013), ash (ASM048), crude protein (CP) and intramuscular fat (IMF). Intramuscular fat was determined by ether extraction in 2012 and 2014, where lipids were extracted from 5 g muscle tissue using chloroform and methanol in a ratio of 2:1, using butylated hydroxytoluene (BHT) as an antioxidant. The extracts were dried, weighed and expressed as a percentage fat per 100 g tissue (w/w). Near-infrared spectroscopy (NIRS) was used to determine the IMF percentage in 2011.

4.10. Sarcomere length (SL)

For the determination of sarcomere lengths, or the distance between two consecutive Z lines in the muscular structure (Hegarty & Naudé, 1970), distilled water (dH_2O) was used instead of Ringer Locke solution (Dreyer *et al.*, 1979). The meat samples were scratched along the grain of the fibres and homogenised with an Ultra Turrax T25 (IKA-Werke, Straufen, Germany), before being transferred onto a slide and covered with a cover slip. Excess water was dried off and the slide was cleaned. Measurements of 50 sarcomeres per sample were taken by means of Video Image Analysis (VIA), using an Olympus System microscope, model BX40 at a 1000× magnification equipped with CC12 video camera (Olympus, Tokyo, Japan). Measurements were processed and quantified with the aid of the AnalySIS Life Science software package (Soft Imaging Systems Gmbh, Münster, Germany), using the mean of the 50 sarcomere lengths as the final length for each sample.

4.11. Water-holding capacity (WHC)

Water-holding capacity, or the amount of water expressible by applying an external pressure force (Jauregui *et al.*, 1981), was determined by calculating the ratio of meat area and liquid area after pressing a 400 – 600 mg fresh meat sample on a filter paper (Whatman 4). The samples were sandwiched between two Perspex plates and pressed at a constant pressure of 300 psi for 60 seconds (Irie *et al.*, 1996). The areas were measured by means of Video Image Analysis (VIA), using a CC12 video camera (Olympus, Tokyo, Japan) at a magnification of two. Image processing and calculations were conducted by means of AnalySIS Life Science software package (Soft Imaging Systems Gmbh, Münster, Germany).

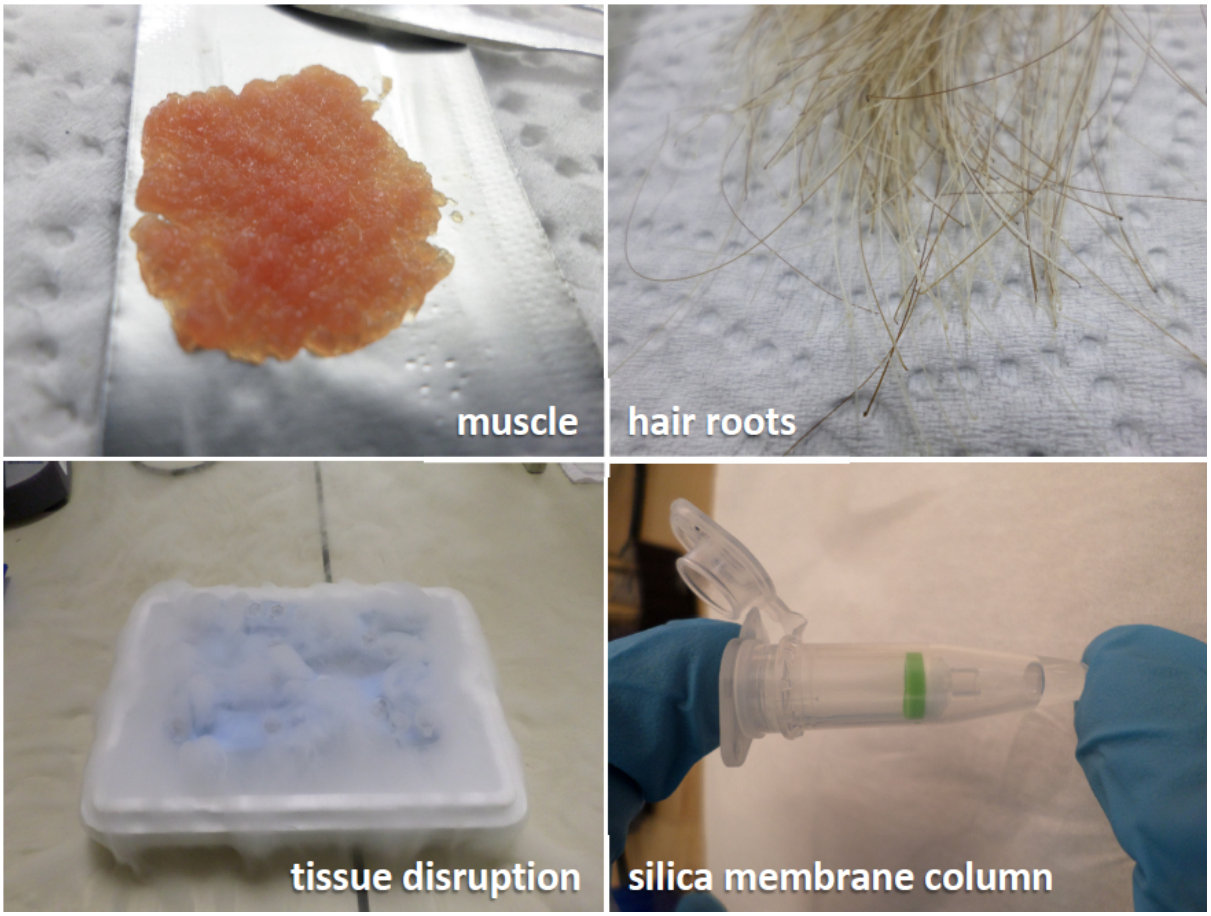
5. DNA extraction and genotyping

5.1. DNA tissue extraction

Frozen, vacuum-sealed meat samples (–20°C) collected at 20 hours post-mortem (2011 and 2012) or hair follicles (2014) were used for genomic DNA extraction using the NucleoSpin® Tissue kit, with silica membrane columns (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). The DNA was extracted according to the manufacturer's instructions (at the Agricultural Research Council, Animal Production, Irene, RSA), with some modifications to the standard protocol, as per the user manual, to enhance muscle DNA yield and concentration. During sample preparation, 75 hair roots were cut, or alternatively 50 mg of muscle tissue was collected from the centre of larger samples (≃5 cm^3), before extensive maceration with a scalpel (Plate 3).

Both muscle and hair samples were drenched with lysis buffer and disrupted with four, repeating cycles of freezing in liquid nitrogen (–196°C) and thawing at 56°C, before the addition of proteinase. Samples were lysed overnight with proteinase K in a water bath at 56°C and extractions performed the following morning. To increase DNA yield and concentration, elution with 100 µL elution buffer as a two-step process (50 µL each) with a short incubation period in-between, was performed. For muscle tissue samples, where DNA yield and concentration tend to be relatively lower, elution buffer was also preheated to 70°C to increase the yield of DNA in the two-step elution, into a final volume of 100 µL buffer.

Plate 3. *Extraction of DNA from tissues of purebred, South African beef cattle.*



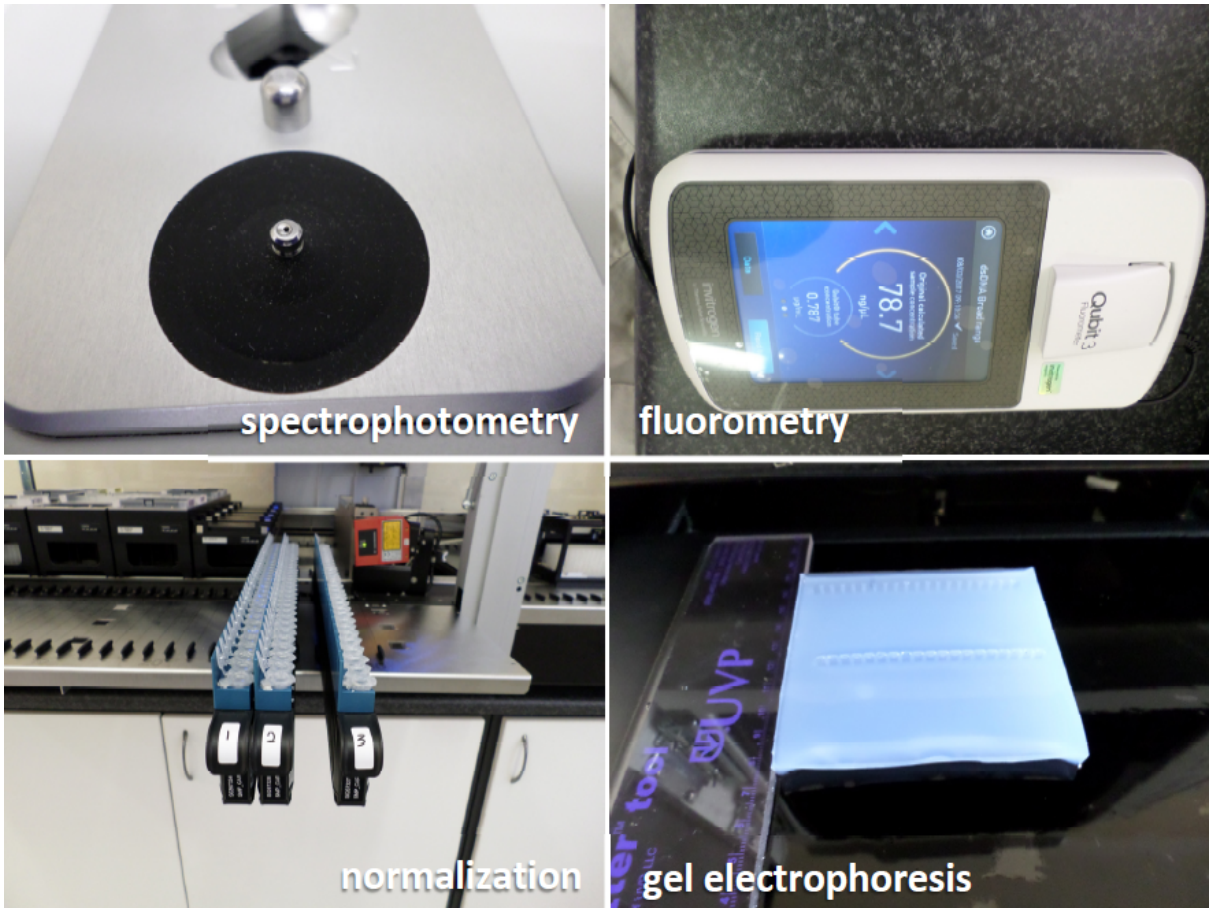
5.2. DNA quality control

5.2.1. NanoDrop (microvolume spectrophotometry)

Following extraction of the DNA, samples were allowed to stabilize overnight at 4°C and an initial estimate of the DNA concentration and quality (Plate 4) was determined with NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at the Agricultural Research Council, Animal Genetics (Irene, RSA). Briefly, 1 µL DNA suspended in elution buffer was pipetted onto a pedestal and the spectrophotometric absorbance compared against the reference solution of elution buffer (hair) or previously preheated elution buffer (muscle). The nucleic acid concentration and ratios of $\frac{A_{260}}{A_{280}}$ and $\frac{A_{260}}{A_{230}}$ were evalueated. Generally the $\frac{A_{260}}{A_{280}}$

ratio should be ≥ 1.80 (1.7 – 1.9), where lower values could indicate contamination of the sample with excessive protein (protein λ_{max} = 280 nm). As a secondary measure of nucleic acid purity, the $\frac{A_{260}}{A_{230}}$ ration should be 1.5 (ideally 1.8, typically 2.0 – 2.2), where lower values could indicate contamination in samples by organic compounds or chaotropic salts (carbohydrates, urea, ethylene diamine tetra-acetic acid (EDTA), phenolate ions). Higher values are generally associated with pipetting errors (empty pedestal), dirty pedestal or a solution dissimilar to the elution buffer (blank, pH or ionic strength different to samples).

Plate 4. *Quality control of extracted DNA from muscle and hair tissues of purebred, South African beef cattle.*



5.2.2. Qubit fluorometry

As a secondary quality control measure for DNA quality, Qubit® 3.0 fluorometric analysis (Thermo Fisher Scientific, Waltham, MA, USA) was run on all the samples at Agricultural Research Council, Biotechnology Platform (Onderstepoort, RSA), to give a more accurate measure of the concentration (and yield) of double-stranded DNA (dsDNA). These observations are more sensitive and more specific than the simple optical density determination. Using the Cubit results for DNA concentrations, samples were normalized to a final dsDNA concentration of ≃50 ng/µL (70 ≤ dsDNA ≥ 37 ng/µL). A total of 26 samples below 37 ng/µL were concentrated by evaporative heat (56°C) to a concentration that fell within the acceptable range, while 54 samples with dsDNA concentrations above 70 ng/µL were diluted with elution buffer to a final concentration 50 ng/µL.

5.2.3. *Electrophoresis*

As a final quality control measure, the normalized samples were evaluated by ethidium bromide agarose gel electrophoresis (Agricultural Research Council, Biotechnology Platform, Onderstepoort, RSA) to determine fragmentation of the dsDNA. Most samples produced clear gel pictures, indicating high-quality (relatively intact) DNA. Samples that exhibited somewhat fragmented DNA, were present in all phases, i.e. long-term storage of muscle samples was not associated with undue degradation of DNA in meat samples at –20°C and samples were all successfully genotyped, with sufficient intact dsDNA for the BovineHD 2 µm welled-bead SNP chip (Illumina, USA).

5.3. *High-density (777K) genotyping*

Animals were individually genotyped using the BovineHD SNP BeadChip (Illumina, San Diego, CA, USA), with variants spread across the entire genome (Plate 5). Intensities were captured using the HiScanSQ platform using the Illumina iScan Control Software (iCS, Illumina, San Diego, CA, USA) and analysed using Genome Studio Software Version 2.0.4 (Illumina, San Diego, CA, USA), with the GenCall (GC) score for successful genotyping set to the default, 0.15. Genotyping and initial analyses to determine the individual alleles (or genotypes) for 777,962 variants in the SNP array, were conducted at the Agricultural Research Council, Biotechnology Platform (Onderstepoort, RSA).

Plate 5. *Genotyping with the BovineHD SNP BeadChip (Illumina®).*

5.4. *Quality control of variant genotypes*

The autosomal genes of the muscle calpain system (Table 1) were identified as important sites for quantitative trait loci (QTL) for beef production and quality. The four genes of the muscle calpain system are the calpain-1 gene (*capn1*) on chromosome 29 (Chr29), the calpain-2 gene (*capn2*) on Chr16, the calpain-3 gene (*capn3*) on Chr10 and the calpastatin gene (*cast*) on Chr7. Only variants within the bounds of the sequences transcripts of these genes, as determined by the Genome Data Viewer of the National Center for Biotechnology Information (NCBI) were selected from the whole-genome data (NCBI, 2021). These sequences stretched between five (*capn*) to 14 (*cast*) kilobases beyond the boundaries of the genes and included 8% of the gene's length in the upstream and downstream untranslated regions (UTR). Filters were applied on the whole-genome data using Genome Studio Software Version 2.0.4 (Illumina, USA):

Table 1. *Description of muscle calpain system genes and reference sequences of the calpain system genes and comparison of the numbers of SNP of 50K, 150K and 777K microarrays.*

Gene	Chr	Description	Start*	End	Length (bp)	Exons	HD 777K# (3.4 kb)	150K# (21.4 kb)	50K# (50.6 kb)
capn1	29	transcript	44,059,777	44,104,001	44,223		12	2	3
		gene	**44,063,463**	**44,100,316**	**36,854**	24			
capn2	16	transcript	27,775,837	27,845,845	70,008		27	1	2
		gene	**27,781,671**	**27,840,011**	**58,341**	21			
capn3	10	transcript	37,823,064	37,891,571	68,507		18	5	3
		gene	**37,828,773**	**37,885,862**	**57,090**	24			
cast	7	transcript	98,431,182	98,594,904	163,722		77	9	3
		gene	**98,444,826**	**98,581,260**	**136,435**	36			
						TOTAL SNP:	**134**	**17**	**11**

* NCBI Genome Data Viewer (http://www.ncbi.nlm.nih.gov) using the Bos_taurus_UMD_3.1.1 transcript (units = bp) (NCBI, 2021).
# The total number of variants that fall within the range, before quality control using common Illumina SNP arrays, the values in parenthesis represent the mean distance between single nucleotide polymorphism
bp - base pairs; Chr – bovine chromosome; HD 777K - BovineHD SNP BeadChip array (relatively higher density genotyping), 50K - BovineSNP50 BeadChip array, 150K - GGP Bovine 150K array variants - including single nucleotide variants that were not polymorphic.

PLINK reports were generated using the Genome Studio plug-in, version 2.1.4, which generates *.ped and *.map files for the top strand alleles for further analyses in PLINK v1.90b3 64-bit (Purcell *et al.*, 2007). All the alleles were matched to the SNP designation from the Illumina final report. PLINK (Purcell *et al.*, 2007) was used to apply quality control measures (Anderson *et al.*, 2010) to the total number of variants located within these genes and the results of the quality control were reported in (Chapter 3). The PLINK and Genome Studio output files were used to record basic genetic information from individual samples and from individual variants. Quality control of variants and individuals were applied *post hoc*, following simple association and mixed linear model association analyses of all variants and individuals.

5.4.1. *Genotyping rate*

The genotyping rate (call rate) is an important measure of DNA quality and is alternatively expressed as "missingness" (or no call rate). It was evaluated for the maximum individual no call rate set to 5% and the

maximum variant no call rate set to 5%. Within the individual samples (or bulls), SNP exhibited a ≥96.3% (range 129 - 134) successful genotyping rate and within the individual SNP, samples had a ≥97.5% (range 162 - 166) successful genotypes. None of the SNP in the analyses was disqualified by these criteria for quality control.

5.4.2. Deviation from Hardy-Weinberg equilibrium

Markers that greatly deviated from Hardy-Weinberg distribution ($P_{HWdev} \leq 1\times10^{-4}$) were excluded from statistical analyses. A Hardy-Weinberg exact test was performed as part of the quality control of variants, which compares the observed genotypic frequencies from the frequencies expected for Hardy-Weinberg equilibrium. One variant in the *capn2* gene exhibited excessive deviation from a Hardy-Weinberg distribution and was excluded from further analyses (Table 2).

5.4.3. Single nucleotide variants versus *–polymorphisms (minor allelic frequency)*

Markers with extremely low rare allelic frequencies that result in a high rate of false-positives in association analyses (Tabangin *et al.*, 2009) were excluded from the results. Four variants; one in the *cast* gene, two in the *capn2* gene and one in the *capn3* gene were found not to be polymorphic with very low minor allelic frequencies (MAF), while the remaining variants can now be called SNP with confidence (Table 2).

Table 2. *Variants that failed genotyping quality control.*

Marker name	Chr	Position	Location	SNP	Reason for exclusion
BovineHD1000031347	10	37,827,639	rs135945111	[A/G]	Hardy-Weinberg deviation ($P_{HWdev}=5.71\times10^{-10}$)
BovineHD0700028753	7	98,510,114	rs136632100	[T/C]	MAF (0.6%)
BovineHD1600007779	16	27,829,558	rs137097425	[T/C]	MAF (3.3%)
BovineHD1600007774	16	27,819,457	rs137478059	[A/G]	MAF (4.2%)
BovineHD1000011715	10	37,837,623	rs109290773	[T/C]	MAF (4.8%)

Chr - Chromosome; MAF – minor allelic frequency; SNP – single nucleotide polymorphism.

5.4.4. The total number of SNP: 134 or 83?

Linkage disequilibrium (LD) between SNP represents an association between the alleles at different loci that is not random, but where alleles are coupled more often than by chance (where chance represent linkage equilibrium) (Lewontin, 1964, 1995). Measures of LD between SNP were evaluated within genes following quality control of variants, in order to avoid redundancy when correcting the *p*-values of single-SNP-single-trait gene-based association analyses. To determine the number of independent SNP (within each of the genes), pairwise exclusion of SNP (Gabriel *et al.*, 2002) was determined that exhibited $r^2 > 0.95$ using PLINK (Purcell *et al.*, 2007), with a 10 kb, 100 bp-sliding window. A total of 51 SNP were found to be "not independent" or in strong LD with nearby SNP, eliminating 40 SNP from the *cast* gene (leaving $36+1_{MAF}$), four SNP from the *capn3* gene (leaving $12+1_{MAF}+1_{HWdev}$), seven from the *capn2* gene (leaving $17+3_{MAF}$) and none from the *capn1* gene (remains 12 SNP).

The exclusion of dependent SNP means that 83 independent SNP were present within the total of 134 SNP genotyped, following quality control. A correction of *p*-values using the method for multiple testing of

Bonferroni (Dunn, 1961), can be modified based on the number of independent SNP. A simple Bonferroni correction would yield a *p*-value for significance of 3.73×10^{-4} (α=0.05÷134) and potentially increase the false-negative rate. However, based on independent SNP, significance would be $p\leq6.02\times10^{-4}$ (α=0.05÷83) for associations determined using only the information of each independent SNP on multiple traits.

All dependent and independent SNP were included in later evaluation of LD and determination of haplotype blocks using the default settings of the method by Gabriel *et al*. (2002) in Haploview 4.2 (Broad Institute, USA) (Barrett *et al.*, 2005). This method uses confidence intervals, instead of point determinations to eliminate the overestimation of linkage disequilibrium of Lewontin's D-prime, by using an upper confidence threshold of 0.98. Results using r^2 as a measure of LD, could be considered more accurate for multiple loci (Hill, 1974) and were also performed along with the measures of D'. These determinations however, had little effect on the results of the LD analyses and the detail of these values (D' and r^2) were reported in Chapter 3.

5.5. Breed stratification (principal components analysis)

Data from all 777,962 variants were loaded in PLINK and subjected to quality control, including 735,293 autosomal SNP with a total genotyping rate of 99.10%. None of the bulls was removed according to the individual call rate threshold, while 25,891 variants were removed due to the variant call threshold (95%). A further 34,443 variants failed the Hardy-Weinberg exact test and 47,851 variants exhibited MAF <5%. This left 627,108 SNP, which were used to generate a variance-standardised genomic relationship matrix (VSGRM). These data were used to perform a principal components analysis (PCA) (Patterson *et al.*, 2006), using autosomal SNP to determine the stratification of breeds, using principal component 1 (PC1) and PC2 and PC3, while PC4 was included in the association analyses as fixed effects or covariates (depending on model). These data were visualised using Orange 2.7 (Slovenia) and Microsoft® Excel (Microsoft Corporation, USA) and reported in Chapter 3.

5.6. Tender alleles

Alleles that were favourable for tenderness was determined using means for genotypes of individual SNP for tenderness traits. The allele (homozygous genotype) with the lowest WBSF and shortest MFL was classified as the favourable or "tender" allele and labelled with an asterisk (A*/C*/G*/T*). For SNP that were not significantly correlated with WBSF or MFL, results were often ambiguous. In these instances, the means for proteolytic enzymatic activity were also compared, where the allele most frequently linked to tender genotypes in these phenotypes was selected as the "tender" allele, if the frequency of the selected allele was ≥16/32 traits. These would include alleles with lower calpastatin inhibition (absolute or relative) and/or with greater calpain protease activity. Determinations assume additive effects of genotypes and linear models are generally applied in association analyses, although not all associations would conform to these simple models. However, addition of non-additive effects failed to improve the accuracy of association analyses of beef growth, carcass and quality traits (Bolormaa *et al.*, 2015) and we are confident that the allocation of tender alleles would not unduly affect the regional association analyses conducted here.

6. Statistical analyses

6.1. Simple data analyses for preliminary results (SAS and PLINK)

Preliminary data analyses were conducted with an analysis of variance (ANOVA) using Statistical Analysis System (SAS, Cary, NY, USA) software (SAS, 2010). The variance effects of SNP genotypes, breeds, post-slaughter treatments and slaughter group effects on the dependent variables or phenotypes were determined, using a linear model. Genotype, breed and treatment effects were included as fixed effects, along with the two-way interactions of genotype with breed, as well as genotype with treatment. Least square means (LSM) of the phenotypes (dependent variables) were estimated and compared at a significance level of α=0.05. These analyses were used for a poster presentation at the International Congress of Meat Science and Technology (ICoMST) (Basson *et al.*, 2016) and to determine LSM for results reported in Chapter 4.

Association analyses were performed using PLINK version 1.9 (Purcell *et al.*, 2007), to provisionally validate the Illumina BovineHD SNP BeadChip for use in South African beef and determine whether enough informative SNP were present in all breeds for meaningful analyses. This utilises a single-trait-single-SNP candidate gene-based association analysis, or least squares regression of the effects of genotypes (the number of copies of alleles) on the beef production and quality phenotypes. Associations were determined in pooled data (for breeds, or for treatment), within each treatment group and within each breed separately. The G×E functionality was used to test interactions between the genotypes and breeds, as well as genotypes and treatments. A Bonferroni correction of *p*-values of 4.39×10^{-4} was used to determine significance (α=0.05) of data analyses for two poster presentations at ICoMST (Basson *et al.*, 2018; Frylinck *et al.*, 2018).

6.2. Mixed linear model association with Benjamini-Hochberg correction (GCTA)

Mixed linear model association (MLMA) analyses (Yang *et al.*, 2014) were conducted using Genome-wide Complex Trait Analysis (GCTA) software (Yang *et al.*, 2011); version 1.92.2 (University of Queensland, Queensland, Australia), which was more suited to complex trait analyses than either PLINK or SAS, using the formula:

$$\boldsymbol{y} = \boldsymbol{a} + \boldsymbol{bx} + \boldsymbol{g} + \boldsymbol{e}$$

In this model, *y* (phenotype) is determined by *a* (the phenotypic mean), *b* (the additive fixed effect of the candidate SNP), *x* (the genotype of the candidate SNP), *g* (the polygenic random effects accumulated from all SNPs captured by the genomic relationship matrix) and *e* (the residual, random effect or error). The model was run within each of the breeds, in pooled data with all breeds as covariates, or with single breeds as covariates to test the interaction of genotypes with breeds. The G×E functionality was also used to test the association of genotypes with ES treatment and much like the analyses in PLINK, limited evidence was found that genotypes interacted with the application of electrical stimulation in beef. This model was used in the data analyses for data reported in (Chapter 4), where correction for multiple testing was determined using Benjamini-Hochberg false discovery rate adjustment, to control false positive associations (Benjamini & Hochberg, 1995).

6.3. Mixed linear model association using principal components 1 – 4, with optimised p*-value correction for each phenotype (FarmCPU and GAPIT)*

Permutation analyses offer a more powerful tool to eliminate false positive associations, or identify false negative associations, by re-assigning SNP to the model of the candidate SNP (permutation), to break the association with genotype and determine the optimal level of probability for each phenotype. The Fixed and random model Circulating Probability Unification (or FarmCPU) software (Liu *et al.*, 2016) that iteratively fits fixed and random models to the data, was used to determine optimised *p*-values. The mean optimal *p*-value for all phenotypes was $p \leq 0.0013$, which was similar to the level of adjustment for putative effects in adjusted Bonferroni correction (α=0.10, ÷ 83 independent SNP). However, this adjustment would only correctly identify 53% of the optimised *p*-values for individual phenotypes and blanket adjustments for all phenotypes were abandoned in favour of the individual optimised *p*-value for all software and models used. These data were reported in Chapter 5.

In addition to this, association analyses were performed on the data within the NS and the ES treatment groups separately using the FarmCPU software, incorporating the principal components from the genomic relationship matrix of the 777K genotypes as covariates in the model. Instead of step-wise testing of other markers that could induce more false negative results (like the MLMM analysis), *p*-values are unified iteratively by fitting fixed– and random effects models to improve the statistical power of the association analyses (Kaler *et al.*, 2020).

The Genome Association and Prediction Integrated Tools package (GAPIT) in R-project (Wang & Zhang, 2021) offers increasingly more complex models for association analyses of SNP data. All these models were run with the first four principal components of the VSGRM matrix (777K) as covariates, using the Model.selection functionality to determine the principal components to include in the models. The generalised linear model (GLM) model of GAPIT performs single-SNP analyses on phenotypes (Prince *et al.*, 2006), including the fixed effect of population structure with the Mixed Linear Model (MLM) (Yu *et al.*, 2006). Although these analyses were useful to confirm associations of SNP with beef growth, carcass and quality traits, the models were a poor fit for the data from some phenotypes (i.e. failed to account for population structure), according to quantile-quantile (QQ) plots. Association analyses performed with Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway (BLINK) (Huang *et al.*, 2018) that identified independent SNP effects and Multi-Locus Mixed Model (MLMM) (Segura *et al.*, 2012) that performs step-wise adjustment for other markers, improved the model fit for association analyses identifying quantitative trait nucleotides (QTN) for beef traits. These two models (BLINK and MLMM) did however not estimate allele effects at the time of analyses and the effects of genotypes on traits were estimated from the MLM model, when found to be significant. Optimised *p*-values from FarmCPU analyses were used to adjust *p*-values for all the data models, with the optimised *p*-value considered a significant association (candidate SNP), while putative effects were considered at a *p*-value that was 2×optimal *p*-value (probative SNP).

As associations of SNPs with a variety of beef quality traits, especially intermediary metabolic effects, could be confounded by a covariant effect of the trait with meat pH, additional analyses were conducted on the associations with energy metabolism. The models were modified to include the observations of pH (1 h, 3 h, 6 h

and 20 h), where generally, only pH_u (20 h) showed a substantial (but small) covariate effect. Including pH_u as a covariate in the models (in addition to PC1–4), did not significantly affect the outcome of the genomic association analyses and it did not seem that these associations were confounded by pH_u.

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Addendum B: Supplementary material for Chapter 3

1. Supplementary figure

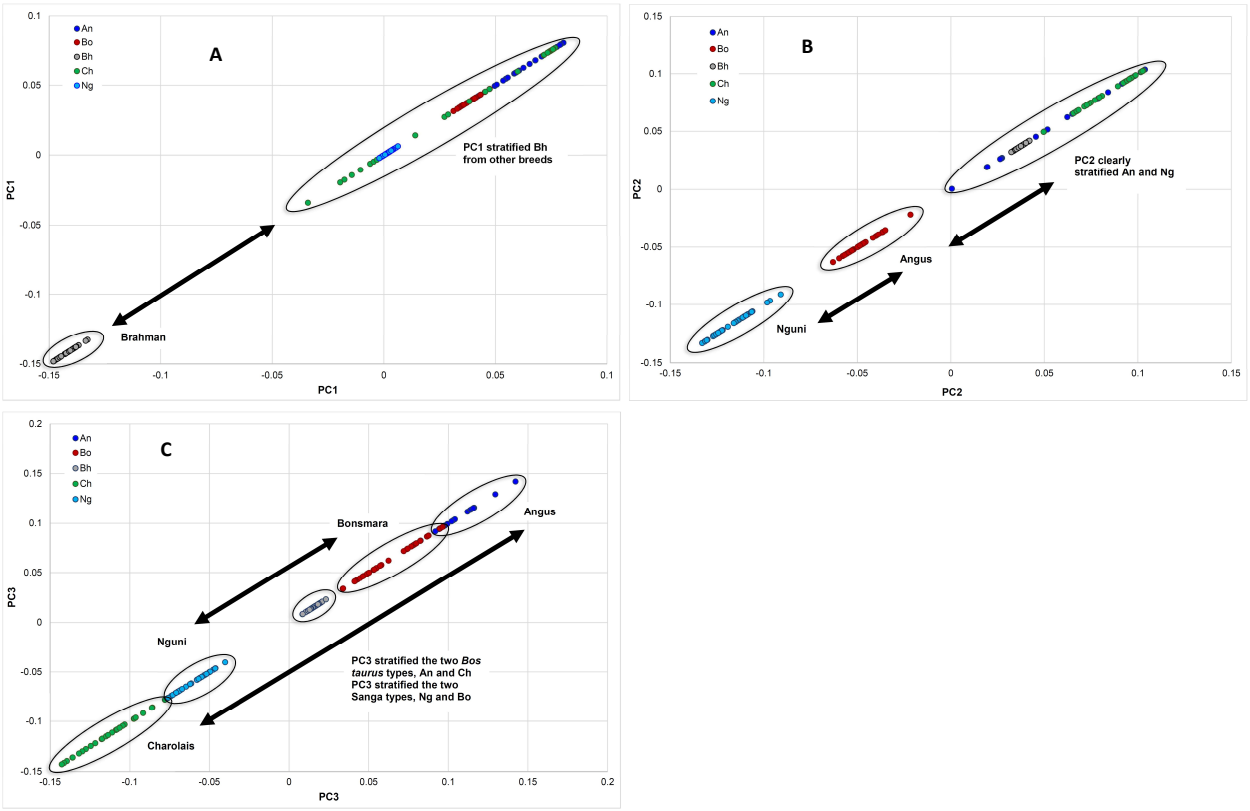

Figure S1. *Breed stratification of South African purebred bulls (n = 166) through principal components analysis (PCA) of 777K Illumina® SNP BeadChip, using single components PC1 (29.34%), PC2 (11.42%) and PC3 (5.34%); An – Angus; Bo – Bonsmara; Bh – Brahman; Ch – Charolais; Ng – Nguni;* ***A****: PC1;* ***B****: PC2;* ***C****: PC3.*

2. Supplementary tables

Table S1. *Minor allelic and genotypic frequencies for SNV identified within the range of the muscle calpain system genes, pooled for South African beef breeds, using Illumina® BovineHD SNP BeadChip (777K).*

Illumina name *cast* gene	Chr	Position	Location	SNV code	[SNP]	MAF (A1%)	A1A1 [1]	A1A2 [1]	A2A2 [1]
BovineHD0700028721	7	98,431,222	rs110062870	*7_721*	[T/G]	41.5%	GG(35)	GT(67)	TT(63)
BovineHD0700028722	7	98,432,644	rs134932261	*7_722*	[T/C]	42.5%	CC(36)	CT(69)	TT(61)
BovineHD0700028723	7	98,435,292	rs135825068	*7_723*	[T/C]	42.5%	TT(36)	TC(69)	CC(61)
BovineHD0700028724	7	98,439,177	rs133855588	*7_724*	[A/G]	17.2%	AA(10)	AG(37)	GG(119)
BovineHD0700028725	7	98,442,449	rs110473478	*7_725*	[T/C]	42.5%	TT(36)	TC(69)	CC(61)
BovineHD0700028726	7	98,448,831	rs132707186	*7_726*	[A/G]	19.3%	GG(11)	GA(42)	AA(113)
BovineHD0700028727	7	98,449,505	rs110540286	*7_727*	[T/C]	19.3%	TT(11)	TC(42)	CC(113)
BovineHD0700028728	7	98,450,117	rs135049475	*7_728*	[T/C]	42.5%	TT(36)	TC(69)	CC(61)
BovineHD0700028729	7	98,454,085	rs109679745	*7_729*	[A/G]	35.5%	GG(24)	GA(70)	AA(72)
BovineHD0700028730	7	98,457,153	rs134804900	*7_730*	[T/C]	23.2%	CC(13)	CT(51)	TT(102)
BovineHD0700028731	7	98,463,330	rs136084722	*7_731*	[A/G]	13.6%	AA(9)	AG(27)	GG(130)
BovineHD0700028732	7	98,466,806	rs133432068	*7_732*	[T/C]	35.5%	CC(24)	CT(70)	TT(72)
BovineHD0700028733	7	98,467,371	rs109932018	*7_733*	[T/C]	35.2%	TT(23)	TC(71)	CC(72)
BovineHD0700028734	7	98,467,934	rs137677027	*7_734*	[T/G]	35.2%	GG(23)	GT(71)	TT(72)
BovineHD0700028735	7	98,471,546	rs109106772	*7_735*	[A/G]	35.5%	AA(24)	AG(70)	GG(72)
BovineHD0700028736	7	98,473,634	rs137217365	*7_736*	[T/G]	27.7%	GG(19)	GT(54)	TT(93)
BovineHD0700028737	7	98,474,995	rs135811099	*7_737*	[T/G]	25.0%	GG(12)	GT(59)	TT(95)
BovineHD0700028738	7	98,476,556	rs109970923	*7_738*	[T/C]	32.8%	CC(21)	CT(67)	TT(78)
BovineHD0700028739	7	98,480,585	rs134897496	*7_739*	[T/C]	20.8%	CC(7)	CT(55)	TT(104)
BovineHD0700028740	7	98,481,274	rs135802918	*7_740*	[A/C]	20.8%	AA(7)	AC(55)	CC(104)
BovineHD4100006349	7	98,482,074	rs41596487	*7_349*	[T/C]	18.4%	CC(6)	CT(49)	TT(111)
BovineHD0700028741	7	98,484,691	rs136939207	*7_741*	[A/C]	21.7%	AA(7)	AC(51)	CC(107)
BovineHD0700028742	7	98,488,255	rs135233602	*7_742*	[T/C]	19.9%	TT(7)	TC(52)	CC(107)
BovineHD0700028743	7	98,492,079	rs137781929	*7_743*	[A/G]	14.2%	GG(5)	GA(37)	AA(123)
BovineHD0700028744	7	98,492,868	rs133927728	*7_744*	[A/G]	14.2%	AA(5)	AG(37)	GG(124)
ARS-BFGL-NGS-43901	7	98,498,047	rs109804679	*7_901*	[T/G]	11.8%	TT(5)	TG(29)	GG(132)
BovineHD0700028746	7	98,498,729	rs135497848	*7_746*	[A/G]	11.8%	AA(5)	AG(29)	GG(132)
BovineHD0700028747	7	98,499,702	rs110707037	*7_747*	[A/C]	21.4%	AA(7)	AC(57)	CC(102)
BovineHD0700028748	7	98,502,599	rs134762812	*7_748*	[T/C]	17.8%	CC(5)	CT(49)	TT(112)
BovineHD0700028749	7	98,506,739	rs136046573	*7_749*	[A/G]	17.2%	AA(5)	AG(47)	GG(114)
BovineHD0700028750	7	98,507,574	rs132701334	*7_750*	[T/G]	20.8%	TT(7)	TG(55)	GG(104)
BovineHD0700028751	7	98,508,282	rs110178428	*7_751*	[A/C]	21.4%	CC(7)	CA(57)	AA(102)
BovineHD0700028752	7	98,508,931	rs135644323	*7_752*	[T/C]	21.4%	CC(7)	CT(57)	TT(102)
BovineHD0700028753	7	98,510,114	rs136632100	*7_753*	[T/C]	0.6%	TT(0)	TC(2)	CC(164)
BovineHD0700028754	7	98,511,880	rs134385243	*7_754*	[T/G]	13.9%	GG(3)	GT(40)	TT(123)
BovineHD0700028755	7	98,512,675	rs135405624	*7_755*	[A/G]	21.4%	GG(7)	GA(57)	AA(102)
BovineHD0700028756	7	98,513,190	rs133290603	*7_756*	[A/G]	21.4%	AA(7)	AG(57)	GG(102)
BovineHD0700028757	7	98,520,428	rs110241720	*7_757*	[T/C]	21.5%	CC(7)	CT(56)	TT(100)
BovineHD0700028758	7	98,524,220	rs135682399	*7_758*	[A/G]	21.4%	AA(7)	AG(57)	GG(102)
BovineHD0700028759	7	98,526,859	rs133488081	*7_759*	[T/C]	7.3%	CC(2)	CT(21)	TT(143)
BovineHD0700028760	7	98,531,321	rs137215768	*7_760*	[A/G]	20.8%	GG(7)	GA(54)	AA(104)
BovineHD0700028761	7	98,531,781	rs136056291	*7_761*	[A/G]	9.0%	GG(3)	GA(24)	AA(139)
BovineHD0700028762	7	98,532,654	rs109938220	*7_762*	[T/C]	20.5%	CC(6)	CT(56)	TT(104)
ARS-USMARC-670	7	98,534,197	rs109677393	*7_670*	[A/G]	20.8%	GG(7)	GA(55)	AA(104)
BovineHD0700028763	7	98,534,736	rs135465452	*7_763*	[T/C]	17.1%	TT(5)	TC(48)	CC(113)
BovineHD0700028764	7	98,537,976	rs109102936	*7_764*	[T/C]	6.6%	CC(2)	CT(18)	TT(146)
BovineHD0700028765	7	98,540,675	rs133997237	*7_765*	[T/C]	6.9%	TT(2)	TC(19)	CC(145)

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Illumina name *cast* gene	Chr	Position	Location	SNV code	[SNP]	MAF (A1%)	A1A1 [1]	A1A2 [1]	A2A2 [1]
BovineHD0700028766	7	98,541,844	rs135693211	*7_766*	[T/G]	20.5%	TT(8)	TG(52)	GG(106)
BovineHD0700028767	7	98,545,774	rs136646587	*7_767*	[T/C]	16.9%	CC(4)	CT(48)	TT(114)
BovineHD0700028768	7	98,547,086	rs110136749	*7_768*	[T/C]	20.5%	CC(8)	CT(52)	TT(106)
BovineHD0700028772	7	98,553,659	rs109087212	*7_772*	[T/C]	20.8%	TT(8)	TC(53)	CC(105)
BovineHD0700028773	7	98,554,459	rs109384915	*7_773*	[A/G]	20.2%	GG(8)	GA(51)	AA(107)
BovineHD0700028774	7	98,557,529	rs134041938	*7_774*	[A/G]	15.4%	AA(3)	AG(45)	GG(118)
BovineHD0700028775	7	98,560,223	rs110733539	*7_775*	[A/G]	18.4%	GG(7)	GA(47)	AA(112)
BovineHD0700028776	7	98,562,742	rs110050430	*7_776*	[A/G]	25.3%	GG(13)	GA(57)	AA(95)
BovineHD0700028777	7	98,563,418	rs110380498	*7_777*	[T/C]	20.6%	CC(8)	CT(53)	TT(105)
ARS-USMARC-116	7	98,566,391	rs109354718	*7_116*	[A/G]	16.6%	GG(5)	GA(45)	AA(116)
BovineHD0700028778	7	98,570,487	rs137558115	*7_778*	[T/G]	22.6%	TT(10)	TG(55)	GG(101)
BovineHD0700028779	7	98,571,597	rs133343732	*7_779*	[T/G]	22.4%	TT(10)	TG(55)	GG(101)
BovineHD0700028780	7	98,574,139	rs135279064	*7_780*	[T/C]	22.6%	TT(10)	TC(55)	CC(101)
BovineHD0700028781	7	98,574,903	rs109843102	*7_781*	[T/C]	22.6%	TT(10)	TC(55)	CC(101)
BovineHD0700028782	7	98,575,799	rs109562954	*7_782*	[T/G]	22.4%	GG(10)	GT(54)	TT(101)
BovineHD0700028783	7	98,576,940	rs110031829	*7_783*	[T/C]	17.5%	CC(5)	CT(48)	TT(113)
BovineHD0700028784	7	98,578,836	rs136073124	*7_784*	[A/G]	21.1%	AA(8)	AG(54)	GG(104)
BovineHD4100006350	7	98,579,574	rs41255587	*7_350*	[A/G]	21.7%	AA(10)	AG(52)	GG(104)
BovineHD0700028786	7	98,585,027	rs110883024	*7_786*	[T/C]	23.2%	TT(10)	TC(57)	CC(99)
BovineHD0700028787	7	98,585,930	rs135444809	*7_787*	[T/C]	23.0%	TT(10)	TC(56)	CC(99)
BovineHD0700028788	7	98,586,431	rs136572376	*7_788*	[A/C]	21.1%	CC(10)	CA(50)	AA(106)
BovineHD0700028789	7	98,589,740	rs133712793	*7_789*	[T/C]	26.5%	CC(13)	CT(62)	TT(91)
BovineHD0700028790	7	98,591,048	rs135081549	*7_790*	[T/C]	17.5%	TT(4)	TC(50)	CC(112)
BovineHD0700028791	7	98,591,826	rs109226849	*7_791*	[T/C]	25.8%	CC(11)	CT(64)	TT(91)
BovineHD0700028792	7	98,592,979	rs110049532	*7_792*	[T/C]	20.3%	CC(6)	CT(56)	TT(104)
BovineHD0700028793	7	98,593,984	rs137368766	*7_793*	[T/C]	19.9%	CC(7)	CT(52)	TT(107)
BovineHD0700028794	7	98,594,835	rs108962480	*7_794*	[T/C]	14.4%	CC(4)	CT(48)	TT(110)
						21.6%	**(36.1%)**	**(30.8%)**	**(33.1%)**

Illumina name *capn3* gene	Chr	Position	Location	SNV code	[SNP]	MAF (A1%)	A1A1 [1]	A1A2 [1]	A2A2 [1]
BovineHD1000011712	10	37,824,551	rs110439595	*10_712*	[A/G]	9.6%	GG(1)	GA(30)	AA(135)
BovineHD1000031347	10	37,827,639	rs135945111	*10_347*	[A/G]	23.8%	GG(25)	GA(29)	AA(112)
ARS-BFGL-NGS-13350	10	37,830,642	rs109425380	*10_350*	[T/C]	16.9%	TT(2)	TC(52)	CC(112)
BovineHD1000011713	10	37,833,640	rs109318676	*10_713*	[A/G]	9.3%	AA(1)	AG(29)	GG(136)
BovineHD1000011714	10	37,836,009	rs109372443	*10_714*	[T/C]	18.7%	CC(8)	CT(46)	TT(112)
BovineHD1000011715	10	37,837,623	rs109290773	*10_715*	[T/C]	4.8%	TT(1)	TC(14)	CC(151)
BovineHD1000011716	10	37,838,570	rs109050259	*10_716*	[A/G]	16.9%	GG(2)	GA(52)	AA(112)
BovineHD1000011718	10	37,843,006	rs109819443	*10_718*	[T/G]	32.5%	TT(20)	TG(68)	GG(78)
BovineHD1000011719	10	37,846,946	rs110452450	*10_719*	[A/G]	43.4%	GG(34)	GA(76)	AA(56)
Hapmap47063-BTA-62293	10	37,852,123	rs41644730	*10_293*	[T/C]	35.8%	TT(26)	TC(67)	CC(73)
BovineHD1000011720	10	37,860,216	rs135593461	*10_720*	[A/G]	35.5%	GG(22)	GA(74)	AA(70)
BovineHD1000011721	10	37,864,319	rs109239406	*10_721*	[A/G]	44.0%	AA(36)	AG(74)	GG(56)
BovineHD1000011722	10	37,869,021	rs134085397	*10_722*	[A/G]	43.7%	AA(34)	AG(77)	GG(55)
BovineHD1000011723	10	37,870,339	rs110307662	*10_723*	[T/C]	18.1%	TT(4)	TC(52)	CC(110)
BovineHD1000011725	10	37,876,596	rs110994653	*10_725*	[T/C]	13.9%	TT(4)	TC(38)	CC(124)
BovineHD1000011726	10	37,877,303	rs109324755	*10_726*	[A/G]	15.1%	GG(4)	GA(42)	AA(120)
BovineHD1000011727	10	37,885,702	rs109474612	*10_727*	[T/G]	12.0%	TT(4)	TG(38)	GG(124)
BovineHD1000011728	10	37,887,422	rs133467689	*10_728*	[T/C]	13.9%	CC(4)	CT(38)	TT(124)
						22.7%	**(32.6%)**	**(29.8%)**	**(37.6%)**

Illumina name *capn2* gene	Chr	Position	Location	SNV code	[SNP]	MAF (A1%)	A1A1 [1]	A1A2 [1]	A2A2 [1]
BovineHD1600007758	16	27,781,212	rs132720146	*16_758*	[T/C]	33.8%	TT(25)	TC(63)	CC(78)
BovineHD1600007759	16	27,786,368	rs134412163	*16_759*	[A/G]	47.6%	GG(42)	GA(74)	AA(50)
BovineHD1600007760	16	27,789,803	rs135593510	*16_760*	[A/G]	42.4%	GG(37)	GA(65)	AA(63)
BovineHD1600007761	16	27,793,409	rs133177149	*16_761*	[T/C]	43.9%	CC(38)	CT(67)	TT(58)
BovineHD1600007762	16	27,798,419	rs134504287	*16_762*	[A/G]	12.8%	AA(7)	AG(33)	GG(125)
BovineHD1600007763	16	27,801,014	rs137662975	*16_763*	[T/C]	47.9%	CC(43)	CT(73)	TT(50)
BovineHD1600007764	16	27,803,013	rs133410746	*16_764*	[A/G]	20.8%	GG(9)	GA(51)	AA(106)
ARS-BFGL-NGS-102216	16	27,805,751	rs109891270	*16_216*	[A/G]	31.0%	GG(21)	GA(61)	AA(84)
BovineHD1600007765	16	27,808,861	rs137301729	*16_765*	[A/G]	32.8%	GG(23)	GA(63)	AA(80)
BovineHD1600007766	16	27,810,205	rs136452103	*16_766*	[A/G]	45.5%	GG(42)	GA(67)	AA(57)
BovineHD1600007767	16	27,810,903	rs133882716	*16_767*	[T/C]	32.2%	CC(22)	CT(63)	TT(81)
BovineHD1600007768	16	27,811,695	rs135482143	*16_768*	[T/C]	21.7%	CC(10)	CT(52)	TT(104)
BovineHD1600007769	16	27,812,241	rs136468320	*16_769*	[T/C]	21.7%	CC(10)	CT(52)	TT(104)
BovineHD4100012416	16	27,812,915	rs41634656	*16_416*	[T/C]	26.5%	CC(18)	CT(52)	TT(96)
BovineHD1600007770	16	27,813,621	rs133943882	*16_770*	[A/G]	36.5%	GG(28)	GA(65)	AA(73)
BovineHD1600007771	16	27,814,339	rs134927379	*16_771*	[A/G]	32.8%	GG(24)	GA(61)	AA(81)
BovineHD1600007772	16	27,815,334	rs137711991	*16_772*	[A/G]	47.9%	GG(43)	GA(73)	AA(50)
BovineHD1600007773	16	27,817,199	rs133964632	*16_773*	[T/C]	27.7%	TT(14)	TC(64)	CC(88)
BovineHD1600007774	16	27,819,457	rs137478059	*16_774*	[A/G]	4.2%	AA(0)	AG(14)	GG(152)
BovineHD1600007775	16	27,820,470	rs132858981	*16_775*	[T/C]	27.7%	TT(14)	TC(64)	CC(88)
BovineHD1600007776	16	27,823,039	rs134023967	*16_776*	[A/G]	34.3%	AA(22)	AG(70)	GG(74)
BovineHD1600007777	16	27,826,235	rs135263847	*16_777*	[T/C]	19.9%	TT(9)	TC(50)	CC(106)
BovineHD1600007778	16	27,828,773	rs132895734	*16_778*	[A/G]	20.8%	GG(9)	GA(51)	AA(106)
BovineHD1600007779	16	27,829,558	rs137097425	*16_779*	[T/C]	3.3%	TT(0)	TC(11)	CC(155)
ARS-BFGL-NGS-85727	16	27,833,776	rs109978733	*16_727*	[A/C]	3.2%	AA(0)	AC(19)	CC(147)
BovineHD1600007780	16	27,836,737	rs135646764	*16_780*	[A/G]	20.8%	GG(15)	GA(39)	AA(112)
BovineHD1600007781	16	27,843,498	rs136736277	*16_781*	[T/C]	28.9%	CC(17)	CT(62)	TT(87)
						28.5%	**(34.2%)**	**(32.8%)**	**(33.0%)**

Illumina name *capn1* gene	Chr	Position	Location	SNV code	[SNP]	MAF (A1%)	A1A1 [1]	A1A2 [1]	A2A2 [1]
BovineHD2900013183	29	44,067,207	rs17872099	*29_183*	[T/C]	46.4%	CC(37)	CT(80)	TT(49)
BovineHD2900013184	29	44,067,968	rs17871986	*29_184*	[A/G]	33.7%	AA(23)	AG(66)	GG(77)
CAPN1_1	29	44,069,063	rs17872000	*29_316*	[C/G]	19.6%	CC(10)	CG(45)	GG(111)
BovineHD2900013185	29	44,070,926	rs110104039	*29_185*	[A/G]	28.3%	AA(23)	AG(48)	GG(95)
BovineHD2900013186	29	44,076,213	rs137225062	*29_186*	[A/C]	38.1%	AA(32)	AC(63)	CC(71)
BovineHD2900013187	29	44,081,056	rs135658374	*29_187*	[T/C]	37.3%	CC(33)	CT(57)	TT(76)
UA-IFASA-1370	29	44,085,769	rs17871058	*29_370*	[A/G]	23.5%	AA(12)	AG(54)	GG(100)
CAPN1_2	29	44,087,629	rs17872050	*29_4751*	[T/C]	49.1%	TT(50)	TC(63)	CC(53)
BovineHD2900013188	29	44,088,897	rs17870847	*29_188*	[A/G]	41.3%	GG(36)	GA(65)	AA(65)
BovineHD2900013189	29	44,093,671	rs134827338	*29_189*	[T/C]	36.7%	CC(29)	CT(63)	TT(74)
BovineHD2900013190	29	44,097,970	rs135736399	*29_190*	[A/G]	40.4%	AA(38)	AG(58)	GG(70)
BovineHD2900013191	29	44,101,417	rs133641190	*29_191*	[T/C]	31.9%	CC(23)	CT(60)	TT(83)
						35.5%	**(26.8%)**	**(36.3%)**	**(36.9%)**

[1] Numbers in parenthesis indicate the number of bulls (n = 166) with the specific genotype; A1 - the minor allele; A2 - the major allele; Chr - chromosome; MAF - minor allelic frequency; [SNP] - alleles of the variant; SNV - single nucleotide variant; SNV code - CHR_last 3 digits of Illumina name; SNV that failed quality control (MAF<0.05, failed Hardy-Weinberg exact test) are highlighted in red.

Table S2. *Minor allelic frequency for single nucleotide variants of the muscle calpain system genes, stratified by South African beef breeds, genotyped using the Illumina® BovineHD SNP BeadChip (777K).*

SNV	Chr	Position	Location	MAF	Angus	Bonsmara	Brahman	Charolais	Nguni
7_721	7	98,431,222	rs110062870	41.5%	18.5%	41.4%	45.7%	42.2%	54.3%
7_722	7	98,432,644	rs134932261	42.5%	18.5%	41.4%	48.6%	44.1%	54.3%
7_723	7	98,435,292	rs135825068	42.5%	18.5%	41.4%	48.6%	44.1%	54.3%
7_724	7	98,439,177	rs133855588	17.2%	3.7%	31.4%	0.0%	17.7%	30.0%
7_725	7	98,442,449	rs110473478	42.5%	18.5%	41.4%	48.6%	44.1%	54.3%
7_726	7	98,448,831	rs132707186	19.3%	14.8%	8.6%	32.9%	14.7%	24.3%
7_727	7	98,449,505	rs110540286	19.3%	14.8%	8.6%	32.9%	14.7%	24.3%
7_728	7	98,450,117	rs135049475	42.5%	18.5%	41.4%	48.6%	44.1%	54.3%
7_729	7	98,454,085	rs109679745	35.5%	18.5%	41.4%	25.7%	39.7%	48.6%
7_730	7	98,457,153	rs134804900	23.2%	3.7%	32.9%	15.7%	29.4%	30.0%
7_731	7	98,463,330	rs136084722	13.6%	1.9%	31.4%	0.0%	4.4%	27.1%
7_732	7	98,466,806	rs133432068	35.5%	18.5%	41.4%	25.7%	39.7%	48.6%
7_733	7	98,467,371	rs109932018	35.2%	18.5%	40.0%	25.7%	39.7%	48.6%
7_734	7	98,467,934	rs137677027	35.2%	18.5%	40.0%	25.7%	39.7%	48.6%
7_735	7	98,471,546	rs109106772	35.5%	18.5%	41.4%	25.7%	39.7%	48.6%
7_736	7	98,473,634	rs137217365	27.7%	16.7%	40.0%	10.0%	23.5%	45.7%
7_737	7	98,474,995	rs135811099	25.0%	16.7%	40.0%	21.4%	14.7%	30.0%
7_738	7	98,476,556	rs109970923	32.8%	16.7%	40.0%	25.7%	29.4%	48.6%
7_739	7	98,480,585	rs134897496	20.8%	16.7%	10.0%	22.9%	32.4%	21.4%
7_740	7	98,481,274	rs135802918	20.8%	16.7%	10.0%	22.9%	32.4%	21.4%
7_349	7	98,482,074	rs41596487	18.4%	16.7%	10.0%	12.9%	30.9%	21.4%
7_741	7	98,484,691	rs136939207	21.7%	18.0%	12.1%	21.9%	32.8%	22.1%
7_742	7	98,488,255	rs135233602	19.9%	16.7%	10.0%	20.0%	30.9%	21.4%
7_743	7	98,492,079	rs137781929	14.2%	16.7%	8.6%	15.7%	28.8%	2.9%
7_744	7	98,492,868	rs133927728	14.2%	16.7%	8.6%	15.7%	27.9%	2.9%
7_901	7	98,498,047	rs109804679	11.8%	16.7%	8.6%	5.7%	26.5%	2.9%
7_746	7	98,498,729	rs135497848	11.8%	16.7%	8.6%	5.7%	26.5%	2.9%
7_747	7	98,499,702	rs110707037	21.4%	16.7%	10.0%	25.7%	35.3%	18.6%
7_748	7	98,502,599	rs134762812	17.8%	16.7%	10.0%	12.9%	30.9%	18.6%
7_749	7	98,506,739	rs136046573	17.2%	16.7%	8.6%	12.9%	29.4%	18.6%
7_750	7	98,507,574	rs132701334	20.8%	16.7%	10.0%	22.9%	35.3%	18.6%
7_751	7	98,508,282	rs110178428	21.4%	16.7%	10.0%	25.7%	35.3%	18.6%
7_752	7	98,508,931	rs135644323	21.4%	16.7%	10.0%	25.7%	35.3%	18.6%
7_753	7	98,510,114	rs136632100	0.6%	0.0%	0.0%	1.4%	1.5%	0.0%
7_754	7	98,511,880	rs134385243	13.9%	13.0%	2.9%	17.1%	20.6%	15.7%
7_755	7	98,512,675	rs135405624	21.4%	16.7%	10.0%	25.7%	35.3%	18.6%
7_756	7	98,513,190	rs133290603	21.4%	16.7%	10.0%	25.7%	35.3%	18.6%
7_757	7	98,520,428	rs110241720	21.5%	16.7%	10.0%	25.0%	37.5%	18.6%
7_758	7	98,524,220	rs135682399	21.4%	16.7%	10.0%	25.7%	35.3%	18.6%
7_759	7	98,526,859	rs133488081	7.3%	3.7%	7.1%	7.4%	14.7%	2.9%
7_760	7	98,531,321	rs137215768	20.8%	14.8%	10.0%	25.7%	33.8%	18.6%
7_761	7	98,531,781	rs136056291	9.0%	14.8%	8.6%	5.7%	14.7%	2.9%
7_762	7	98,532,654	rs109938220	20.5%	14.8%	10.0%	24.3%	33.8%	18.6%
7_670	7	98,534,197	rs109677393	20.8%	14.8%	10.0%	25.7%	33.8%	18.6%
7_763	7	98,534,736	rs135465452	17.1%	14.8%	11.4%	21.2%	19.1%	18.6%
7_764	7	98,537,976	rs109102936	6.6%	11.1%	1.4%	10.0%	11.8%	0.0%
7_765	7	98,540,675	rs133997237	6.9%	11.1%	1.4%	8.6%	14.7%	0.0%

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SNV	Chr	Position	Location	MAF	Angus	Bonsmara	Brahman	Charolais	Nguni
7_766	7	98,541,844	rs135693211	20.5%	14.8%	8.6%	25.7%	33.8%	18.6%
7_767	7	98,545,774	rs136646587	16.9%	14.8%	8.6%	20.0%	22.1%	18.6%
7_768	7	98,547,086	rs110136749	20.5%	14.8%	8.6%	25.7%	33.8%	18.6%
7_769	7	98,551,183	rs137371179	15.9%	3.7%	7.1%	24.3%	23.4%	18.6%
7_770	7	98,551,927	rs132851516	20.6%	14.8%	8.6%	25.0%	35.3%	18.6%
7_771	7	98,552,632	rs109712965	20.8%	14.8%	8.6%	25.7%	35.3%	18.6%
7_772	7	98,553,659	rs109087212	20.8%	14.8%	8.6%	25.7%	35.3%	18.6%
7_773	7	98,554,459	rs109384915	20.2%	14.8%	8.6%	24.3%	33.8%	18.6%
7_774	7	98,557,529	rs134041938	15.4%	3.7%	7.1%	22.9%	22.1%	18.6%
7_775	7	98,560,223	rs110733539	18.4%	14.8%	8.6%	18.6%	30.9%	18.6%
7_776	7	98,562,742	rs110050430	25.3%	40.7%	10.0%	23.5%	37.9%	18.6%
7_777	7	98,563,418	rs110380498	20.6%	13.5%	8.6%	25.7%	35.3%	18.6%
7_116	7	98,566,391	rs109354718	16.6%	3.7%	7.1%	25.7%	25.0%	18.6%
7_778	7	98,570,487	rs137558115	22.6%	14.8%	8.6%	35.7%	33.8%	18.6%
7_779	7	98,571,597	rs133343732	22.4%	14.8%	8.6%	35.7%	33.3%	18.6%
7_780	7	98,574,139	rs135279064	22.6%	14.8%	8.6%	34.3%	35.3%	18.6%
7_781	7	98,574,903	rs109843102	22.6%	14.8%	8.6%	34.3%	35.3%	18.6%
7_782	7	98,575,799	rs109562954	22.4%	14.8%	8.6%	34.3%	35.3%	17.7%
7_783	7	98,576,940	rs110031829	17.5%	14.8%	8.6%	21.4%	23.5%	18.6%
7_784	7	98,578,836	rs136073124	21.1%	14.8%	8.6%	37.1%	25.0%	18.6%
7_350	7	98,579,574	rs41255587	21.7%	14.8%	8.6%	31.4%	33.8%	18.6%
7_786	7	98,585,027	rs110883024	23.2%	14.8%	8.6%	37.1%	35.3%	18.6%
7_787	7	98,585,930	rs135444809	23.0%	14.8%	8.6%	37.1%	34.9%	18.6%
7_788	7	98,586,431	rs136572376	21.1%	14.8%	8.6%	30.0%	32.4%	18.6%
7_789	7	98,589,740	rs133712793	26.5%	38.9%	10.0%	31.4%	36.8%	18.6%
7_790	7	98,591,048	rs135081549	17.5%	14.8%	8.6%	21.4%	23.5%	18.6%
7_791	7	98,591,826	rs109226849	25.8%	40.7%	10.0%	35.7%	27.3%	18.6%
7_792	7	98,592,979	rs110049532	20.3%	14.8%	8.6%	23.5%	35.3%	18.6%
7_793	7	98,593,984	rs137368766	19.9%	14.8%	8.6%	32.9%	23.5%	18.6%
7_794	7	98,594,835	rs108962480	14.4%	10.0%	6.1%	19.7%	16.7%	18.6%
cast			**mean; deviation**	**21.9%**	**15.6% -6.3%**	**15.1% -6.8%**	**24.6% +2.7%**	**30.3% +8.4%**	**22.8% +0.9%**

SNV	Chr	Position	Location	MAF	Angus	Bonsmara	Brahman	Charolais	Nguni
10_712	10	37,824,551	rs110439595	9.6%	20.4%	8.6%	1.4%	19.1%	1.4%
10_347	10	37,827,639	rs135945111	23.8%	9.3%	1.4%	84.3%	11.8%	8.6%
10_350	10	37,830,642	rs109425380	16.9%	20.4%	14.3%	20.0%	22.1%	8.6%
10_713	10	37,833,640	rs109318676	9.3%	20.4%	8.6%	0.0%	19.1%	1.4%
10_714	10	37,836,009	rs109372443	18.7%	22.2%	15.7%	24.3%	23.5%	8.6%
10_715	10	37,837,623	rs109290773	4.8%	1.9%	7.1%	0.0%	13.2%	1.4%
10_716	10	37,838,570	rs109050259	16.9%	20.4%	14.3%	20.0%	22.1%	8.6%
10_718	10	37,843,006	rs109819443	32.5%	16.7%	60.0%	22.9%	19.1%	40.0%
10_719	10	37,846,946	rs110452450	43.4%	37.0%	68.6%	24.3%	36.8%	48.6%
10_293	10	37,852,123	rs41644730	35.8%	50.0%	30.0%	5.7%	48.5%	48.6%
10_720	10	37,860,216	rs135593461	35.5%	38.9%	50.0%	21.4%	27.9%	40.0%
10_721	10	37,864,319	rs109239406	44.0%	20.4%	50.0%	78.6%	26.5%	38.6%
10_722	10	37,869,021	rs134085397	43.7%	59.3%	51.4%	41.4%	32.4%	37.1%
10_723	10	37,870,339	rs110307662	18.1%	9.3%	28.6%	2.9%	30.9%	17.1%
10_725	10	37,876,596	rs110994653	13.9%	22.2%	15.7%	2.9%	13.2%	17.1%
10_726	10	37,877,303	rs109324755	15.1%	22.2%	15.7%	2.9%	13.2%	22.9%
10_727	10	37,885,702	rs109474612	12.0%	22.2%	14.7%	1.5%	9.7%	14.1%
10_728	10	37,887,422	rs133467689	13.9%	22.2%	15.7%	2.9%	13.2%	17.1%
capn3			**mean; deviation**	**23.7%**	**25.5% +1.8%**	**27.3% +3.5%**	**21.0% -2.7%**	**22.9% -0.8%**	**22.3% -1.5%**

SNV	Chr	Position	Location	MAF	Angus	Bonsmara	Brahman	Charolais	Nguni
16_758	16	27,781,212	rs132720146	33.8%	13.0%	48.5%	61.4%	22.7%	18.6%
16_759	16	27,786,368	rs134412163	47.6%	27.8%	61.4%	65.7%	39.7%	38.6%
16_760	16	27,789,803	rs135593510	42.4%	18.5%	57.1%	65.7%	29.7%	34.3%
16_761	16	27,793,409	rs133177149	43.9%	19.2%	58.8%	58.6%	42.4%	34.3%
16_762	16	27,798,419	rs134504287	12.8%	19.2%	4.5%	0.0%	36.4%	5.9%
16_763	16	27,801,014	rs137662975	47.9%	25.9%	61.4%	65.7%	42.7%	38.6%
16_764	16	27,803,013	rs133410746	20.8%	14.8%	42.9%	8.6%	17.7%	18.6%
16_216	16	27,805,751	rs109891270	31.0%	18.5%	57.1%	22.9%	20.6%	32.9%
16_765	16	27,808,861	rs137301729	32.8%	14.8%	51.4%	44.3%	23.5%	25.7%
16_766	16	27,810,205	rs136452103	45.5%	20.4%	58.6%	65.7%	42.7%	34.3%
16_767	16	27,810,903	rs133882716	32.2%	14.8%	51.4%	41.4%	23.5%	25.7%
16_768	16	27,811,695	rs135482143	21.7%	14.8%	42.9%	12.9%	17.7%	18.6%
16_769	16	27,812,241	rs136468320	21.7%	14.8%	42.9%	12.9%	17.7%	18.6%
16_416	16	27,812,915	rs41634656	26.5%	14.8%	51.4%	18.6%	19.1%	25.7%
16_770	16	27,813,621	rs133943882	36.5%	18.5%	57.1%	44.3%	25.0%	32.9%
16_771	16	27,814,339	rs134927379	32.8%	14.8%	51.4%	42.9%	25.0%	25.7%
16_772	16	27,815,334	rs137711991	47.9%	25.9%	61.4%	65.7%	42.7%	38.6%
16_773	16	27,817,199	rs133964632	27.7%	14.8%	42.9%	34.3%	25.0%	18.6%
16_774	16	27,819,457	rs137478059	4.2%	5.6%	2.9%	0.0%	13.2%	0.0%
16_775	16	27,820,470	rs132858981	27.7%	14.8%	42.9%	34.3%	25.0%	18.6%
16_776	16	27,823,039	rs134023967	34.3%	14.8%	42.9%	51.4%	39.7%	18.6%
16_777	16	27,826,235	rs135263847	19.9%	14.8%	42.7%	11.4%	13.6%	15.6%
16_778	16	27,828,773	rs132895734	20.8%	14.8%	42.9%	11.4%	14.7%	18.6%
16_779	16	27,829,558	rs137097425	3.3%	1.9%	1.4%	0.0%	13.2%	0.0%
16_727	16	27,833,776	rs109978733	3.2%	0.0%	0.0%	12.1%	3.7%	0.0%
16_780	16	27,836,737	rs135646764	20.8%	5.6%	14.3%	42.9%	23.5%	14.3%
16_781	16	27,843,498	rs136736277	28.9%	9.3%	48.6%	40.0%	16.2%	25.7%
capn2			**mean; deviation**	**31.6%**	**16.6% -14.9%**	**47.4% +15.8%**	**38.5% +6.9%**	**26.9% -4.6%**	**24.9% -6.7%**

SNV	Chr	Position	Location	MAF	Angus	Bonsmara	Brahman	Charolais	Nguni
29_183	29	44,067,207	rs17872099	46.4%	55.6%	64.3%	24.3%	60.3%	30.0%
29_184	29	44,067,968	rs17871986	33.7%	18.5%	17.1%	74.3%	32.4%	22.9%
29_316	29	44,069,063	rs17872000	19.6%	25.9%	18.6%	0.0%	7.4%	47.1%
29_185	29	44,070,926	rs110104039	28.3%	25.9%	48.6%	0.0%	8.8%	57.1%
29_186	29	44,076,213	rs137225062	38.1%	27.8%	26.5%	80.0%	28.8%	24.3%
29_187	29	44,081,056	rs135658374	37.3%	22.2%	12.9%	90.0%	31.8%	25.7%
29_370	29	44,085,769	rs17871058	23.5%	37.0%	32.9%	11.4%	35.3%	4.3%
29_4751	29	44,087,629	rs17872050	49.1%	40.7%	34.3%	90.0%	48.5%	30.0%
29_188	29	44,088,897	rs17870847	41.3%	37.0%	32.9%	75.7%	44.1%	15.7%
29_189	29	44,093,671	rs134827338	36.7%	22.2%	22.9%	78.6%	25.8%	30.0%
29_190	29	44,097,970	rs135736399	40.4%	29.6%	24.3%	90.0%	25.0%	30.0%
29_191	29	44,101,417	rs133641190	31.9%	22.2%	22.9%	67.1%	25.0%	20.0%
capn1			**mean; deviation**	**35.5%**	**30.4% -5.1%**	**29.8% -5.7%**	**56.8% +21.3%**	**31.1% -4.4%**	**28.1% -7.4%**

Chr - chromosome; MAF - pooled minor allelic frequency for all breeds; SNV - single nucleotide variant coded as CHR_last 3 digits of Illumina name; SNV that failed quality control (MAF<0.05, failed Hardy-Weinberg exact test) are highlighted in red; deviation of >10% from pooled data were highlighted in grey and greater than 20% in yellow; Where the allele designated A1 in pooled data was not the minor allele in the specific breed, text was edited to bold and underlined.

Table S3. *Linkage disequilibrium (r^2) of the muscle calpain system genes (*capn1*,* capn2*,* capn3 *and* cast*) in South African beef breeds, genotyped using the Illumina® BovineHD SNP BeadChip (777K).*

SNV	Chr	Position	Location	Mean [1]	Angus	Bonsmara	Brahman	Charolais	Nguni
7_721	7	98,431,222	rs110062870	0.330	0.741	0.285	0.274	0.459	0.293
7_722	7	98,432,644	rs134932261	0.368	0.741	0.285	0.346	0.544	0.293
7_723	7	98,435,292	rs135825068	0.368	0.741	0.285	0.346	0.544	0.293
7_724	7	98,439,177	rs133855588	0.081	[2] n/a	0.176	n/a	0.142	0.180
7_725	7	98,442,449	rs110473478	0.368	0.741	0.285	0.346	0.544	0.293
7_726	7	98,448,831	rs132707186	0.235	0.807	0.722	0.123	0.075	0.355
7_727	7	98,449,505	rs110540286	0.235	0.807	0.722	0.123	0.075	0.355
7_728	7	98,450,117	rs135049475	0.368	0.741	0.285	0.346	0.544	0.293
7_729	7	98,454,085	rs109679745	0.415	0.741	0.285	0.608	0.602	0.331
7_730	7	98,457,153	rs134804900	0.119	n/a	0.165	0.283	0.321	0.180
7_731	7	98,463,330	rs136084722	0.095	n/a	0.176	n/a	n/a	0.159
7_732	7	98,466,806	rs133432068	0.415	0.741	0.285	0.608	0.602	0.331
7_733	7	98,467,371	rs109932018	0.418	0.741	0.290	0.608	0.602	0.331
7_734	7	98,467,934	rs137677027	0.418	0.741	0.290	0.608	0.602	0.331
7_735	7	98,471,546	rs109106772	0.415	0.741	0.285	0.608	0.602	0.331
7_736	7	98,473,634	rs137217365	0.206	0.737	0.290	0.302	0.261	0.218
7_737	7	98,474,995	rs135811099	0.148	0.737	0.290	0.477	0.070	0.165
7_738	7	98,476,556	rs109970923	0.341	0.737	0.290	0.608	0.365	0.331
7_739	7	98,480,585	rs134897496	0.591	0.796	0.686	0.554	0.606	0.639
7_740	7	98,481,274	rs135802918	0.591	0.796	0.686	0.554	0.606	0.639
7_349	7	98,482,074	rs41596487	0.509	0.796	0.686	0.293	0.565	0.639
7_741	7	98,484,691	rs136939207	0.580	0.796	0.686	0.534	0.594	0.630
7_742	7	98,488,255	rs135233602	0.582	0.796	0.686	0.534	0.594	0.639
7_743	7	98,492,079	rs137781929	0.429	0.796	0.722	0.425	0.514	n/a
7_744	7	98,492,868	rs133927728	0.429	0.796	0.722	0.425	0.518	n/a
7_901	7	98,498,047	rs109804679	0.358	0.796	0.722	0.202	0.485	n/a
7_746	7	98,498,729	rs135497848	0.358	0.796	0.722	0.202	0.485	n/a
7_747	7	98,499,702	rs110707037	0.631	0.796	0.686	0.608	0.668	0.719
7_748	7	98,502,599	rs134762812	0.523	0.796	0.686	0.293	0.565	0.719
7_749	7	98,506,739	rs136046573	0.539	0.796	0.722	0.353	0.556	0.719
7_750	7	98,507,574	rs132701334	0.610	0.796	0.686	0.515	0.668	0.719
7_751	7	98,508,282	rs110178428	0.631	0.796	0.686	0.608	0.668	0.719
7_752	7	98,508,931	rs135644323	0.631	0.796	0.686	0.608	0.668	0.719
7_753	7	98,510,114	rs136632100	n/a	n/a	n/a	n/a	n/a	n/a
7_754	7	98,511,880	rs134385243	0.320	0.571	n/a	0.309	0.260	0.590
7_755	7	98,512,675	rs135405624	0.631	0.796	0.686	0.608	0.668	0.719
7_756	7	98,513,190	rs133290603	0.631	0.796	0.686	0.608	0.668	0.719
7_757	7	98,520,428	rs110241720	0.631	0.796	0.686	0.603	0.672	0.719
7_758	7	98,524,220	rs135682399	0.631	0.796	0.686	0.608	0.668	0.719
7_759	7	98,526,859	rs133488081	0.258	n/a	0.606	0.240	0.336	n/a
7_760	7	98,531,321	rs137215768	0.630	0.807	0.686	0.608	0.660	0.719
7_761	7	98,531,781	rs136056291	0.320	0.807	0.722	0.202	0.336	n/a
7_762	7	98,532,654	rs109938220	0.624	0.807	0.686	0.576	0.661	0.719
7_670	7	98,534,197	rs109677393	0.630	0.807	0.686	0.608	0.661	0.719
7_763	7	98,534,736	rs135465452	0.536	0.807	0.587	0.577	0.422	0.719
7_764	7	98,537,976	rs109102936	0.147	0.587	n/a	0.145	0.179	n/a
7_765	7	98,540,675	rs133997237	0.161	0.587	n/a	0.109	0.234	n/a

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SNV	Chr	Position	Location	Mean [1]	Angus	Bonsmara	Brahman	Charolais	Nguni
7_766	7	98,541,844	rs135693211	0.612	0.807	0.722	0.556	0.616	0.719
7_767	7	98,545,774	rs136646587	0.553	0.807	0.722	0.505	0.459	0.719
7_768	7	98,547,086	rs110136749	0.612	0.807	0.722	0.556	0.616	0.719
7_769	7	98,551,183	rs137371179	0.452	n/a	0.606	0.577	0.363	0.719
7_770	7	98,551,927	rs132851516	0.607	0.807	0.722	0.556	0.603	0.719
7_771	7	98,552,632	rs109712965	0.607	0.807	0.722	0.556	0.603	0.719
7_772	7	98,553,659	rs109087212	0.607	0.807	0.722	0.556	0.603	0.719
7_773	7	98,554,459	rs109384915	0.616	0.807	0.722	0.577	0.616	0.719
7_774	7	98,557,529	rs134041938	0.472	n/a	0.606	0.548	0.459	0.719
7_775	7	98,560,223	rs110733539	0.572	0.807	0.722	0.453	0.557	0.719
7_776	7	98,562,742	rs110050430	0.462	0.249	0.607	0.571	0.580	0.719
7_777	7	98,563,418	rs110380498	0.607	0.807	0.722	0.556	0.603	0.719
7_116	7	98,566,391	rs109354718	0.457	n/a	0.606	0.556	0.402	0.719
7_778	7	98,570,487	rs137558115	0.600	0.807	0.722	0.518	0.661	0.719
7_779	7	98,571,597	rs133343732	0.600	0.807	0.722	0.518	0.661	0.719
7_780	7	98,574,139	rs135279064	0.581	0.807	0.722	0.440	0.647	0.719
7_781	7	98,574,903	rs109843102	0.581	0.807	0.722	0.440	0.647	0.719
7_782	7	98,575,799	rs109562954	0.580	0.807	0.722	0.440	0.647	0.718
7_783	7	98,576,940	rs110031829	0.543	0.807	0.722	0.468	0.458	0.719
7_784	7	98,578,836	rs136073124	0.529	0.807	0.722	0.514	0.438	0.719
7_350	7	98,579,574	rs41255587	0.571	0.807	0.722	0.395	0.632	0.719
7_786	7	98,585,027	rs110883024	0.594	0.807	0.722	0.514	0.647	0.719
7_787	7	98,585,930	rs135444809	0.593	0.807	0.722	0.514	0.645	0.719
7_788	7	98,586,431	rs136572376	0.578	0.807	0.722	0.405	0.648	0.719
7_789	7	98,589,740	rs133712793	0.453	0.267	0.607	0.410	0.624	0.719
7_790	7	98,591,048	rs135081549	0.531	0.807	0.722	0.436	0.437	0.719
7_791	7	98,591,826	rs109226849	0.405	0.249	0.607	0.471	0.457	0.719
7_792	7	98,592,979	rs110049532	0.596	0.807	0.722	0.461	0.647	0.719
7_793	7	98,593,984	rs137368766	0.514	0.807	0.722	0.418	0.437	0.719
7_794	7	98,594,835	rs108962480	0.534	0.807	0.718	0.436	0.442	0.719
				0.475	**0.757**	**0.601**	**0.460**	**0.520**	**0.595**

SNV	Chr	Position	Location	Mean	Angus	Bonsmara	Brahman	Charolais	Nguni
10_712	10	37,824,551	rs110439595	0.224	0.313	0.264	n/a	0.448	n/a
10_347	10	37,827,639	rs135945111	n/a	n/a	n/a	n/a	n/a	n/a
10_350	10	37,830,642	rs109425380	0.226	0.313	0.339	0.314	0.417	0.182
10_713	10	37,833,640	rs109318676	0.218	0.313	0.264	n/a	0.448	n/a
10_714	10	37,836,009	rs109372443	0.201	0.290	0.307	0.247	0.393	0.182
10_715	10	37,837,623	rs109290773	n/a	n/a	0.263	n/a	0.457	n/a
10_716	10	37,838,570	rs109050259	0.226	0.313	0.339	0.314	0.417	0.182
10_718	10	37,843,006	rs109819443	0.128	0.122	0.261	0.281	0.124	0.174
10_719	10	37,846,946	rs110452450	0.194	0.304	0.215	0.321	0.299	0.231
10_293	10	37,852,123	rs41644730	0.137	0.263	0.213	0.047	0.218	0.223
10_720	10	37,860,216	rs135593461	0.181	0.258	0.284	0.262	0.192	0.260
10_721	10	37,864,319	rs109239406	0.159	0.125	0.313	0.079	0.174	0.239
10_722	10	37,869,021	rs134085397	0.131	0.097	0.298	0.114	0.214	0.244
10_723	10	37,870,339	rs110307662	0.147	0.037	0.255	n/a	0.191	0.256
10_725	10	37,876,596	rs110994653	0.248	0.246	0.339	n/a	0.457	0.256
10_726	10	37,877,303	rs109324755	0.231	0.246	0.339	n/a	0.457	0.215
10_727	10	37,885,702	rs109474612	0.248	0.246	0.339	n/a	0.457	0.256
10_728	10	37,887,422	rs133467689	0.248	0.246	0.339	n/a	0.457	0.256
				0.197	**0.233**	**0.292**	**0.220**	**0.342**	**0.225**

SNV	Chr	Position	Location	Mean [1]	Angus	Bonsmara	Brahman	Charolais	Nguni
16_758	16	27,781,212	rs132720146	0.341	0.429	0.583	0.339	0.149	0.422
16_759	16	27,786,368	rs134412163	0.443	0.457	0.566	0.405	0.393	0.539
16_760	16	27,789,803	rs135593510	0.411	0.516	0.621	0.405	0.209	0.608
16_761	16	27,793,409	rs133177149	0.486	0.595	0.608	0.386	0.450	0.608
16_762	16	27,798,419	rs134504287	0.075	0.048	n/a	n/a	0.210	0.028
16_763	16	27,801,014	rs137662975	0.460	0.492	0.566	0.405	0.451	0.539
16_764	16	27,803,013	rs133410746	0.427	0.704	0.615	0.208	0.380	0.610
16_216	16	27,805,751	rs109891270	0.436	0.646	0.625	0.251	0.428	0.620
16_765	16	27,808,861	rs137301729	0.520	0.704	0.659	0.397	0.489	0.655
16_766	16	27,810,205	rs136452103	0.488	0.613	0.613	0.405	0.451	0.608
16_767	16	27,810,903	rs133882716	0.515	0.704	0.659	0.368	0.489	0.655
16_768	16	27,811,695	rs135482143	0.455	0.704	0.615	0.230	0.475	0.610
16_769	16	27,812,241	rs136468320	0.455	0.704	0.615	0.230	0.475	0.610
16_416	16	27,812,915	rs41634656	0.462	0.704	0.659	0.217	0.466	0.655
16_770	16	27,813,621	rs133943882	0.501	0.646	0.625	0.397	0.493	0.620
16_771	16	27,814,339	rs134927379	0.516	0.704	0.659	0.371	0.493	0.655
16_772	16	27,815,334	rs137711991	0.460	0.492	0.566	0.405	0.451	0.539
16_773	16	27,817,199	rs133964632	0.482	0.704	0.615	0.321	0.493	0.610
16_774	16	27,819,457	rs137478059	n/a	0.241	n/a	n/a	0.402	n/a
16_775	16	27,820,470	rs132858981	0.482	0.704	0.615	0.321	0.493	0.610
16_776	16	27,823,039	rs134023967	0.443	0.704	0.615	0.310	0.435	0.610
16_777	16	27,826,235	rs135263847	0.439	0.704	0.615	0.209	0.425	0.610
16_778	16	27,828,773	rs132895734	0.441	0.704	0.615	0.209	0.439	0.610
16_779	16	27,829,558	rs137097425	n/a	n/a	n/a	n/a	0.402	n/a
16_727	16	27,833,776	rs109978733	n/a	n/a	n/a	0.084	n/a	n/a
16_780	16	27,836,737	rs135646764	0.112	0.128	0.088	0.206	0.158	0.133
16_781	16	27,843,498	rs136736277	0.342	0.429	0.583	0.172	0.140	0.655
				0.425	**0.567**	**0.591**	**0.302**	**0.398**	**0.559**

SNV	Chr	Position	Location	Mean [1]	Angus	Bonsmara	Brahman	Charolais	Nguni
29_183	29	44,067,207	rs17872099	0.088	0.231	0.201	0.389	0.100	0.091
29_184	29	44,067,968	rs17871986	0.189	0.081	0.085	0.401	0.084	0.376
29_316	29	44,069,063	rs17872000	0.181	0.244	0.125	n/a	0.106	0.262
29_185	29	44,070,926	rs110104039	0.226	0.244	0.233	n/a	0.109	0.337
29_186	29	44,076,213	rs137225062	0.263	0.035	0.424	0.479	0.131	0.421
29_187	29	44,081,056	rs135658374	0.291	0.169	0.039	0.424	0.330	0.466
29_370	29	44,085,769	rs17871058	0.119	0.339	0.436	0.199	0.191	n/a
29_4751	29	44,087,629	rs17872050	0.412	0.389	0.459	0.424	0.299	0.548
29_188	29	44,088,897	rs17870847	0.298	0.339	0.436	0.145	0.262	0.262
29_189	29	44,093,671	rs134827338	0.373	0.305	0.419	0.486	0.328	0.548
29_190	29	44,097,970	rs135736399	0.394	0.356	0.432	0.424	0.328	0.548
29_191	29	44,101,417	rs133641190	0.310	0.305	0.419	0.323	0.328	0.330
				0.262	**0.253**	**0.309**	**0.369**	**0.216**	**0.381**

[1] Mean – pooled data for all breeds; [2] n/a – LD could not be determined (one allele was fixed), or single nucleotide variant failed quality control; Chr – chromosome; SNV – single nucleotide variant coded as CHR_last 3 digits of Illumina name.

***Table S4**. Individual linkage disequilibrium between SNPs of the muscle calpain system genes (*capn1, capn2, capn3 *and* cast *genes) in South African beef breeds, genotyped using the Illumina® BovineHD SNP BeadChip (777K).*

A–

***cast* D' (0.85)**

r^2 (0.48)

	7_721	7_722	7_723	7_724	7_725	7_726	7_727	7_728	7_729	7_730	7_731	7_732	7_733	7_734	7_735	7_736	7_737	7_738	7_739	7_740	7_349	7_741	7_742	7_743	7_744	7_901	7_746	7_747	7_748	7_749	7_750	7_751	7_752	7_754	7_755	7_756	7_757	7_758	7_759	7_760
7_721		1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	0.89	1.00	0.94	0.94	0.94	0.94	0.98	0.95	0.93	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.88	1.00	1.00	0.88	0.88	0.88	0.85	0.88	0.88	0.88	0.88	1.00	0.88
7_722	0.95		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
7_723	0.95	1.00		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
7_724	0.29	0.28	0.28		1.00	1.00	1.00	1.00	1.00	0.98	1.00	1.00	1.00	1.00	1.00	0.76	0.72	0.82	0.16	0.16	0.03	0.06	0.06	0.02	0.02	0.11	0.11	0.11	0.04	0.03	0.06	0.11	0.11	0.01	0.11	0.11	0.09	0.11	0.45	0.06
7_725	0.95	1.00	1.00	0.28		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
7_726	0.34	0.32	0.32	0.05	0.32		1.00	1.00	0.39	1.00	1.00	0.39	0.40	0.40	0.39	0.43	0.21	0.44	0.57	0.57	0.59	0.55	0.56	0.37	0.37	0.42	0.42	0.52	0.57	0.60	0.50	0.52	0.52	0.40	0.52	0.52	0.52	0.52	0.46	0.50
7_727	0.34	0.32	0.32	0.05	0.32	1.00		1.00	0.39	1.00	1.00	0.39	0.40	0.40	0.39	0.43	0.21	0.44	0.57	0.57	0.59	0.55	0.56	0.37	0.37	0.42	0.42	0.52	0.57	0.60	0.50	0.52	0.52	0.40	0.52	0.52	0.52	0.52	0.46	0.50
7_728	0.95	1.00	1.00	0.28	1.00	0.32	0.32		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
7_729	0.69	0.75	0.75	0.38	0.75	0.07	0.07	0.75		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
7_730	0.34	0.41	0.41	0.65	0.41	0.07	0.07	0.41	0.55		1.00	1.00	0.98	0.98	1.00	0.51	0.68	0.81	0.22	0.22	0.14	0.18	0.18	0.30	0.31	0.18	0.18	0.30	0.16	0.12	0.27	0.30	0.30	0.32	0.30	0.30	0.31	0.30	0.17	0.30
7_731	0.22	0.21	0.21	0.76	0.21	0.04	0.04	0.21	0.28	0.52		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
7_732	0.69	0.75	0.75	0.38	0.75	0.07	0.07	0.75	1.00	0.55	0.28		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
7_733	0.68	0.74	0.74	0.38	0.74	0.07	0.07	0.74	0.99	0.53	0.29	0.99		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
7_734	0.68	0.74	0.74	0.38	0.74	0.07	0.07	0.74	0.99	0.53	0.29	0.99	1.00		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
7_735	0.69	0.75	0.75	0.38	0.75	0.07	0.07	0.75	1.00	0.55	0.28	1.00	0.99	0.99		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
7_736	0.52	0.52	0.52	0.31	0.52	0.12	0.12	0.52	0.70	0.21	0.41	0.70	0.70	0.70	0.70		0.79	1.00	0.49	0.49	0.63	0.53	0.54	0.34	0.34	0.52	0.52	0.43	0.62	0.63	0.46	0.43	0.43	0.32	0.43	0.43	0.42	0.43	0.71	0.44
7_737	0.43	0.45	0.45	0.32	0.45	0.03	0.03	0.45	0.61	0.42	0.47	0.61	0.61	0.61	0.61	0.54		1.00	0.35	0.35	0.27	0.31	0.31	0.44	0.44	0.32	0.32	0.34	0.26	0.24	0.31	0.34	0.34	0.19	0.34	0.34	0.35	0.34	0.44	0.34
7_738	0.60	0.66	0.66	0.28	0.66	0.09	0.09	0.66	0.89	0.40	0.32	0.89	0.90	0.90	0.89	0.78	0.68		0.79	0.79	0.76	0.78	0.78	0.67	0.67	0.60	0.60	0.80	0.75	0.74	0.79	0.80	0.80	0.70	0.80	0.80	0.80	0.80	1.00	0.82
7_739	0.38	0.36	0.36	0.00	0.36	0.30	0.30	0.36	0.48	0.04	0.04	0.48	0.48	0.48	0.48	0.16	0.10	0.34		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.96	1.00	1.00	0.93	0.96	0.96	0.91	0.96	0.96	0.96	0.96	1.00	0.93
7_740	0.38	0.36	0.36	0.00	0.36	0.30	0.30	0.36	0.48	0.04	0.04	0.48	0.48	0.48	0.48	0.16	0.10	0.34	1.00		1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.96	1.00	1.00	0.93	0.96	0.96	0.91	0.96	0.96	0.96	0.96	1.00	0.93
7_349	0.32	0.31	0.31	0.00	0.31	0.33	0.33	0.31	0.41	0.01	0.04	0.41	0.41	0.41	0.41	0.23	0.05	0.27	0.86	0.86		0.94	0.94	0.81	0.81	1.00	1.00	0.96	1.00	0.96	0.96	0.96	0.96	0.74	0.96	0.96	0.96	0.96	0.94	0.91
7_741	0.37	0.35	0.35	0.00	0.35	0.29	0.29	0.35	0.48	0.03	0.04	0.48	0.48	0.48	0.48	0.20	0.08	0.33	0.94	0.94	0.79		1.00	1.00	1.00	1.00	1.00	0.96	0.93	1.00	0.92	0.96	0.96	0.84	0.96	0.96	0.96	0.96	1.00	0.92
7_742	0.36	0.34	0.34	0.00	0.34	0.30	0.30	0.34	0.45	0.03	0.04	0.45	0.46	0.46	0.45	0.19	0.07	0.31	0.95	0.95	0.80	1.00		1.00	1.00	1.00	1.00	0.96	0.94	1.00	0.92	0.96	0.96	0.82	0.96	0.96	0.96	0.96	1.00	0.92
7_743	0.24	0.22	0.22	0.00	0.22	0.09	0.09	0.22	0.30	0.05	0.03	0.30	0.30	0.30	0.30	0.05	0.10	0.15	0.63	0.63	0.48	0.66	0.66		1.00	1.00	1.00	1.00	0.81	0.87	0.94	1.00	1.00	0.37	1.00	1.00	1.00	1.00	1.00	0.94
7_744	0.24	0.22	0.22	0.00	0.22	0.09	0.09	0.22	0.30	0.05	0.03	0.30	0.30	0.30	0.30	0.05	0.10	0.15	0.63	0.63	0.48	0.66	0.67	1.00		1.00	1.00	1.00	0.81	0.87	0.94	1.00	1.00	0.37	1.00	1.00	1.00	1.00	1.00	0.94
7_901	0.19	0.18	0.18	0.01	0.18	0.10	0.10	0.18	0.24	0.02	0.02	0.24	0.25	0.25	0.24	0.09	0.04	0.10	0.51	0.51	0.59	0.53	0.54	0.81	0.81		1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.29	1.00	1.00	1.00	1.00	0.95	0.93
7_746	0.19	0.18	0.18	0.01	0.18	0.10	0.10	0.18	0.24	0.02	0.02	0.24	0.25	0.25	0.24	0.09	0.04	0.10	0.51	0.51	0.59	0.53	0.54	0.81	0.81	1.00		1.00	1.00	1.00	1.00	1.00	1.00	0.29	1.00	1.00	1.00	1.00	0.95	0.93
7_747	0.30	0.37	0.37	0.00	0.37	0.24	0.24	0.37	0.49	0.08	0.04	0.49	0.50	0.50	0.49	0.13	0.09	0.36	0.89	0.89	0.76	0.85	0.84	0.61	0.61	0.49	0.49		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
7_748	0.31	0.29	0.29	0.00	0.29	0.29	0.29	0.29	0.39	0.02	0.03	0.39	0.40	0.40	0.39	0.21	0.05	0.25	0.82	0.82	0.96	0.76	0.76	0.50	0.50	0.62	0.62	0.79		0.96	1.00	1.00	1.00	0.74	1.00	1.00	1.00	1.00	0.94	0.96
7_749	0.30	0.28	0.28	0.00	0.28	0.31	0.31	0.28	0.38	0.01	0.03	0.38	0.38	0.38	0.38	0.21	0.04	0.23	0.79	0.79	0.84	0.83	0.84	0.60	0.60	0.64	0.64	0.76	0.88		0.95	1.00	1.00	0.63	1.00	1.00	1.00	1.00	1.00	0.95
7_750	0.29	0.36	0.36	0.00	0.36	0.23	0.23	0.36	0.48	0.06	0.04	0.48	0.48	0.48	0.48	0.15	0.08	0.34	0.86	0.86	0.79	0.82	0.80	0.56	0.56	0.51	0.51	0.96	0.82	0.72		1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	0.96
7_751	0.30	0.37	0.37	0.00	0.37	0.24	0.24	0.37	0.49	0.08	0.04	0.49	0.50	0.50	0.49	0.13	0.09	0.36	0.89	0.89	0.76	0.85	0.84	0.61	0.61	0.49	0.49	1.00	0.79	0.76	0.96		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
7_752	0.30	0.37	0.37	0.00	0.37	0.24	0.24	0.37	0.49	0.08	0.04	0.49	0.50	0.50	0.49	0.13	0.09	0.36	0.89	0.89	0.76	0.85	0.84	0.61	0.61	0.49	0.49	1.00	0.79	0.76	0.96	1.00		1.00	1.00	1.00	1.00	1.00	1.00	1.00
7_754	0.17	0.22	0.22	0.00	0.22	0.11	0.11	0.22	0.29	0.05	0.03	0.29	0.30	0.30	0.29	0.04	0.02	0.16	0.51	0.51	0.39	0.45	0.44	0.14	0.14	0.07	0.07	0.59	0.41	0.31	0.61	0.59	0.59		1.00	1.00	1.00	1.00	1.00	0.94
7_755	0.30	0.37	0.37	0.00	0.37	0.24	0.24	0.37	0.49	0.08	0.04	0.49	0.50	0.50	0.49	0.13	0.09	0.36	0.89	0.89	0.76	0.85	0.84	0.61	0.61	0.49	0.49	1.00	0.79	0.76	0.96	1.00	1.00	0.59		1.00	1.00	1.00	1.00	1.00
7_756	0.30	0.37	0.37	0.00	0.37	0.24	0.24	0.37	0.49	0.08	0.04	0.49	0.50	0.50	0.49	0.13	0.09	0.36	0.89	0.89	0.76	0.85	0.84	0.61	0.61	0.49	0.49	1.00	0.79	0.76	0.96	1.00	1.00	0.59	1.00		1.00	1.00	1.00	1.00
7_757	0.30	0.37	0.37	0.00	0.37	0.24	0.24	0.37	0.50	0.08	0.04	0.50	0.50	0.50	0.50	0.13	0.10	0.36	0.89	0.89	0.75	0.85	0.84	0.62	0.62	0.50	0.50	1.00	0.79	0.76	0.96	1.00	1.00	0.59	1.00	1.00		1.00	1.00	1.00
7_758	0.30	0.37	0.37	0.00	0.37	0.24	0.24	0.37	0.49	0.08	0.04	0.49	0.50	0.50	0.49	0.13	0.09	0.36	0.89	0.89	0.76	0.85	0.84	0.61	0.61	0.49	0.49	1.00	0.79	0.76	0.96	1.00	1.00	0.59	1.00	1.00	1.00		1.00	1.00
7_759	0.11	0.11	0.11	0.00	0.11	0.07	0.07	0.11	0.14	0.01	0.01	0.14	0.15	0.15	0.14	0.10	0.05	0.16	0.30	0.30	0.31	0.32	0.32	0.48	0.48	0.53	0.53	0.29	0.32	0.38	0.26	0.29	0.29	0.01	0.29	0.29	0.30	0.29		1.00
7_760	0.29	0.36	0.36	0.00	0.36	0.23	0.23	0.36	0.48	0.08	0.04	0.48	0.48	0.48	0.48	0.13	0.09	0.36	0.86	0.86	0.72	0.82	0.80	0.56	0.56	0.44	0.44	0.96	0.75	0.72	0.93	0.96	0.96	0.54	0.96	0.96	0.96	0.96	0.30	
7_761	0.14	0.14	0.14	0.01	0.14	0.16	0.16	0.14	0.18	0.00	0.02	0.18	0.18	0.18	0.18	0.18	0.10	0.20	0.38	0.38	0.44	0.40	0.40	0.60	0.60	0.75	0.75	0.37	0.46	0.48	0.38	0.37	0.37	0.01	0.37	0.37	0.37	0.37	0.71	0.38
7_762	0.28	0.35	0.35	0.00	0.35	0.24	0.24	0.35	0.47	0.08	0.04	0.47	0.47	0.47	0.47	0.14	0.10	0.35	0.84	0.84	0.73	0.80	0.79	0.57	0.57	0.45	0.45	0.95	0.77	0.73	0.91	0.95	0.95	0.52	0.95	0.95	0.95	0.95	0.31	0.98
7_670	0.29	0.36	0.36	0.00	0.36	0.23	0.23	0.36	0.48	0.08	0.04	0.48	0.48	0.48	0.48	0.13	0.09	0.36	0.86	0.86	0.72	0.82	0.80	0.56	0.56	0.44	0.44	0.96	0.75	0.72	0.93	0.96	0.96	0.54	0.96	0.96	0.96	0.96	0.30	1.00
7_763	0.25	0.26	0.26	0.04	0.26	0.31	0.31	0.26	0.35	0.00	0.03	0.35	0.36	0.36	0.35	0.22	0.12	0.40	0.72	0.72	0.57	0.75	0.76	0.45	0.45	0.32	0.32	0.74	0.60	0.64	0.72	0.74	0.74	0.33	0.74	0.74	0.73	0.74	0.38	0.77
7_764	0.06	0.10	0.10	0.01	0.10	0.01	0.01	0.10	0.13	0.06	0.01	0.13	0.13	0.13	0.13	0.00	0.04	0.02	0.20	0.20	0.18	0.14	0.13	0.22	0.22	0.17	0.17	0.26	0.19	0.17	0.20	0.26	0.26	0.35	0.26	0.26	0.27	0.26	0.00	0.27
7_765	0.03	0.08	0.08	0.02	0.08	0.00	0.00	0.08	0.11	0.05	0.01	0.11	0.11	0.11	0.11	0.00	0.01	0.02	0.16	0.16	0.14	0.18	0.17	0.28	0.28	0.23	0.23	0.24	0.14	0.21	0.19	0.24	0.24	0.28	0.24	0.24	0.24	0.24	0.00	0.25
7_766	0.24	0.31	0.31	0.00	0.31	0.24	0.24	0.31	0.39	0.04	0.04	0.39	0.40	0.40	0.39	0.12	0.05	0.29	0.74	0.74	0.60	0.80	0.79	0.57	0.57	0.45	0.45	0.84	0.63	0.73	0.81	0.84	0.84	0.42	0.84	0.84	0.84	0.84	0.31	0.87
7_767	0.25	0.28	0.28	0.02	0.28	0.35	0.35	0.28	0.34	0.00	0.03	0.34	0.35	0.35	0.34	0.23	0.09	0.39	0.67	0.67	0.54	0.70	0.71	0.46	0.47	0.32	0.32	0.71	0.56	0.66	0.67	0.71	0.71	0.26	0.71	0.71	0.71	0.71	0.39	0.74
7_768	0.24	0.31	0.31	0.00	0.31	0.24	0.24	0.31	0.39	0.04	0.04	0.39	0.40	0.40	0.39	0.12	0.05	0.29	0.74	0.74	0.60	0.80	0.79	0.57	0.57	0.45	0.45	0.84	0.63	0.73	0.81	0.84	0.84	0.42	0.84	0.84	0.84	0.84	0.31	0.87
7_769	0.17	0.23	0.23	0.01	0.23	0.20	0.20	0.23	0.29	0.03	0.03	0.29	0.30	0.30	0.29	0.12	0.04	0.33	0.51	0.51	0.35	0.56	0.55	0.29	0.29	0.16	0.16	0.62	0.37	0.46	0.58	0.62	0.62	0.17	0.62	0.62	0.62	0.62	0.43	0.65
7_770	0.25	0.31	0.31	0.00	0.31	0.25	0.25	0.31	0.38	0.04	0.04	0.38	0.38	0.38	0.38	0.11	0.05	0.27	0.72	0.72	0.58	0.78	0.77	0.57	0.57	0.45	0.45	0.82	0.61	0.72	0.79	0.82	0.82	0.40	0.82	0.82	0.82	0.82	0.31	0.86
7_771	0.25	0.31	0.31	0.00	0.31	0.25	0.25	0.31	0.38	0.04	0.04	0.38	0.38	0.38	0.38	0.12	0.04	0.27	0.72	0.72	0.59	0.78	0.77	0.56	0.56	0.44	0.44	0.83	0.62	0.72	0.79	0.83	0.83	0.41	0.83	0.83	0.82	0.83	0.30	0.86
7_772	0.25	0.31	0.31	0.00	0.31	0.25	0.25	0.31	0.38	0.04	0.04	0.38	0.38	0.38	0.38	0.12	0.04	0.27	0.72	0.72	0.59	0.78	0.77	0.56	0.56	0.44	0.44	0.83	0.62	0.72	0.79	0.83	0.83	0.41	0.83	0.83	0.82	0.83	0.30	0.86
7_773	0.26	0.32	0.32	0.00	0.32	0.25	0.25	0.32	0.41	0.04	0.04	0.41	0.42	0.42	0.41	0.13	0.05	0.30	0.75	0.75	0.61	0.81	0.80	0.58	0.58	0.46	0.46	0.86	0.64	0.75	0.82	0.86	0.86	0.43	0.86	0.86	0.86	0.86	0.31	0.89
7_774	0.18	0.25	0.25	0.02	0.25	0.23	0.23	0.25	0.30	0.03	0.03	0.30	0.31	0.31	0.30	0.14	0.05	0.35	0.52	0.52	0.38	0.56	0.55	0.30	0.30	0.16	0.16	0.63	0.40	0.49	0.59	0.63	0.63	0.17	0.63	0.63	0.63	0.63	0.44	0.66
7_775	0.30	0.28	0.28	0.00	0.28	0.28	0.28	0.28	0.36	0.02	0.04	0.36	0.36	0.36	0.36	0.16	0.04	0.25	0.79	0.79	0.70	0.83	0.83	0.58	0.58	0.52	0.52	0.76	0.73	0.77	0.79	0.76	0.76	0.39	0.76	0.76	0.75	0.76	0.31	0.79
7_776	0.11	0.15	0.15	0.01	0.15	0.16	0.16	0.15	0.23	0.01	0.05	0.23	0.24	0.24	0.23	0.05	0.01	0.14	0.59	0.59	0.48	0.64	0.64	0.49	0.49	0.40	0.40	0.68	0.51	0.61	0.65	0.68	0.68	0.35	0.68	0.68	0.68	0.68	0.24	0.65
7_777	0.24	0.31	0.31	0.00	0.31	0.25	0.25	0.31	0.38	0.04	0.04	0.38	0.38	0.38	0.38	0.11	0.04	0.27	0.72	0.72	0.58	0.78	0.77	0.55	0.55	0.43	0.43	0.82	0.61	0.72	0.79	0.82	0.82	0.40	0.82	0.82	0.82	0.82	0.31	0.86
7_116	0.16	0.23	0.23	0.01	0.23	0.21	0.21	0.23	0.26	0.02	0.03	0.26	0.27	0.27	0.26	0.11	0.03	0.31	0.49	0.49	0.34	0.53	0.52	0.26	0.26	0.14	0.14	0.59	0.36	0.44	0.55	0.59	0.59	0.16	0.59	0.59	0.59	0.59	0.40	0.62
7_778	0.33	0.40	0.40	0.00	0.40	0.32	0.32	0.40	0.36	0.04	0.05	0.36	0.37	0.37	0.36	0.08	0.05	0.27	0.73	0.73	0.61	0.69	0.69	0.50	0.50	0.39	0.39	0.83	0.64	0.64	0.80	0.83	0.83	0.45	0.83	0.83	0.83	0.83	0.28	0.87
7_779	0.33	0.39	0.39	0.00	0.39	0.32	0.32	0.39	0.36	0.04	0.05	0.36	0.36	0.36	0.36	0.08	0.06	0.26	0.73	0.73	0.60	0.69	0.68	0.51	0.51	0.40	0.40	0.83	0.64	0.64	0.80	0.83	0.83	0.45	0.83	0.83	0.83	0.83	0.28	0.86
7_780	0.35	0.37	0.37	0.00	0.37	0.35	0.35	0.37	0.32	0.03	0.05	0.32	0.32	0.32	0.32	0.08	0.03	0.23	0.73	0.73	0.61	0.68	0.69	0.50	0.50	0.39	0.39	0.77	0.64	0.64	0.73	0.77	0.77	0.39	0.77	0.77	0.76	0.77	0.28	0.80
7_781	0.35	0.37	0.37	0.00	0.37	0.35	0.35	0.37	0.32	0.03	0.05	0.32	0.32	0.32	0.32	0.08	0.03	0.23	0.73	0.73	0.61	0.68	0.69	0.50	0.50	0.39	0.39	0.77	0.64	0.64	0.73	0.77	0.77	0.39	0.77	0.77	0.76	0.77	0.28	0.80
7_782	0.35	0.38	0.38	0.00	0.38	0.35	0.35	0.38	0.32	0.03	0.05	0.32	0.32	0.32	0.32	0.08	0.04	0.22	0.73	0.73	0.60	0.67	0.68	0.51	0.51	0.40	0.40	0.76	0.64	0.64	0.73	0.76	0.76	0.39	0.76	0.76	0.76	0.76	0.28	0.80
7_783	0.26	0.29	0.29	0.02	0.29	0.33	0.33	0.29	0.36	0.01	0.03	0.36	0.36	0.36	0.36	0.21	0.09	0.41	0.70	0.70	0.61	0.63	0.64	0.38	0.38	0.31	0.31	0.74	0.64	0.56	0.77	0.74	0.74	0.36	0.74	0.74	0.74	0.74	0.33	0.77
7_784	0.28	0.34	0.34	0.04	0.34	0.39	0.39	0.34	0.28	0.00	0.04	0.28	0.28	0.28	0.28	0.11	0.08	0.33	0.58	0.58	0.45	0.53	0.53	0.33	0.33	0.22	0.22	0.68	0.48	0.48	0.64	0.68	0.68	0.31	0.68	0.68	0.67	0.68	0.30	0.71
7_350	0.33	0.35	0.35	0.00	0.35	0.35	0.35	0.35	0.29	0.02	0.04	0.29	0.30	0.30	0.29	0.10	0.02	0.20	0.68	0.68	0.55	0.72	0.73	0.53	0.53	0.41	0.41	0.71	0.58	0.68	0.68	0.71	0.71	0.33	0.71	0.71	0.71	0.71	0.29	0.74
7_786	0.32	0.39	0.39	0.01	0.39	0.33	0.33	0.39	0.34	0.03	0.05	0.34	0.34	0.34	0.34	0.07	0.04	0.25	0.71	0.71	0.59	0.66	0.66	0.48	0.48	0.38	0.38	0.80	0.62	0.62	0.77	0.80	0.80	0.44	0.80	0.80	0.80	0.80	0.27	0.84
7_787	0.32	0.38	0.38	0.01	0.38	0.32	0.32	0.38	0.33	0.04	0.05	0.33	0.34	0.34	0.33	0.07	0.05	0.24	0.70	0.70	0.58	0.66	0.66	0.49	0.49	0.38	0.38	0.80	0.61	0.62	0.77	0.80	0.80	0.43	0.80	0.80	0.80	0.80	0.27	0.83
7_788	0.34	0.36	0.36	0.00	0.36	0.34	0.34	0.36	0.32	0.02	0.04	0.32	0.33	0.33	0.32	0.11	0.03	0.23	0.71	0.71	0.57	0.75	0.76	0.55	0.55	0.43	0.43	0.74	0.60	0.71	0.71	0.74	0.74	0.34	0.74	0.74	0.74	0.74	0.30	0.77
7_789	0.20	0.21	0.21	0.01	0.21	0.25	0.25	0.21	0.20	0.01	0.06	0.20	0.20	0.20	0.20	0.04	0.01	0.12	0.61	0.61	0.54	0.56	0.57	0.46	0.46	0.37	0.37	0.64	0.57	0.58	0.61	0.64	0.64	0.33	0.64	0.64	0.63	0.64	0.22	0.61
7_790	0.20	0.26	0.26	0.02	0.26	0.33	0.33	0.26	0.31	0.00	0.03	0.31	0.32	0.32	0.31	0.20	0.08	0.36	0.57	0.57	0.51	0.61	0.61	0.38	0.38	0.31	0.31	0.67	0.53	0.56	0.70	0.67	0.67	0.30	0.67	0.67	0.67	0.67	0.33	0.70
7_791	0.13	0.18	0.18	0.06	0.18	0.28	0.28	0.18	0.14	0.00	0.06	0.14	0.15	0.15	0.14	0.04	0.03	0.16	0.46	0.46	0.38	0.41	0.42	0.31	0.31	0.22	0.22	0.54	0.41	0.41	0.51	0.54	0.54	0.25	0.54	0.54	0.53	0.54	0.23	0.51
7_792	0.26	0.33	0.33	0.00	0.33	0.26	0.26	0.33	0.39	0.05	0.04	0.39	0.40	0.40	0.39	0.12	0.06	0.28	0.73	0.73	0.69	0.69	0.68	0.51	0.51	0.46	0.46	0.84	0.73	0.66	0.87	0.84	0.84	0.48	0.84	0.84	0.84	0.84	0.27	0.87
7_793	0.25	0.31	0.31	0.03	0.31	0.41	0.41	0.31	0.24	0.00	0.04	0.24	0.25	0.25	0.24	0.14	0.06	0.29	0.52	0.52	0.41	0.56	0.55	0.36	0.36	0.25	0.25	0.61	0.43	0.52	0.58	0.61	0.61	0.23	0.61	0.61	0.61	0.61	0.33	0.64
7_794	0.16	0.22	0.22	0.01	0.22	0.29	0.29	0.22	0.26	0.00	0.03	0.26	0.26	0.26	0.26	0.16	0.05	0.30	0.50	0.50	0.44	0.55	0.54	0.29	0.29	0.22	0.22	0.60	0.47	0.50	0.63	0.60	0.60	0.27	0.60	0.60	0.60	0.60	0.31	0.63

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7_761	7_762	7_670	7_763	7_764	7_765	7_766	7_767	7_768	7_769	7_770	7_771	7_772	7_773	7_774	7_775	7_776	7_777	7_116	7_778	7_779	7_780	7_781	7_782	7_783	7_784	7_350	7_786	7_787	7_788	7_789	7_790	7_791	7_792	7_793	7_794	
1.00	0.87	0.88	0.92	0.76	0.53	0.81	0.92	0.81	0.79	0.81	0.81	0.81	0.84	0.82	0.96	0.48	0.81	0.76	0.89	0.89	0.92	0.92	0.92	0.92	0.85	0.91	0.86	0.86	0.94	0.62	0.80	0.52	0.84	0.84	0.79	7_721
1.00	1.00	1.00	0.96	1.00	0.88	0.94	1.00	0.94	0.96	0.94	0.94	0.94	0.97	1.00	0.96	0.57	0.94	0.92	1.00	1.00	0.97	0.97	0.97	1.00	0.97	0.97	0.97	0.97	1.00	0.66	0.96	0.61	0.97	0.97	0.95	7_722
1.00	1.00	1.00	0.96	1.00	0.88	0.94	1.00	0.94	0.96	0.94	0.94	0.94	0.97	1.00	0.96	0.57	0.94	0.92	1.00	1.00	0.97	0.97	0.97	1.00	0.97	0.97	0.97	0.97	1.00	0.66	0.96	0.61	0.97	0.97	0.95	7_723
0.68	0.03	0.06	1.00	0.12	0.21	0.03	0.67	0.03	0.48	0.04	0.05	0.05	0.00	0.63	0.02	0.26	0.04	0.53	0.27	0.26	0.27	0.27	0.26	0.70	0.82	0.21	0.31	0.30	0.16	0.39	0.70	0.87	0.09	0.79	0.52	7_724
1.00	1.00	1.00	0.96	1.00	0.88	0.94	1.00	0.94	0.96	0.94	0.94	0.94	0.97	1.00	0.96	0.57	0.94	0.92	1.00	1.00	0.97	0.97	0.97	1.00	0.97	0.97	0.97	0.97	1.00	0.66	0.96	0.61	0.97	0.97	0.95	7_725
0.63	0.51	0.50	0.60	0.19	0.11	0.51	0.64	0.51	0.50	0.53	0.53	0.53	0.51	0.55	0.54	0.48	0.52	0.51	0.63	0.62	0.65	0.65	0.66	0.61	0.66	0.64	0.65	0.64	0.62	0.62	0.61	0.64	0.54	0.65	0.62	7_726
0.63	0.51	0.50	0.60	0.19	0.11	0.51	0.64	0.51	0.50	0.53	0.53	0.53	0.51	0.55	0.54	0.48	0.52	0.51	0.63	0.62	0.65	0.65	0.66	0.61	0.66	0.64	0.65	0.64	0.62	0.62	0.61	0.64	0.54	0.65	0.62	7_727
1.00	1.00	1.00	0.96	1.00	0.88	0.94	1.00	0.94	0.96	0.94	0.94	0.94	0.97	1.00	0.96	0.57	0.94	0.92	1.00	1.00	0.97	0.97	0.97	1.00	0.97	0.97	0.97	0.97	1.00	0.66	0.96	0.61	0.97	0.97	0.95	7_728
1.00	1.00	1.00	0.97	1.00	0.90	0.92	0.97	0.92	0.92	0.89	0.89	0.89	0.94	0.96	0.94	0.62	0.89	0.86	0.83	0.83	0.78	0.78	0.77	0.97	0.75	0.77	0.78	0.78	0.81	0.55	0.90	0.47	0.92	0.73	0.87	7_729
0.00	0.30	0.30	0.08	0.48	0.45	0.21	0.08	0.21	0.21	0.21	0.20	0.20	0.23	0.21	0.15	0.10	0.21	0.17	0.20	0.21	0.16	0.16	0.17	0.09	0.07	0.13	0.18	0.19	0.15	0.08	0.05	0.16	0.23	0.03	0.03	7_730
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	7_731
1.00	1.00	1.00	0.97	1.00	0.90	0.92	0.97	0.92	0.92	0.89	0.89	0.89	0.94	0.96	0.94	0.62	0.89	0.86	0.83	0.83	0.78	0.78	0.77	0.97	0.75	0.77	0.78	0.78	0.81	0.55	0.90	0.47	0.92	0.73	0.87	7_732
1.00	1.00	1.00	0.97	1.00	0.90	0.92	0.97	0.92	0.93	0.89	0.89	0.89	0.95	0.96	0.94	0.62	0.89	0.86	0.83	0.83	0.78	0.78	0.78	0.97	0.76	0.77	0.79	0.78	0.81	0.56	0.90	0.48	0.92	0.74	0.87	7_733
1.00	1.00	1.00	0.97	1.00	0.90	0.92	0.97	0.92	0.93	0.89	0.89	0.89	0.95	0.96	0.94	0.62	0.89	0.86	0.83	0.83	0.78	0.78	0.78	0.97	0.76	0.77	0.79	0.78	0.81	0.56	0.90	0.48	0.92	0.74	0.87	7_734
1.00	1.00	1.00	0.97	1.00	0.90	0.92	0.97	0.92	0.92	0.89	0.89	0.89	0.94	0.96	0.94	0.62	0.89	0.86	0.83	0.83	0.78	0.78	0.77	0.97	0.75	0.77	0.78	0.78	0.81	0.55	0.90	0.47	0.92	0.73	0.87	7_735
0.83	0.45	0.44	0.65	0.18	0.02	0.43	0.65	0.43	0.49	0.41	0.41	0.41	0.44	0.54	0.52	0.24	0.41	0.46	0.33	0.32	0.33	0.33	0.32	0.61	0.40	0.37	0.30	0.30	0.40	0.21	0.61	0.21	0.42	0.46	0.57	7_736
0.56	0.35	0.34	0.43	0.44	0.16	0.25	0.38	0.25	0.28	0.24	0.23	0.23	0.26	0.30	0.24	0.10	0.22	0.23	0.24	0.25	0.20	0.20	0.21	0.38	0.31	0.16	0.22	0.23	0.19	0.09	0.35	0.16	0.28	0.28	0.29	7_737
1.00	0.82	0.82	0.97	0.38	0.34	0.74	0.97	0.74	0.93	0.71	0.72	0.72	0.76	0.96	0.73	0.46	0.71	0.87	0.67	0.67	0.62	0.62	0.61	0.97	0.77	0.60	0.63	0.63	0.64	0.40	0.91	0.47	0.73	0.76	0.88	7_738
1.00	0.93	0.93	0.95	0.87	0.75	0.87	0.93	0.87	0.84	0.85	0.85	0.85	0.88	0.87	0.96	0.87	0.85	0.80	0.90	0.90	0.90	0.90	0.90	0.93	0.77	0.85	0.90	0.90	0.85	0.92	0.84	0.78	0.87	0.74	0.82	7_739
1.00	0.93	0.93	0.95	0.87	0.75	0.87	0.93	0.87	0.84	0.85	0.85	0.85	0.88	0.87	0.96	0.87	0.85	0.80	0.90	0.90	0.90	0.90	0.90	0.93	0.77	0.85	0.90	0.90	0.85	0.92	0.84	0.78	0.87	0.74	0.82	7_740
1.00	0.92	0.91	0.79	0.75	0.64	0.83	0.77	0.83	0.65	0.82	0.83	0.83	0.83	0.69	0.84	0.85	0.82	0.62	0.89	0.89	0.89	0.89	0.89	0.80	0.73	0.82	0.89	0.88	0.83	0.93	0.73	0.77	0.89	0.67	0.72	7_349
1.00	0.90	0.92	0.95	0.72	0.80	0.90	0.93	0.90	0.87	0.90	0.90	0.90	0.90	0.89	0.96	0.93	0.90	0.82	0.90	0.89	0.89	0.89	0.89	0.86	0.75	0.90	0.89	0.89	0.90	0.91	0.86	0.76	0.84	0.75	0.85	7_741
1.00	0.90	0.92	0.95	0.68	0.75	0.90	0.93	0.90	0.85	0.90	0.90	0.90	0.90	0.87	0.96	0.93	0.90	0.81	0.90	0.90	0.90	0.90	0.90	0.87	0.76	0.90	0.90	0.90	0.90	0.91	0.84	0.77	0.84	0.74	0.83	7_742
1.00	0.94	0.94	0.75	0.71	0.78	0.94	0.76	0.94	0.57	0.94	0.94	0.94	0.94	0.57	0.89	1.00	0.94	0.56	0.94	0.94	0.94	0.94	0.94	0.70	0.73	0.94	0.94	0.94	0.94	1.00	0.70	0.80	0.89	0.74	0.61	7_743
1.00	0.94	0.94	0.75	0.71	0.78	0.94	0.76	0.94	0.57	0.94	0.94	0.94	0.94	0.57	0.89	1.00	0.94	0.56	0.94	0.94	0.94	0.94	0.94	0.70	0.73	0.94	0.94	0.94	0.94	1.00	0.70	0.80	0.89	0.74	0.61	7_744
1.00	0.93	0.93	0.70	0.56	0.64	0.93	0.70	0.93	0.47	0.93	0.93	0.93	0.93	0.47	0.93	1.00	0.93	0.46	0.93	0.93	0.93	0.93	0.93	0.70	0.67	0.93	0.93	0.93	0.93	1.00	0.70	0.76	0.93	0.68	0.60	7_901
1.00	0.93	0.93	0.70	0.56	0.64	0.93	0.70	0.93	0.47	0.93	0.93	0.93	0.93	0.47	0.93	1.00	0.93	0.46	0.93	0.93	0.93	0.93	0.93	0.70	0.67	0.93	0.93	0.93	0.93	1.00	0.70	0.76	0.93	0.68	0.60	7_746
1.00	1.00	1.00	0.98	1.00	0.94	0.94	0.98	0.94	0.95	0.92	0.93	0.93	0.96	0.97	0.96	0.92	0.92	0.90	0.94	0.94	0.91	0.91	0.91	0.98	0.83	0.85	0.94	0.94	0.87	0.92	0.93	0.83	0.94	0.82	0.91	7_747
1.00	0.96	0.96	0.80	0.75	0.64	0.87	0.77	0.87	0.65	0.86	0.87	0.87	0.87	0.69	0.87	0.90	0.86	0.62	0.93	0.93	0.93	0.93	0.93	0.81	0.77	0.86	0.93	0.93	0.86	0.98	0.74	0.82	0.93	0.71	0.72	7_748
1.00	0.95	0.95	0.80	0.69	0.77	0.95	0.82	0.95	0.71	0.95	0.95	0.95	0.95	0.75	0.91	1.00	0.95	0.68	0.95	0.95	0.95	0.95	0.95	0.76	0.79	0.95	0.95	0.95	0.95	1.00	0.76	0.84	0.91	0.79	0.73	7_749
1.00	0.96	0.96	0.95	0.87	0.81	0.91	0.93	0.91	0.90	0.89	0.89	0.89	0.92	0.92	0.96	0.92	0.89	0.85	0.94	0.94	0.90	0.90	0.90	0.98	0.81	0.85	0.94	0.94	0.85	0.92	0.93	0.83	0.94	0.78	0.91	7_750
1.00	1.00	1.00	0.98	1.00	0.94	0.94	0.98	0.94	0.95	0.92	0.93	0.93	0.96	0.97	0.96	0.92	0.92	0.90	0.94	0.94	0.91	0.91	0.91	0.98	0.83	0.85	0.94	0.94	0.87	0.92	0.93	0.83	0.94	0.82	0.91	7_751
1.00	1.00	1.00	0.98	1.00	0.94	0.94	0.98	0.94	0.95	0.92	0.93	0.93	0.96	0.97	0.96	0.92	0.92	0.90	0.94	0.94	0.91	0.91	0.91	0.98	0.83	0.85	0.94	0.94	0.87	0.92	0.93	0.83	0.94	0.82	0.91	7_752
0.09	0.91	0.94	0.65	0.89	0.78	0.82	0.57	0.82	0.45	0.82	0.82	0.82	0.82	0.43	0.74	0.86	0.82	0.45	0.91	0.90	0.84	0.84	0.84	0.69	0.72	0.75	0.90	0.90	0.76	0.86	0.62	0.75	0.88	0.60	0.56	7_754
1.00	1.00	1.00	0.98	1.00	0.94	0.94	0.98	0.94	0.95	0.92	0.93	0.93	0.96	0.97	0.96	0.92	0.92	0.90	0.94	0.94	0.91	0.91	0.91	0.98	0.83	0.85	0.94	0.94	0.87	0.92	0.93	0.83	0.94	0.82	0.91	7_755
1.00	1.00	1.00	0.98	1.00	0.94	0.94	0.98	0.94	0.95	0.92	0.93	0.93	0.96	0.97	0.96	0.92	0.92	0.90	0.94	0.94	0.91	0.91	0.91	0.98	0.83	0.85	0.94	0.94	0.87	0.92	0.93	0.83	0.94	0.82	0.91	7_756
1.00	1.00	1.00	0.98	1.00	0.94	0.94	0.98	0.94	0.95	0.92	0.92	0.92	0.96	0.97	0.96	0.92	0.92	0.90	0.94	0.94	0.91	0.91	0.90	0.98	0.83	0.85	0.94	0.94	0.87	0.92	0.93	0.83	0.94	0.82	0.91	7_757
1.00	1.00	1.00	0.98	1.00	0.94	0.94	0.98	0.94	0.95	0.92	0.93	0.93	0.96	0.97	0.96	0.92	0.92	0.90	0.94	0.94	0.91	0.91	0.91	0.98	0.83	0.85	0.94	0.94	0.87	0.92	0.93	0.83	0.94	0.82	0.91	7_758
0.95	1.00	1.00	1.00	0.82	0.05	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00	0.94	1.00	0.93	7_759
1.00	1.00	1.00	0.98	1.00	0.94	0.94	0.98	0.94	0.95	0.93	0.93	0.93	0.96	0.97	0.96	0.92	0.93	0.90	0.98	0.98	0.94	0.94	0.94	0.98	0.85	0.89	0.98	0.98	0.89	0.92	0.93	0.83	0.94	0.82	0.91	7_760
	1.00	1.00	1.00	0.26	0.34	1.00	1.00	1.00	0.70	1.00	1.00	1.00	1.00	0.70	1.00	1.00	1.00	0.69	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	7_761
0.39		1.00	0.95	1.00	0.94	0.93	0.98	0.93	0.92	0.92	0.93	0.93	0.94	0.97	0.94	0.92	0.92	0.88	0.98	0.98	0.94	0.94	0.94	0.96	0.85	0.88	0.98	0.98	0.89	0.94	0.93	0.85	0.94	0.82	0.91	7_762
0.38	0.98		0.98	1.00	0.94	0.94	0.98	0.94	0.95	0.93	0.93	0.93	0.96	0.97	0.96	0.92	0.93	0.90	0.98	0.98	0.94	0.94	0.94	0.98	0.85	0.89	0.98	0.98	0.89	0.92	0.93	0.83	0.94	0.82	0.91	7_670
0.49	0.74	0.77		0.39	0.48	0.95	0.93	0.95	0.90	0.95	0.95	0.95	0.95	0.92	0.91	0.95	0.95	0.86	0.95	0.95	0.93	0.93	0.93	0.91	0.95	0.93	0.95	0.95	0.93	0.89	0.91	0.92	0.90	0.93	0.89	7_763
0.05	0.28	0.27	0.05		0.85	0.80	0.30	0.80	0.00	0.80	0.80	0.80	0.81	0.31	0.57	0.79	0.79	0.07	1.00	1.00	0.86	0.86	0.86	0.37	0.59	0.67	1.00	1.00	0.67	0.85	0.29	0.61	0.87	0.39	0.10	7_764
0.09	0.25	0.25	0.08	0.69		1.00	0.46	1.00	0.20	1.00	1.00	1.00	0.94	0.16	0.65	0.93	1.00	0.24	0.94	0.94	0.87	0.87	0.87	0.35	0.61	0.87	1.00	1.00	0.81	0.79	0.52	0.63	0.88	0.62	0.41	7_765
0.39	0.86	0.87	0.74	0.18	0.29		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.98	1.00	0.98	0.96	0.96	0.94	0.94	0.94	0.93	0.85	0.94	0.98	0.98	0.92	0.89	0.98	0.82	0.92	0.86	0.97	7_766
0.49	0.75	0.74	0.85	0.03	0.08	0.79		1.00	0.91	1.00	1.00	1.00	1.00	0.95	0.91	1.00	1.00	0.87	1.00	1.00	1.00	1.00	1.00	0.96	1.00	1.00	1.00	1.00	1.00	1.00	0.96	1.00	0.95	1.00	0.92	7_767
0.39	0.86	0.87	0.74	0.18	0.29	1.00	0.79		1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.98	1.00	0.98	0.96	0.96	0.94	0.94	0.94	0.93	0.85	0.94	0.98	0.98	0.92	0.89	0.98	0.82	0.92	0.86	0.97	7_768
0.26	0.63	0.65	0.72	0.00	0.02	0.73	0.77	0.73		1.00	1.00	1.00	1.00	1.00	0.85	1.00	1.00	1.00	0.97	0.97	0.92	0.92	0.92	0.88	0.97	0.92	0.97	0.97	0.92	0.88	0.90	0.94	0.90	0.95	0.89	7_769
0.39	0.84	0.86	0.73	0.18	0.29	0.98	0.77	0.98	0.72		1.00	1.00	1.00	1.00	1.00	0.96	1.00	1.00	0.94	0.94	0.94	0.94	0.94	0.93	0.85	0.94	0.98	0.98	0.91	0.87	1.00	0.83	0.94	0.88	1.00	7_770
0.38	0.84	0.86	0.73	0.17	0.28	0.98	0.77	0.98	0.72	1.00		1.00	1.00	1.00	1.00	0.96	1.00	1.00	0.94	0.94	0.94	0.94	0.94	0.93	0.85	0.94	0.98	0.98	0.91	0.87	1.00	0.83	0.94	0.88	1.00	7_771
0.38	0.84	0.86	0.73	0.17	0.28	0.98	0.77	0.98	0.72	1.00	1.00		1.00	1.00	1.00	0.96	1.00	1.00	0.94	0.94	0.94	0.94	0.94	0.93	0.85	0.94	0.98	0.98	0.91	0.87	1.00	0.83	0.94	0.88	1.00	7_772
0.39	0.87	0.89	0.76	0.18	0.26	0.98	0.80	0.98	0.75	0.96	0.96	0.96		1.00	1.00	1.00	1.00	0.95	0.98	0.98	0.94	0.94	0.94	0.93	0.84	0.94	0.98	0.98	0.94	0.91	0.96	0.82	0.92	0.84	0.94	7_773
0.27	0.67	0.66	0.73	0.00	0.01	0.71	0.81	0.71	0.96	0.69	0.69	0.69	0.72		0.85	1.00	1.00	1.00	1.00	1.00	0.95	0.95	0.94	0.90	1.00	0.95	1.00	1.00	0.95	0.94	0.95	1.00	0.95	1.00	0.92	7_774
0.44	0.77	0.79	0.75	0.10	0.14	0.87	0.75	0.87	0.61	0.86	0.86	0.86	0.89	0.58		1.00	1.00	0.81	0.98	0.98	0.98	0.98	0.98	0.89	0.82	0.98	0.98	0.98	0.98	0.95	0.87	0.80	0.96	0.81	0.86	7_775
0.30	0.63	0.65	0.55	0.13	0.19	0.73	0.60	0.73	0.55	0.71	0.71	0.71	0.74	0.53	0.66		0.96	0.95	0.83	0.83	0.79	0.79	0.79	0.92	0.77	0.84	0.80	0.79	0.88	0.92	0.95	0.85	0.89	0.82	0.93	7_776
0.37	0.84	0.86	0.73	0.17	0.28	0.98	0.77	0.98	0.73	1.00	1.00	1.00	0.96	0.70	0.86	0.72		1.00	0.94	0.94	0.94	0.94	0.94	0.93	0.85	0.94	0.98	0.98	0.91	0.87	1.00	0.83	0.94	0.88	1.00	7_777
0.24	0.60	0.62	0.69	0.00	0.02	0.73	0.73	0.73	0.96	0.75	0.76	0.76	0.71	0.91	0.58	0.52	0.77		0.92	0.92	0.92	0.92	0.92	0.84	0.98	0.93	0.98	0.97	0.88	0.83	0.91	0.94	0.90	0.95	0.92	7_116
0.34	0.85	0.87	0.66	0.24	0.22	0.82	0.70	0.82	0.61	0.80	0.80	0.80	0.83	0.62	0.74	0.59	0.80	0.58		1.00	0.97	0.97	0.97	1.00	0.96	0.96	1.00	1.00	1.00	0.94	0.95	0.87	0.96	0.96	0.94	7_778
0.35	0.85	0.86	0.66	0.25	0.23	0.81	0.69	0.81	0.61	0.79	0.80	0.80	0.83	0.62	0.74	0.59	0.79	0.58	1.00		0.97	0.97	0.97	1.00	0.96	0.96	1.00	1.00	1.00	0.94	0.95	0.87	0.96	0.96	0.94	7_779
0.34	0.78	0.80	0.62	0.18	0.19	0.78	0.70	0.78	0.55	0.80	0.80	0.80	0.77	0.56	0.74	0.53	0.80	0.58	0.93	0.93		1.00	1.00	1.00	0.96	1.00	1.00	1.00	1.00	0.94	0.95	0.87	0.96	0.96	0.97	7_780
0.34	0.78	0.80	0.62	0.18	0.19	0.78	0.70	0.78	0.55	0.80	0.80	0.80	0.77	0.56	0.74	0.53	0.80	0.58	0.93	0.93	1.00		1.00	1.00	0.96	1.00	1.00	1.00	1.00	0.94	0.95	0.87	0.96	0.96	0.97	7_781
0.35	0.78	0.80	0.61	0.18	0.20	0.78	0.69	0.78	0.54	0.79	0.80	0.80	0.76	0.55	0.74	0.53	0.79	0.58	0.93	0.93	1.00	1.00		1.00	0.96	1.00	1.00	1.00	1.00	0.94	0.95	0.86	0.96	0.96	0.97	7_782
0.47	0.75	0.77	0.80	0.05	0.04	0.71	0.88	0.71	0.69	0.70	0.70	0.70	0.73	0.70	0.75	0.53	0.70	0.66	0.73	0.72	0.73	0.73	0.72		1.00	0.93	1.00	1.00	0.93	0.97	0.92	0.97	0.98	0.91	0.92	7_783
0.37	0.69	0.71	0.73	0.09	0.11	0.69	0.76	0.69	0.67	0.70	0.71	0.71	0.67	0.68	0.57	0.46	0.70	0.71	0.85	0.85	0.85	0.85	0.85	0.79		0.91	1.00	1.00	0.87	0.90	1.00	0.98	0.86	1.00	1.00	7_784
0.36	0.73	0.74	0.65	0.11	0.21	0.83	0.73	0.83	0.58	0.84	0.84	0.84	0.81	0.59	0.78	0.58	0.84	0.62	0.88	0.88	0.95	0.95	0.95	0.66	0.79		1.00	1.00	1.00	0.94	0.95	0.86	0.90	0.96	0.97	7_350
0.33	0.82	0.84	0.64	0.24	0.25	0.82	0.67	0.82	0.59	0.83	0.84	0.84	0.80	0.60	0.71	0.56	0.83	0.62	0.97	0.97	0.97	0.97	0.97	0.70	0.89	0.92		1.00	1.00	0.91	1.00	0.87	1.00	1.00	1.00	7_786
0.33	0.82	0.83	0.64	0.24	0.25	0.82	0.67	0.82	0.59	0.83	0.83	0.83	0.80	0.60	0.71	0.56	0.83	0.62	0.97	0.97	0.97	0.97	0.97	0.70	0.88	0.92	1.00		1.00	0.91	1.00	0.87	1.00	1.00	1.00	7_787
0.37	0.76	0.77	0.68	0.12	0.18	0.82	0.76	0.82	0.60	0.81	0.81	0.81	0.84	0.61	0.81	0.60	0.81	0.57	0.92	0.92	0.92	0.92	0.92	0.69	0.76	0.97	0.89	0.88		0.98	0.91	0.85	0.86	0.92	0.91	7_788
0.28	0.63	0.61	0.46	0.14	0.13	0.57	0.56	0.57	0.40	0.55	0.55	0.55	0.58	0.44	0.57	0.79	0.56	0.38	0.72	0.72	0.72	0.72	0.72	0.56	0.59	0.68	0.69	0.69	0.71		0.89	0.92	0.91	0.91	0.90	7_789
0.47	0.71	0.70	0.81	0.03	0.09	0.79	0.88	0.79	0.73	0.80	0.81	0.81	0.76	0.78	0.71	0.56	0.80	0.78	0.66	0.65	0.66	0.66	0.65	0.84	0.79	0.70	0.70	0.70	0.65	0.47		1.00	1.00	1.00	1.00	7_790
0.29	0.53	0.51	0.51	0.08	0.09	0.50	0.58	0.50	0.48	0.51	0.51	0.51	0.49	0.52	0.41	0.70	0.52	0.50	0.62	0.62	0.62	0.62	0.62	0.57	0.73	0.58	0.65	0.65	0.56	0.82	0.60		0.87	1.00	1.00	7_791
0.39	0.89	0.87	0.65	0.21	0.23	0.85	0.71	0.85	0.59	0.87	0.87	0.87	0.84	0.63	0.80	0.61	0.87	0.62	0.81	0.81	0.81	0.81	0.81	0.78	0.72	0.76	0.85	0.85	0.72	0.59	0.82	0.55		0.84	1.00	7_792
0.40	0.65	0.64	0.75	0.04	0.12	0.72	0.82	0.72	0.69	0.73	0.74	0.74	0.70	0.73	0.59	0.49	0.73	0.73	0.78	0.78	0.78	0.78	0.78	0.71	0.93	0.83	0.82	0.82	0.79	0.57	0.85	0.71	0.68		1.00	7_793
0.41	0.64	0.63	0.77	0.00	0.06	0.73	0.85	0.73	0.77	0.75	0.75	0.75	0.70	0.80	0.66	0.50	0.75	0.81	0.59	0.60	0.61	0.61	0.61	0.80	0.74	0.65	0.64	0.65	0.60	0.42	0.98	0.56	0.77	0.81		7_794

B

r² (0.20)	*capn3* D' (0.57)																	
	10_712	10_350	10_713	10_714	10_716	10_718	10_719	10_293	10_720	10_721	10_722	10_723	10_725	10_726	10_727	10_728
10_712		1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.21	1.00	0.00	0.42	0.40	0.38	0.34	0.40
10_350	0.53		1.00	1.00	1.00	0.07	0.95	0.90	0.28	0.26	0.08	0.21	0.28	0.26	0.22	0.28
10_713	0.97	0.51		1.00	1.00	1.00	1.00	1.00	0.27	1.00	0.07	0.40	0.38	0.36	0.31	0.38
10_714	0.47	0.88	0.45		1.00	0.05	0.72	0.92	0.21	0.27	0.05	0.16	0.25	0.23	0.19	0.25
10_716	0.53	1.00	0.51	0.88		0.07	0.95	0.90	0.28	0.26	0.08	0.21	0.28	0.26	0.22	0.28
10_718	0.05	0.00	0.05	0.00	0.00		0.98	0.96	0.78	0.70	0.71	0.12	0.03	0.01	0.05	0.03
10_719	0.14	0.24	0.13	0.16	0.24	0.61		1.00	0.92	0.39	0.58	0.17	0.39	0.32	0.40	0.39
10_293	0.06	0.09	0.06	0.11	0.09	0.25	0.43		1.00	1.00	0.76	0.33	0.07	0.14	0.05	0.07
10_720	0.01	0.03	0.01	0.02	0.03	0.54	0.61	0.31		0.70	0.95	1.00	1.00	1.00	1.00	1.00
10_721	0.08	0.01	0.08	0.01	0.01	0.30	0.15	0.44	0.34		0.65	1.00	1.00	1.00	1.00	1.00
10_722	0.00	0.00	0.00	0.00	0.00	0.31	0.33	0.25	0.64	0.42		1.00	0.35	0.42	0.25	0.35
10_723	0.08	0.04	0.07	0.02	0.04	0.00	0.01	0.04	0.12	0.17	0.17		0.71	0.63	0.66	0.71
10_725	0.10	0.06	0.09	0.05	0.06	0.00	0.03	0.00	0.09	0.13	0.02	0.37		1.00	1.00	1.00
10_726	0.09	0.06	0.07	0.04	0.06	0.00	0.02	0.01	0.10	0.14	0.02	0.32	0.91		1.00	1.00
10_727	0.08	0.04	0.07	0.02	0.04	0.00	0.03	0.00	0.08	0.11	0.01	0.30	1.00	0.89		1.00
10_728	0.10	0.06	0.09	0.05	0.06	0.00	0.03	0.00	0.09	0.13	0.02	0.37	1.00	0.91	1.00	

C

r² (0.47)	*capn2* D' (0.91)																												
	16_758	16_759	16_760	16_761	16_762	16_763	16_764	16_216	16_765	16_766	16_767	16_768	16_769	16_416	16_770	16_771	16_772	16_773	16_775	16_776	16_777	16_778	16_780	16_781
16_758		1.00	0.93	0.87	1.00	0.96	0.62	0.52	0.73	0.96	0.73	0.58	0.58	0.65	0.68	0.73	0.96	0.68	0.68	0.68	0.63	0.62	0.60	0.88
16_759	0.56		0.94	0.94	1.00	0.96	1.00	1.00	1.00	0.95	1.00	1.00	1.00	1.00	1.00	1.00	0.96	1.00	1.00	0.92	1.00	1.00	0.87	0.95
16_760	0.62	0.71		0.93	1.00	1.00	0.77	0.83	0.86	1.00	0.86	0.75	0.75	0.79	0.84	0.84	1.00	0.80	0.80	0.83	0.76	0.77	1.00	0.98
16_761	0.49	0.77	0.81		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.88	0.93
16_762	0.08	0.14	0.11	0.12		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
16_763	0.51	0.92	0.79	0.85	0.14		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
16_764	0.19	0.29	0.21	0.33	0.04	0.29		1.00	0.95	1.00	0.95	0.96	0.96	0.96	0.95	0.95	1.00	0.96	0.96	0.97	0.96	0.96	0.87	0.70
16_216	0.23	0.50	0.42	0.57	0.07	0.49	0.58		0.80	1.00	0.79	1.00	1.00	0.98	0.97	0.79	1.00	0.71	0.71	0.59	1.00	1.00	0.22	0.67
16_765	0.52	0.54	0.49	0.62	0.08	0.53	0.49	0.59		1.00	1.00	1.00	1.00	0.98	1.00	0.99	1.00	0.98	0.98	0.78	1.00	1.00	0.46	0.73
16_766	0.57	0.83	0.87	0.94	0.13	0.91	0.31	0.54	0.59		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
16_767	0.49	0.52	0.48	0.60	0.07	0.52	0.50	0.59	0.97	0.57		1.00	1.00	0.98	1.00	0.99	1.00	0.98	0.98	0.77	0.98	0.98	0.42	0.72
16_768	0.18	0.31	0.21	0.35	0.04	0.30	0.88	0.62	0.57	0.33	0.58		1.00	0.98	1.00	0.98	1.00	1.00	1.00	1.00	0.96	0.96	0.64	0.69
16_769	0.18	0.31	0.21	0.35	0.04	0.30	0.88	0.62	0.57	0.33	0.58	1.00		0.98	1.00	0.98	1.00	1.00	1.00	1.00	0.96	0.96	0.64	0.69
16_416	0.30	0.40	0.31	0.46	0.06	0.39	0.67	0.78	0.71	0.43	0.73	0.74	0.74		1.00	1.00	1.00	0.77	0.77	0.75	0.96	0.96	0.03	0.75
16_770	0.41	0.63	0.56	0.73	0.09	0.62	0.41	0.74	0.85	0.69	0.83	0.48	0.48	0.63		1.00	1.00	1.00	1.00	0.72	1.00	1.00	0.60	0.70
16_771	0.52	0.54	0.47	0.62	0.08	0.53	0.49	0.57	0.97	0.59	0.95	0.54	0.54	0.74	0.85		1.00	0.98	0.98	0.78	1.00	1.00	0.44	0.73
16_772	0.51	0.92	0.79	0.85	0.14	1.00	0.29	0.49	0.53	0.91	0.52	0.30	0.30	0.39	0.62	0.53		1.00	1.00	1.00	1.00	1.00	1.00	1.00
16_773	0.35	0.42	0.33	0.48	0.06	0.42	0.63	0.43	0.76	0.46	0.78	0.72	0.72	0.56	0.67	0.76	0.42		1.00	1.00	0.98	0.98	0.19	0.56
16_775	0.35	0.42	0.33	0.48	0.06	0.42	0.63	0.43	0.76	0.46	0.78	0.72	0.72	0.56	0.67	0.76	0.42	1.00		1.00	0.98	0.98	0.19	0.56
16_776	0.45	0.49	0.49	0.66	0.08	0.57	0.48	0.30	0.57	0.63	0.54	0.53	0.53	0.38	0.47	0.57	0.57	0.73	0.73		1.00	1.00	0.39	0.72
16_777	0.19	0.28	0.20	0.33	0.04	0.28	0.92	0.59	0.54	0.31	0.52	0.87	0.87	0.68	0.46	0.54	0.28	0.64	0.64	0.49		1.00	0.87	0.73
16_778	0.20	0.29	0.21	0.33	0.04	0.29	0.93	0.58	0.54	0.31	0.53	0.88	0.88	0.67	0.46	0.54	0.29	0.66	0.66	0.50	1.00		0.87	0.73
16_780	0.19	0.22	0.36	0.26	0.04	0.29	0.05	0.03	0.11	0.31	0.10	0.03	0.03	0.00	0.17	0.10	0.29	0.02	0.02	0.08	0.05	0.05		0.24
16_781	0.62	0.41	0.54	0.45	0.06	0.44	0.32	0.41	0.44	0.49	0.44	0.33	0.33	0.49	0.35	0.44	0.44	0.29	0.29	0.40	0.34	0.34	0.04	

D

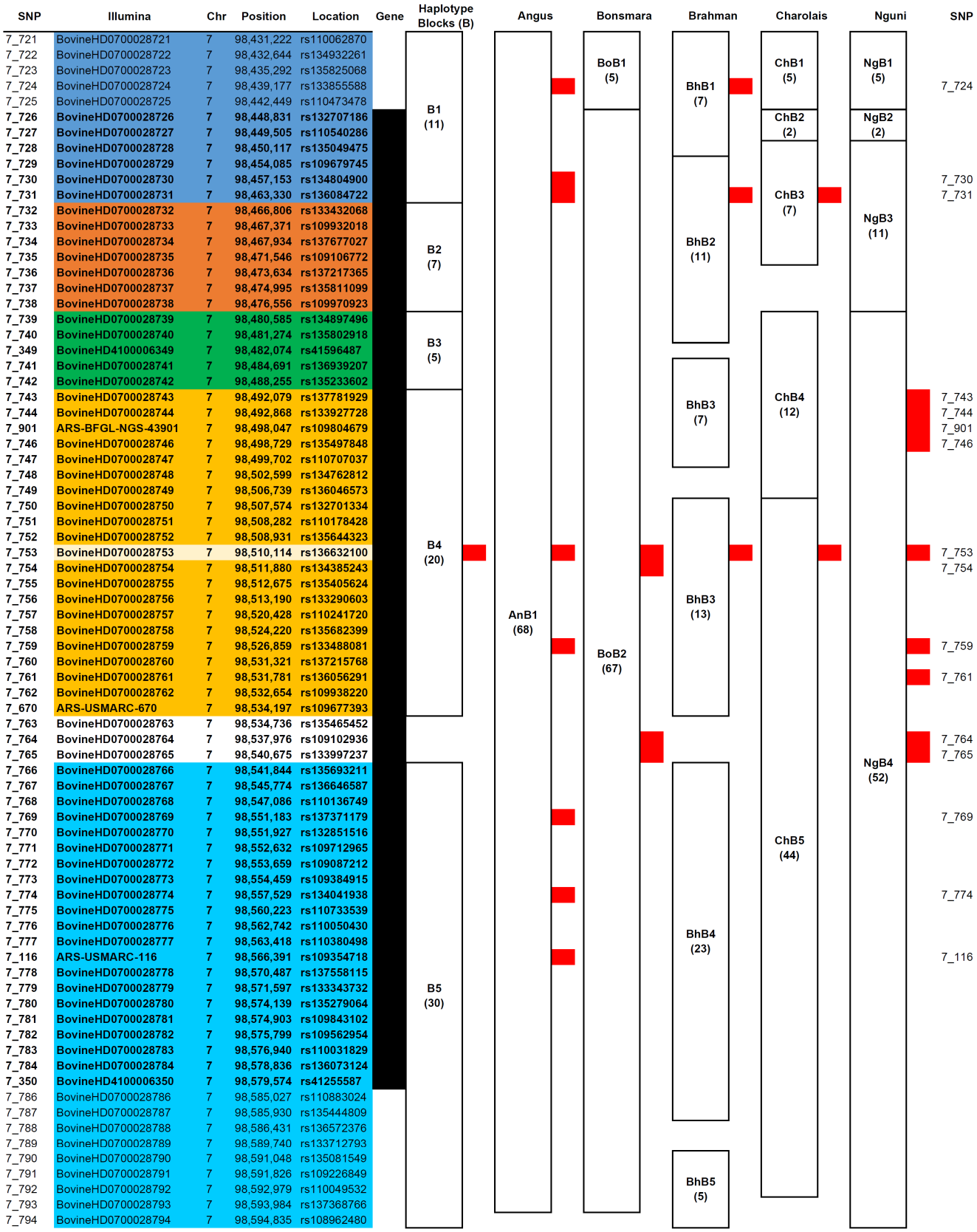
r² (0.26)	*capn1* D' (0.69)											
	29_183	29_184	29_316	29_185	29_186	29_187	29_370	29_4751	29_188	29_189	29_190	29_191
29_183		1.00	1.00	0.27	0.21	0.27	0.76	0.01	0.09	0.13	0.11	0.11
29_184	0.44		1.00	1.00	0.49	0.56	0.71	0.56	0.38	0.45	0.48	0.38
29_316	0.21	0.12		1.00	0.83	0.95	1.00	0.92	0.96	0.94	0.95	0.93
29_185	0.03	0.20	0.62		0.89	0.93	0.84	0.90	0.91	0.96	0.97	0.96
29_186	0.02	0.20	0.11	0.19		0.50	0.25	0.84	0.62	0.74	0.69	0.69
29_187	0.04	0.28	0.13	0.21	0.24		0.03	0.97	0.59	0.69	0.84	0.67
29_370	0.21	0.08	0.08	0.09	0.03	0.00		1.00	1.00	0.14	0.28	0.20
29_4751	0.00	0.17	0.20	0.31	0.46	0.57	0.32		1.00	1.00	1.00	1.00
29_188	0.01	0.10	0.16	0.23	0.34	0.29	0.44	0.73		0.62	0.66	0.61
29_189	0.01	0.18	0.13	0.21	0.51	0.47	0.01	0.59	0.32		1.00	1.00
29_190	0.01	0.17	0.15	0.25	0.43	0.62	0.04	0.70	0.42	0.85		1.00
29_191	0.01	0.13	0.10	0.17	0.36	0.36	0.03	0.49	0.25	0.82	0.69	

Individual linkage disequilibrium values for all SNPs within each of the genes are given as D' above the diagonal and r^2 below the diagonal; D'(number); r^2 (number) – the average linkage disequilibrium for each gene; **A** – the *cast* gene; **B** – the *capn3* gene; **C** – the *capn2* gene; **D** – the *capn1* gene.

Table S5. *Haplotype blocks of the muscle calpain system genes in South African beef breeds genotyped using the Illumina® BovineHD SNP BeadChip (777K).*

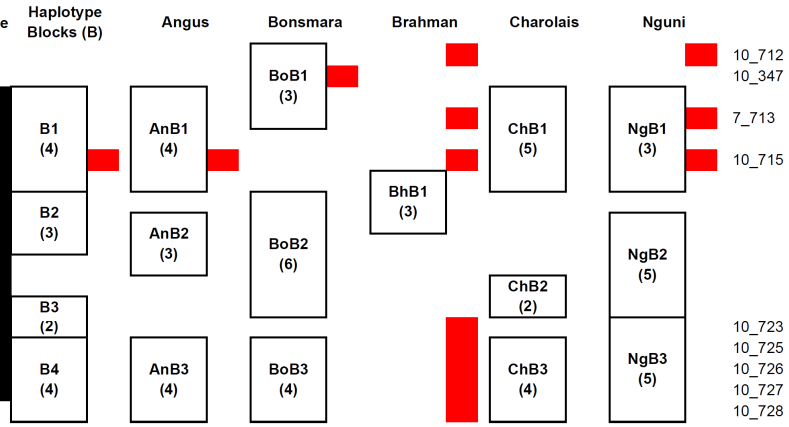
The cast gene (7:98,444,826-98,581,260)

SNP	Illumina	Chr	Position	Location	Gene
7_721	BovineHD0700028721	7	98,431,222	rs110062870	
7_722	BovineHD0700028722	7	98,432,644	rs134932261	
7_723	BovineHD0700028723	7	98,435,292	rs135825068	
7_724	BovineHD0700028724	7	98,439,177	rs133855588	
7_725	BovineHD0700028725	7	98,442,449	rs110473478	
7_726	**BovineHD0700028726**	**7**	**98,448,831**	**rs132707186**	
7_727	**BovineHD0700028727**	**7**	**98,449,505**	**rs110540286**	
7_728	**BovineHD0700028728**	**7**	**98,450,117**	**rs135049475**	
7_729	**BovineHD0700028729**	**7**	**98,454,085**	**rs109679745**	
7_730	**BovineHD0700028730**	**7**	**98,457,153**	**rs134804900**	
7_731	**BovineHD0700028731**	**7**	**98,463,330**	**rs136084722**	
7_732	**BovineHD0700028732**	**7**	**98,466,806**	**rs133432068**	
7_733	**BovineHD0700028733**	**7**	**98,467,371**	**rs109932018**	
7_734	**BovineHD0700028734**	**7**	**98,467,934**	**rs137677027**	
7_735	**BovineHD0700028735**	**7**	**98,471,546**	**rs109106772**	
7_736	**BovineHD0700028736**	**7**	**98,473,634**	**rs137217365**	
7_737	**BovineHD0700028737**	**7**	**98,474,995**	**rs135811099**	
7_738	**BovineHD0700028738**	**7**	**98,476,556**	**rs109970923**	
7_739	**BovineHD0700028739**	**7**	**98,480,585**	**rs134897496**	
7_740	**BovineHD0700028740**	**7**	**98,481,274**	**rs135802918**	
7_349	**BovineHD4100006349**	**7**	**98,482,074**	**rs41596487**	
7_741	**BovineHD0700028741**	**7**	**98,484,691**	**rs136939207**	
7_742	**BovineHD0700028742**	**7**	**98,488,255**	**rs135233602**	
7_743	**BovineHD0700028743**	**7**	**98,492,079**	**rs137781929**	
7_744	**BovineHD0700028744**	**7**	**98,492,868**	**rs133927728**	
7_901	**ARS-BFGL-NGS-43901**	**7**	**98,498,047**	**rs109804679**	
7_746	**BovineHD0700028746**	**7**	**98,498,729**	**rs135497848**	
7_747	**BovineHD0700028747**	**7**	**98,499,702**	**rs110707037**	
7_748	**BovineHD0700028748**	**7**	**98,502,599**	**rs134762812**	
7_749	**BovineHD0700028749**	**7**	**98,506,739**	**rs136046573**	
7_750	**BovineHD0700028750**	**7**	**98,507,574**	**rs132701334**	
7_751	**BovineHD0700028751**	**7**	**98,508,282**	**rs110178428**	
7_752	**BovineHD0700028752**	**7**	**98,508,931**	**rs135644323**	
7_753	**BovineHD0700028753**	**7**	**98,510,114**	**rs136632100**	
7_754	**BovineHD0700028754**	**7**	**98,511,880**	**rs134385243**	
7_755	**BovineHD0700028755**	**7**	**98,512,675**	**rs135405624**	
7_756	**BovineHD0700028756**	**7**	**98,513,190**	**rs133290603**	
7_757	**BovineHD0700028757**	**7**	**98,520,428**	**rs110241720**	
7_758	**BovineHD0700028758**	**7**	**98,524,220**	**rs135682399**	
7_759	**BovineHD0700028759**	**7**	**98,526,859**	**rs133488081**	
7_760	**BovineHD0700028760**	**7**	**98,531,321**	**rs137215768**	
7_761	**BovineHD0700028761**	**7**	**98,531,781**	**rs136056291**	
7_762	**BovineHD0700028762**	**7**	**98,532,654**	**rs109938220**	
7_670	**ARS-USMARC-670**	**7**	**98,534,197**	**rs109677393**	
7_763	BovineHD0700028763	7	98,534,736	rs135465452	
7_764	BovineHD0700028764	7	98,537,976	rs109102936	
7_765	BovineHD0700028765	7	98,540,675	rs133997237	
7_766	**BovineHD0700028766**	**7**	**98,541,844**	**rs135693211**	
7_767	**BovineHD0700028767**	**7**	**98,545,774**	**rs136646587**	
7_768	**BovineHD0700028768**	**7**	**98,547,086**	**rs110136749**	
7_769	**BovineHD0700028769**	**7**	**98,551,183**	**rs137371179**	
7_770	**BovineHD0700028770**	**7**	**98,551,927**	**rs132851516**	
7_771	**BovineHD0700028771**	**7**	**98,552,632**	**rs109712965**	
7_772	**BovineHD0700028772**	**7**	**98,553,659**	**rs109087212**	
7_773	**BovineHD0700028773**	**7**	**98,554,459**	**rs109384915**	
7_774	**BovineHD0700028774**	**7**	**98,557,529**	**rs134041938**	
7_775	**BovineHD0700028775**	**7**	**98,560,223**	**rs110733539**	
7_776	**BovineHD0700028776**	**7**	**98,562,742**	**rs110050430**	
7_777	**BovineHD0700028777**	**7**	**98,563,418**	**rs110380498**	
7_116	**ARS-USMARC-116**	**7**	**98,566,391**	**rs109354718**	
7_778	**BovineHD0700028778**	**7**	**98,570,487**	**rs137558115**	
7_779	**BovineHD0700028779**	**7**	**98,571,597**	**rs133343732**	
7_780	**BovineHD0700028780**	**7**	**98,574,139**	**rs135279064**	
7_781	**BovineHD0700028781**	**7**	**98,574,903**	**rs109843102**	
7_782	**BovineHD0700028782**	**7**	**98,575,799**	**rs109562954**	
7_783	**BovineHD0700028783**	**7**	**98,576,940**	**rs110031829**	
7_784	**BovineHD0700028784**	**7**	**98,578,836**	**rs136073124**	
7_350	**BovineHD4100006350**	**7**	**98,579,574**	**rs41255587**	
7_786	BovineHD0700028786	7	98,585,027	rs110883024	
7_787	BovineHD0700028787	7	98,585,930	rs135444809	
7_788	BovineHD0700028788	7	98,586,431	rs136572376	
7_789	BovineHD0700028789	7	98,589,740	rs133712793	
7_790	BovineHD0700028790	7	98,591,048	rs135081549	
7_791	BovineHD0700028791	7	98,591,826	rs109226849	
7_792	BovineHD0700028792	7	98,592,979	rs110049532	
7_793	BovineHD0700028793	7	98,593,984	rs137368766	
7_794	BovineHD0700028794	7	98,594,835	rs108962480	



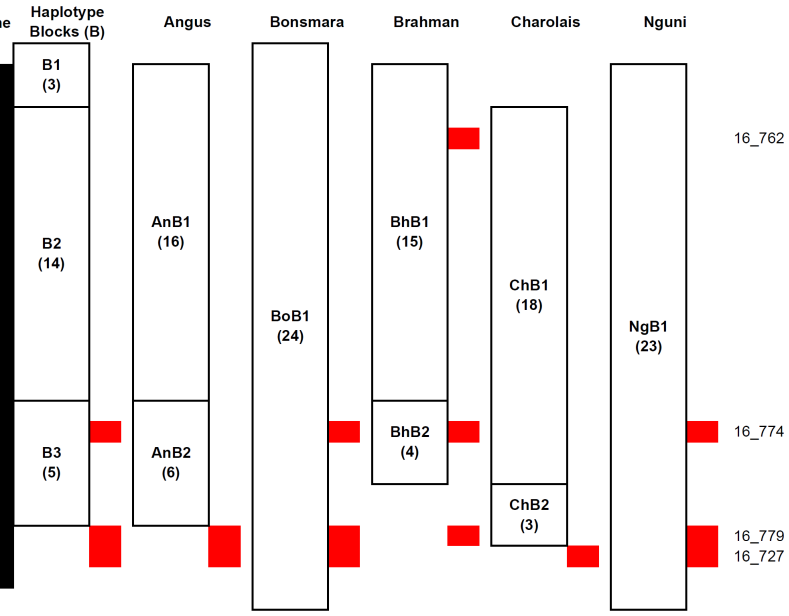
The capn3 *gene (10:37,828,773-37,885,862)*

SNP	Illumina	Chr	Position	Location	Gene
10_712	BovineHD1000011712	10	37,824,551	rs110439595	
10_347	BovineHD1000031347	10	37,827,639	rs135945111	
10_350	ARS-BFGL-NGS-13350	10	37,830,642	rs109425380	
10_713	BovineHD1000011713	10	37,833,640	rs109318676	
10_714	BovineHD1000011714	10	37,836,009	rs109372443	
10_715	BovineHD1000011715	10	37,837,623	rs109290773	
10_716	BovineHD1000011716	10	37,838,570	rs109050259	
10_718	BovineHD1000011718	10	37,843,006	rs109819443	
10_719	BovineHD1000011719	10	37,846,946	rs110452450	
10_293	Hapmap47063-BTA-62293	10	37,852,123	rs41644730	
10_720	BovineHD1000011720	10	37,860,216	rs135593461	
10_721	BovineHD1000011721	10	37,864,319	rs109239406	
10_722	BovineHD1000011722	10	37,869,021	rs134085397	
10_723	BovineHD1000011723	10	37,870,339	rs110307662	
10_725	BovineHD1000011725	10	37,876,596	rs110994653	
10_726	BovineHD1000011726	10	37,877,303	rs109324755	
10_727	BovineHD1000011727	10	37,885,702	rs109474612	
10_728	BovineHD1000011728	10	37,887,422	rs133467689	



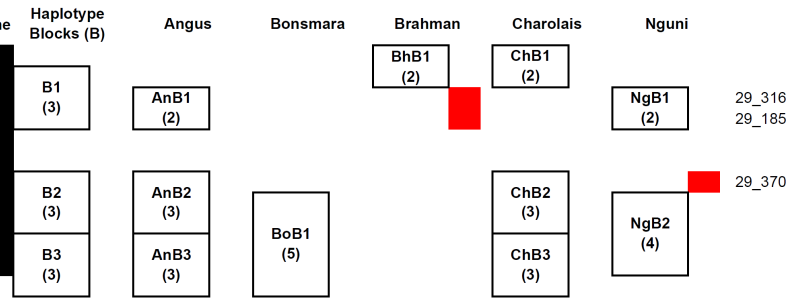
The capn2 *gene (16:27,781,671-27,840,011)*

SNP	Illumina	Chr	Position	Location	Gene
16_758	BovineHD1600007758	16	27,781,212	rs132720146	
16_759	BovineHD1600007759	16	27,786,368	rs134412163	
16_760	BovineHD1600007760	16	27,789,803	rs135593510	
16_761	BovineHD1600007761	16	27,793,409	rs133177149	
16_762	BovineHD1600007762	16	27,798,419	rs134504287	
16_763	BovineHD1600007763	16	27,801,014	rs137662975	
16_764	BovineHD1600007764	16	27,803,013	rs133410746	
16_216	ARS-BFGL-NGS-102216	16	27,805,751	rs109891270	
16_765	BovineHD1600007765	16	27,808,861	rs137301729	
16_766	BovineHD1600007766	16	27,810,205	rs136452103	
16_767	BovineHD1600007767	16	27,810,903	rs133882716	
16_768	BovineHD1600007768	16	27,811,695	rs135482143	
16_769	BovineHD1600007769	16	27,812,241	rs136468320	
16_416	BovineHD4100012416	16	27,812,915	rs41634656	
16_770	BovineHD1600007770	16	27,813,621	rs133943882	
16_771	BovineHD1600007771	16	27,814,339	rs134927379	
16_772	BovineHD1600007772	16	27,815,334	rs137711991	
16_773	BovineHD1600007773	16	27,817,199	rs133964632	
16_774	BovineHD1600007774	16	27,819,457	rs137478059	
16_775	BovineHD1600007775	16	27,820,470	rs132858981	
16_776	BovineHD1600007776	16	27,823,039	rs134023967	
16_777	BovineHD1600007777	16	27,826,235	rs135263847	
16_778	BovineHD1600007778	16	27,828,773	rs132895734	
16_779	BovineHD1600007779	16	27,829,558	rs137097425	
16_727	ARS-BFGL-NGS-85727	16	27,833,776	rs109978733	
16_780	BovineHD1600007780	16	27,836,737	rs135646764	
16_781	BovineHD1600007781	16	27,843,498	rs136736277	



The capn1 *gene (29:44,063,463-44,100,316)*

SNP	Illumina	Chr	Position	Location	Gene
29_183	BovineHD2900013183	29	44,067,207	rs17872099	
29_184	BovineHD2900013184	29	44,067,968	rs17871986	
29_316	CAPN1_1	29	44,069,063	rs17872000	
29_185	BovineHD2900013185	29	44,070,926	rs110104039	
29_186	BovineHD2900013186	29	44,076,213	rs137225062	
29_187	BovineHD2900013187	29	44,081,056	rs135658374	
29_370	UA-IFASA-1370	29	44,085,769	rs17871058	
29_4751	CAPN1_2	29	44,087,629	rs17872050	
29_188	BovineHD2900013188	29	44,088,897	rs17870847	
29_189	BovineHD2900013189	29	44,093,671	rs134827338	
29_190	BovineHD2900013190	29	44,097,970	rs135736399	
29_191	BovineHD2900013191	29	44,101,417	rs133641190	



B1 - B5 – Haplotype blocks identified using pairwise analysis in Haploview 4.2 (Gabriel *et al.*, 2002; Barrett *et al.*, 2005); An – Angus; Bo – Bonsmara; Bh – Brahman; Ch – Charolais; Ng – Nguni; Chr - chromosome; Gene – Blacked-out blocks represent coding regions of the genes (plus 8% of the gene's length upstream and downstream from the reference sequence) (NCBI, 2021) ; Illumina – Illumina name assigned to variant; SNP – SNV code - CHR_last 3 digits of Illumina name; SNV that failed quality control, or were not polymorphic within breeds are highlighted in red.

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Barrett, J.C., Fry, B., Maller, J. & Daly, M.J., 2005. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21: 263–265.

Gabriel, S.B., Schaffner, S.F., Nguyen, H., Moore, J.M., Roy, J., Blumenstiel, B., Higgins, J., DeFelice, M., Lochner, A., Faggart, M., Liu-Cordero, S.N., Rotimi, C., Adeyemo, A., Cooper, R., Ward, R., Lander, E.S., Daly, M.J. & Altshuler, D., 2002. The structure of haplotype blocks in the human genome. Science 296: 2225–2229.

NCBI, 2021. National Center for Biotechnology Information. Genome Data Viewer: Bos taurus assembly UMD_3.1.1. US National Library of Medicine, Maryland, USA https://www.ncbi.nlm.nih.gov.

Addendum C: Supplementary material for Chapter 5

Table S1. *Phenotypic means of detailed growth and carcass traits determined in South African feedlot-finished beef bulls (whole animal or carcass) and carcass, intermediary metabolic energy and meat quality traits for the control (non-electrically stimulated) and electrically stimulated carcass halves, over four ageing periods up to d 20 post-mortem.*

Trait	Animal or whole carcass determinations
Days fed (d)	123 ± 2.82
63 d Average daily gain (kg/d)	1.71 ± 0.05
Live body weight at slaughter (kg)	388 ± 4.26
Total collagen	1.55 ± 0.02
Soluble collagen	0.21 ± 0.01
Insoluble collagen	1.34 ± 0.02
Collagen solubility (%)	13.62 ± 0.34
Dry matter (%)	24.1 ± 0.06
Moisture (%)	75.9 ± 0.41
Ash (%)	1.10 ± 0.01
Crude protein (%)	21.38 ± 0.06
Intramuscular fat (%)	1.32 ± 0.04

Trait	NS	ES	*p*-value [1]
Hot (warm) carcass weight (kg) [2]	109 ± 1.23	110 ± 1.24	–
Cold (chilled) carcass weight (kg)	107 ± 1.20	107 ± 1.22	–
Carcass mass loss percentage	2.20 ± 0.05	2.16 ± 0.04	–
Sarcomere length (µm)	1.90 ± 0.01 [a]	1.88 ± 0.01 [b]	0.0356
pH 1 h	6.69 ± 0.01 [a]	6.27 ± 0.01 [b]	<0.0001
pH 3 h	6.29 ± 0.02 [a]	5.90 ± 0.02 [b]	<0.0001
pH 6 h	5.91 ± 0.02 [a]	5.66 ± 0.01 [b]	<0.0001
pHu 20 h	5.56 ± 0.02 [a]	5.47 ± 0.02 [b]	<0.0001
Temp 1 h (°C)	37.43 ± 0.12	37.65 ± 0.13	0.1883
Temp 3 h (°C)	28.79 ± 0.16 [a]	26.87 ± 0.20 [b]	<0.0001
Temp 6 h (°C)	21.34 ± 0.14 [a]	19.08 ± 0.19 [b]	<0.0001
Temp 20 h (°C)	6.57 ± 0.19 [a]	6.32 ± 0.18 [b]	0.0371
ATP 1 h (µmol/g)	6.30 ± 0.06 [a]	5.78 ± 0.08 [b]	<0.0001
ATP 3 h (µmol/g)	5.92 ± 0.08 [a]	5.15 ± 0.10 [b]	<0.0001
ATP 6 h (µmol/g)	5.15 ± 0.10 [a]	4.05 ± 0.09 [b]	<0.0001
ATP 20 h (µmol/g)	2.69 ± 0.06	2.56 ± 0.06	0.1155
Creatine phosphate 1 h (µmol/g)	3.55 ± 0.09 [a]	2.75 ± 0.05 [b]	<0.0001
Creatine phosphate 3 h (µmol/g)	2.85 ± 0.07 [a]	2.67 ± 0.05 [b]	0.0115
Creatine phosphate 6 h (µmol/g)	2.39 ± 0.05	2.43 ± 0.05	0.5159
Creatine phosphate 20 h (µmol/g)	1.85 ± 0.03 [b]	1.96 ± 0.04 [a]	0.0297
Glucose 1 h (µmol/g)	0.84 ± 0.03 [b]	1.83 ± 0.05 [a]	<0.0001
Glucose 3 h (µmol/g)	0.99 ± 0.05 [b]	1.89 ± 0.06 [a]	<0.0001
Glucose 6 h (µmol/g)	1.33 ± 0.06 [b]	2.36 ± 0.08 [a]	<0.0001
Glucose 20 h (µmol/g)	2.61 ± 0.08 [b]	3.60 ± 0.09 [a]	<0.0001
Glucose 6-phosphate 1 h (µmol/g)	2.98 ± 0.10 [b]	3.39 ± 0.13 [a]	0.0038
Glucose 6-phosphate 3 h (µmol/g)	2.50 ± 0.10 [b]	2.80 ± 0.13 [a]	0.0437
Glucose 6-phosphate 6 h (µmol/g)	2.91 ± 0.15 [b]	3.31 ± 0.16 [a]	0.0427
Glucose 6-phosphate 20 h (µmol/g)	6.99 ± 0.22 [a]	6.32 ± 0.23 [b]	0.0161
Lactate 1 h (µmol/g)	21.31 ± 0.61 [b]	37.27 ± 0.67 [a]	<0.0001
Lactate 3 h (µmol/g)	26.85 ± 0.84 [b]	42.48 ± 0.98 [a]	<0.0001
Lactate 6 h (µmol/g)	35.17 ± 1.00 [b]	50.87 ± 1.01 [a]	<0.0001
Lactate 20 h (µmol/g)	75.84 ± 1.22 [a]	69.34 ± 0.85 [b]	<0.0001
Glycogen 1 h (µmol glycosyl units/g)	33.54 ± 1.05 [a]	28.79 ± 0.99 [b]	0.0002
Glycogen 3 h (µmol glycosyl units/g)	27.64 ± 1.26 [z]	24.99 ± 1.13 [y]	0.0614
Glycogen 6 h (µmol glycosyl units/g)	22.45 ± 1.04 [a]	17.28 ± 1.05 [b]	0.0001
Glycogen 20 h (µmol glycosyl units/g)	9.71 ± 0.54	8.75 ± 0.52	0.1653
Glycolytic potential 1 h (µmol/g)	96.02 ± 2.21 [b]	105.29 ± 2.18 [a]	0.0006
Glycolytic potential 3 h (µmol/g)	89.10 ± 2.80 [b]	101.86 ± 2.57 [a]	<0.0001
Glycolytic potential 6 h (µmol/g)	88.56 ± 2.48 [b]	96.79 ± 2.64 [a]	0.0055
Glycolytic potential 20 h (µmol/g)	114.47 ± 2.04 [a]	106.67 ± 1.91 [b]	0.0014

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Trait	NS	ES	*p*-value [1]
Warner-Bratzler shear force d 3 (kg) [2]	6.72 ± 0.14 [a]	5.58 ± 0.11 [b]	<0.0001
Warner-Bratzler shear force d 9 (kg)	5.44 ± 0.14 [a]	4.22 ± 0.08 [b]	<0.0001
Warner-Bratzler shear force d 14 (kg)	4.61 ± 0.11 [a]	3.72 ± 0.07 [b]	<0.0001
Warner-Bratzler shear force d 20 (kg)	4.27 ± 0.11 [a]	3.47 ± 0.06 [b]	<0.0001
Myofibril fragment length d 3	35.42 ± 0.66	35.60 ± 0.69	0.8308
Myofibril fragment length d 9	26.34 ± 0.39	26.08 ± 0.49	0.6502
Myofibril fragment length d 14	23.98 ± 0.38	23.87 ± 0.43	0.8357
Myofibril fragment length d 20	21.72 ± 0.30	21.60 ± 0.35	0.7788
Calpastatin 1 h (U/g meat)	1.98 ± 0.04	2.03 ± 0.03	0.2241
Calpastatin 20 h (U/g meat)	1.64 ± 0.04 [y]	1.72 ± 0.04 [z]	0.0843
Calpain-1 1 h (U/g meat)	1.40 ± 0.03	1.44 ± 0.03	0.1439
Calpain-1 20 h (U/g meat)	1.10 ± 0.03 [a]	1.02 ± 0.03 [b]	0.0136
Calpain-2 1 h (U/g meat)	0.98 ± 0.01	1.00 ± 0.01	0.1134
Calpain-2 20 h (U/g meat)	1.02 ± 0.01	1.01 ± 0.01	0.6110
Calpastatin/calpain-1 1 h	1.47 ± 0.03	1.45 ± 0.02	0.6410
Calpastatin/calpain-1 20 h	1.58 ± 0.04 [b]	1.87 ± 0.06 [a]	<0.0001
Calpastatin/calpains 1 h	0.84 ± 0.01	0.84 ± 0.01	0.8745
Calpastatin/calpains 20 h	0.78 ± 0.01 [b]	0.85 ± 0.01 [a]	<0.0001
Water-holding capacity d 3	0.39 ± 0.00	0.39 ± 0.00	0.4418
Water-holding capacity d 9	0.37 ± 0.01	0.37 ± 0.01	0.3897
Water-holding capacity d 14	0.35 ± 0.00	0.35 ± 0.00	0.4666
Water-holding capacity d 20	0.38 ± 0.01	0.37 ± 0.00	0.1312
Cooking loss d 3 (%)	21.37 ± 0.29	21.78 ± 0.28	0.1818
Cooking loss d 9 (%)	21.23 ± 0.31	21.46 ± 0.29	0.5226
Cooking loss d 14 (%)	21.01 ± 0.30	21.30 ± 0.29	0.3377
Cooking loss d 20 (%)	21.24 ± 0.33	21.69 ± 0.34	0.1972
L* d 3	39.67 ± 0.26	39.57 ± 0.25	0.6889
L* d 9	38.04 ± 0.30	38.53 ± 0.30	0.1291
L* d 14	38.61 ± 0.31 [b]	39.39 ± 0.31 [a]	0.0216
L* d 20	38.77 ± 0.34 [y]	39.48 ± 0.35 [z]	0.0671
a* d 3	11.12 ± 0.11 [y]	11.37 ± 0.12 [z]	0.0878
a* d 9	11.41 ± 0.25	11.32 ± 0.24	0.6908
a* d 14	12.52 ± 0.18	12.72 ± 0.18	0.3537
a* d 20	12.32 ± 0.16	12.35 ± 0.19	0.8699
b* d 3	10.99 ± 0.23	10.82 ± 0.24	0.5279
b* d 9	10.65 ± 0.29	10.56 ± 0.28	0.6594
b* d 14	10.03 ± 0.30	10.32 ± 0.29	0.1771
b* d 20	10.08 ± 0.31	10.19 ± 0.29	0.6264
C* d 3	15.78 ± 0.20	15.85 ± 0.21	0.7695
C* d 9	15.84 ± 0.31	15.71 ± 0.30	0.6469
C* d 14	16.36 ± 0.25	16.67 ± 0.24	0.2782
C* d 20	16.23 ± 0.24	16.29 ± 0.26	0.8515
H_{ab} d 3	43.72 ± 0.64 [z]	42.59 ± 0.64 [y]	0.0975
H_{ab} d 9	42.54 ± 0.81	42.57 ± 0.81	0.9621
H_{ab} d 14	37.45 ± 0.89	37.99 ± 0.85	0.1609
H_{ab} d 20	37.86 ± 0.90	38.38 ± 0.84	0.2436

[1] *p*-values for the effect of treatment (electrical stimulation) on traits, derived from a generalised linear model (GLM) analysis in SAS (SAS, 2010); [2] All values are given as least squares means (LSM) ± standard errors (SE). Values that were significantly different between treatments ($p \leq 0.05$) were indicated with different ascending letter superscripts (a, b) in each row and those that tended to be significantly different ($p \leq 0.10$) with different descending letter superscripts (z, y).

NS – non-electrically stimulated or the control; ES – electrically stimulated or the treatment.

Table S2. *Description of SNPs and alleles identified in the* capn1*,* capn2*,* capn3 *and* cast *genes in South African beef breeds, genotyped using the Illumina® BovineHD SNP BeadChip (777K).*

#	Illumina name [1]	Chr	Position	Location	SNP	MAF [2]	P_{HWdev} [2]	MA (A1)	TENDER
1	BovineHD0700028721	7	98,431,222	rs110062870	*7_721*	41%	0.0384	G	T*
2	BovineHD0700028722	7	98,432,644	rs134932261	*7_722*	42%	0.0707	C	T*
3	BovineHD0700028723	7	98,435,292	rs135825068	*7_723*	42%	0.0707	T	C*
4	BovineHD0700028724	7	98,439,177	rs133855588	*7_724*	17%	0.0154	A	A*
5	BovineHD0700028725	7	98,442,449	rs110473478	*7_725*	42%	0.0707	T	C*
6	**BovineHD0700028726**	7	98,448,831	rs132707186	*7_726*	19%	0.0337	G	A*
7	**BovineHD0700028727**	7	98,449,505	rs110540286	*7_727*	19%	0.0337	T	C*
8	**BovineHD0700028728**	7	98,450,117	rs135049475	*7_728*	42%	0.0707	T	C*
9	**BovineHD0700028729**	7	98,454,085	rs109679745	*7_729*	36%	0.3688	G	A*
10	**BovineHD0700028730**	7	98,457,153	rs134804900	*7_730*	23%	0.1151	C	C*
11	**BovineHD0700028731**	7	98,463,330	rs136084722	*7_731*	14%	0.0010	A	A*
12	**BovineHD0700028732**	7	98,466,806	rs133432068	*7_732*	36%	0.3688	C	T*
13	**BovineHD0700028733**	7	98,467,371	rs109932018	*7_733*	35%	0.4943	T	C*
14	**BovineHD0700028734**	7	98,467,934	rs137677027	*7_734*	35%	0.4943	G	T*
15	**BovineHD0700028735**	7	98,471,546	rs109106772	*7_735*	36%	0.3688	A	G*
16	**BovineHD0700028736**	7	98,473,634	rs137217365	*7_736*	28%	0.0253	G	G*
17	**BovineHD0700028737**	7	98,474,995	rs135811099	*7_737*	25%	0.6039	G	G*
18	**BovineHD0700028738**	7	98,476,556	rs109970923	*7_738*	33%	0.3374	C	C*
19	**BovineHD0700028739**	7	98,480,585	rs134897496	*7_739*	21%	1.0000	C	T*
20	**BovineHD0700028740**	7	98,481,274	rs135802918	*7_740*	21%	1.0000	A	C*
21	**BovineHD4100006349**	7	98,482,074	rs41596487	*7_349*	18%	0.9832	C	T*
22	**BovineHD0700028741**	7	98,484,691	rs136939207	*7_741*	22%	1.0000	A	C*
23	**BovineHD0700028742**	7	98,488,255	rs135233602	*7_742*	20%	0.9680	T	C*
24	**BovineHD0700028743**	7	98,492,079	rs137781929	*7_743*	14%	0.4222	G	A*
25	**BovineHD0700028744**	7	98,492,868	rs133927728	*7_744*	14%	0.4144	A	G*
26	**ARS-BFGL-NGS-43901**	7	98,498,047	rs109804679	*7_901*	12%	0.1053	T	G*
27	**BovineHD0700028746**	7	98,498,729	rs135497848	*7_746*	12%	0.1053	A	G*
28	**BovineHD0700028747**	7	98,499,702	rs110707037	*7_747*	21%	1.0000	A	C*
29	**BovineHD0700028748**	7	98,502,599	rs134762812	*7_748*	18%	1.0000	C	T*
30	**BovineHD0700028749**	7	98,506,739	rs136046573	*7_749*	17%	1.0000	A	G*
31	**BovineHD0700028750**	7	98,507,574	rs132701334	*7_750*	21%	1.0000	T	G*
32	**BovineHD0700028751**	7	98,508,282	rs110178428	*7_751*	21%	1.0000	C	A*
33	**BovineHD0700028752**	7	98,508,931	rs135644323	*7_752*	21%	1.0000	C	T*
34	**BovineHD0700028753**	7	98,510,114	rs136632100	*7_753*	1%	1.0000	T	C*
35	**BovineHD0700028754**	7	98,511,880	rs134385243	*7_754*	14%	1.0000	G	T*
36	**BovineHD0700028755**	7	98,512,675	rs135405624	*7_755*	21%	1.0000	G	A*
37	**BovineHD0700028756**	7	98,513,190	rs133290603	*7_756*	21%	1.0000	A	G*
38	**BovineHD0700028757**	7	98,520,428	rs110241720	*7_757*	21%	1.0000	C	T*
39	**BovineHD0700028758**	7	98,524,220	rs135682399	*7_758*	21%	1.0000	A	G*
40	**BovineHD0700028759**	7	98,526,859	rs133488081	*7_759*	7%	0.3988	C	T*
41	**BovineHD0700028760**	7	98,531,321	rs137215768	*7_760*	21%	1.0000	G	A*
42	**BovineHD0700028761**	7	98,531,781	rs136056291	*7_761*	9%	0.2592	G	G*
43	**BovineHD0700028762**	7	98,532,654	rs109938220	*7_762*	20%	0.8771	C	T*
44	**ARS-USMARC-670**	7	98,534,197	rs109677393	*7_670*	21%	1.0000	G	A*
45	**BovineHD0700028763**	7	98,534,736	rs135465452	*7_763*	17%	1.0000	T	C*
46	**BovineHD0700028764**	7	98,537,976	rs109102936	*7_764*	7%	0.2935	C	T*
47	**BovineHD0700028765**	7	98,540,675	rs133997237	*7_765*	7%	0.3423	T	C*

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#	Illumina name [1]	Chr	Position	Location	SNP	MAF [2]	P_{HWdev} [2]	MA (A1)	TENDER
48	**BovineHD0700028766**	7	98,541,844	rs135693211	*7_766*	20%	0.7482	T	G*
49	**BovineHD0700028767**	7	98,545,774	rs136646587	*7_767*	17%	0.9662	C	T*
50	**BovineHD0700028768**	7	98,547,086	rs110136749	*7_768*	20%	0.7482	C	T*
51	**BovineHD0700028769**	7	98,551,183	rs137371179	*7_769*	16%	0.7602	A	G*
52	**BovineHD0700028770**	7	98,551,927	rs132851516	*7_770*	21%	0.7636	C	T*
53	**BovineHD0700028771**	7	98,552,632	rs109712965	*7_771*	21%	0.8248	A	C*
54	**BovineHD0700028772**	7	98,553,659	rs109087212	*7_772*	21%	0.8248	T	C*
55	**BovineHD0700028773**	7	98,554,459	rs109384915	*7_773*	20%	0.6738	G	A*
56	**BovineHD0700028774**	7	98,557,529	rs134041938	*7_774*	15%	0.8714	A	A*
57	**BovineHD0700028775**	7	98,560,223	rs110733539	*7_775*	18%	0.5955	G	A*
58	**BovineHD0700028776**	7	98,562,742	rs110050430	*7_776*	25%	0.3832	G	A*
59	**BovineHD0700028777**	7	98,563,418	rs110380498	*7_777*	21%	0.7636	C	T*
60	**ARS-USMARC-116**	7	98,566,391	rs109354718	*7_116*	17%	0.9590	G	A*
61	**BovineHD0700028778**	7	98,570,487	rs137558115	*7_778*	23%	0.6073	T	G*
62	**BovineHD0700028779**	7	98,571,597	rs133343732	*7_779*	22%	0.5522	T	G*
63	**BovineHD0700028780**	7	98,574,139	rs135279064	*7_780*	23%	0.6073	T	C*
64	**BovineHD0700028781**	7	98,574,903	rs109843102	*7_781*	23%	0.6073	T	C*
65	**BovineHD0700028782**	7	98,575,799	rs109562954	*7_782*	22%	0.5522	G	T*
66	**BovineHD0700028783**	7	98,576,940	rs110031829	*7_783*	17%	1.0000	C	T*
67	**BovineHD0700028784**	7	98,578,836	rs136073124	*7_784*	21%	0.9030	A	G*
68	**BovineHD4100006350**	7	98,579,574	rs41255587	*7_350*	22%	0.4098	A	G*
69	BovineHD0700028786	7	98,585,027	rs110883024	*7_786*	23%	0.7582	T	C*
70	BovineHD0700028787	7	98,585,930	rs135444809	*7_787*	23%	0.6985	T	C*
71	BovineHD0700028788	7	98,586,431	rs136572376	*7_788*	21%	0.3019	C	A*
72	BovineHD0700028789	7	98,589,740	rs133712793	*7_789*	27%	0.6994	C	T*
73	BovineHD0700028790	7	98,591,048	rs135081549	*7_790*	17%	0.8187	T	C*
74	BovineHD0700028791	7	98,591,826	rs109226849	*7_791*	26%	1.0000	C	T*
75	BovineHD0700028792	7	98,592,979	rs110049532	*7_792*	20%	0.9385	C	T*
76	BovineHD0700028793	7	98,593,984	rs137368766	*7_793*	20%	0.9680	C	T*
77	BovineHD0700028794	7	98,594,835	rs108962480	*7_794*	14%	0.2632	C	C*

#	Illumina name [1]	Chr	Position	Location	SNP	MAF [2]	P_{HWdev} [2]	MA (A1)	TENDER
78	BovineHD1000011712	10	37,824,551	rs110439595	*10_712*	10%	1.0000	G	G*
79	BovineHD1000031347	10	37,827,639	rs135945111	*10_347*	24%	5.71×10^{-10}	G	A*
80	**ARS-BFGL-NGS-13350**	10	37,830,642	rs109425380	*10_350*	17%	0.2209	T	C*
81	**BovineHD1000011713**	10	37,833,640	rs109318676	*10_713*	9%	1.0000	A	A*
82	**BovineHD1000011714**	10	37,836,009	rs109372443	*10_714*	19%	0.3552	C	T*
83	**BovineHD1000011715**	10	37,837,623	rs109290773	*10_715*	5%	0.6313	T	T*
84	**BovineHD1000011716**	10	37,838,570	rs109050259	*10_716*	17%	0.2209	G	A*
85	**BovineHD1000011718**	10	37,843,006	rs109819443	*10_718*	33%	0.4668	T	T*
86	**BovineHD1000011719**	10	37,846,946	rs110452450	*10_719*	43%	0.4484	G	G*
87	**Hapmap47063-BTA-62293**	10	37,852,123	rs41644730	*10_293*	36%	0.1480	T	T*
88	**BovineHD1000011720**	10	37,860,216	rs135593461	*10_720*	36%	0.8232	G	G*
89	**BovineHD1000011721**	10	37,864,319	rs109239406	*10_721*	44%	0.2666	A	G*
90	**BovineHD1000011722**	10	37,869,021	rs134085397	*10_722*	44%	0.5356	A	A*
91	**BovineHD1000011723**	10	37,870,339	rs110307662	*10_723*	18%	0.6782	T	C*
92	**BovineHD1000011725**	10	37,876,596	rs110994653	*10_725*	14%	0.7649	T	T*
93	**BovineHD1000011726**	10	37,877,303	rs109324755	*10_726*	15%	1.0000	G	G*
94	**BovineHD1000011727**	10	37,885,702	rs109474612	*10_727*	12%	0.3294	T	T*
95	BovineHD1000011728	10	37,887,422	rs133467689	*10_728*	14%	0.7649	C	C*

#	Illumina name [1]	Chr	Position	Location	SNP	MAF [2]	P_{HWdev} [2]	MA (A1)	TENDER
96	BovineHD1600007758	16	27,781,212	rs132720146	*16_758*	34%	0.0429	T	T*
97	**BovineHD1600007759**	16	27,786,368	rs134412163	*16_759*	48%	0.2092	G	G*
98	**BovineHD1600007760**	16	27,789,803	rs135593510	*16_760*	42%	0.0216	G	A*
99	**BovineHD1600007761**	16	27,793,409	rs133177149	*16_761*	44%	0.0460	C	T*
100	**BovineHD1600007762**	16	27,798,419	rs134504287	*16_762*	13%	0.0110	A	A*
101	**BovineHD1600007763**	16	27,801,014	rs137662975	*16_763*	48%	0.1561	C	T*
102	**BovineHD1600007764**	16	27,803,013	rs133410746	*16_764*	21%	0.4943	G	G*
103	**ARS-BFGL-NGS-102216**	16	27,805,751	rs109891270	*16_216*	31%	0.0946	G	G*
104	**BovineHD1600007765**	16	27,808,861	rs137301729	*16_765*	33%	0.0979	G	A*
105	**BovineHD1600007766**	16	27,810,205	rs136452103	*16_766*	45%	0.0222	G	A*
106	**BovineHD1600007767**	16	27,810,903	rs133882716	*16_767*	32%	0.1218	C	T*
107	**BovineHD1600007768**	16	27,811,695	rs135482143	*16_768*	22%	0.4098	C	C*
108	**BovineHD1600007769**	16	27,812,241	rs136468320	*16_769*	22%	0.4098	C	C*
109	**BovineHD4100012416**	16	27,812,915	rs41634656	*16_416*	27%	0.0201	C	C*
110	**BovineHD1600007770**	16	27,813,621	rs133943882	*16_770*	36%	0.0625	G	A*
111	**BovineHD1600007771**	16	27,814,339	rs134927379	*16_771*	33%	0.0453	G	A*
112	**BovineHD1600007772**	16	27,815,334	rs137711991	*16_772*	48%	0.1561	G	A*
113	**BovineHD1600007773**	16	27,817,199	rs133964632	*16_773*	28%	0.7317	T	T*
114	**BovineHD1600007774**	16	27,819,457	rs137478059	*16_774*	4%	1.0000	A	G*
115	**BovineHD1600007775**	16	27,820,470	rs132858981	*16_775*	28%	0.7317	T	T*
116	**BovineHD1600007776**	16	27,823,039	rs134023967	*16_776*	34%	0.4798	A	G*
117	**BovineHD1600007777**	16	27,826,235	rs135263847	*16_777*	20%	0.2713	T	T*
118	**BovineHD1600007778**	16	27,828,773	rs132895734	*16_778*	21%	0.4943	G	G*
119	**BovineHD1600007779**	16	27,829,558	rs137097425	*16_779*	3%	1.0000	T	C*
120	**ARS-BFGL-NGS-85727**	16	27,833,776	rs109978733	*16_727*	3%	1.0000	A	C*
121	**BovineHD1600007780**	16	27,836,737	rs135646764	*16_780*	21%	0.0009	G	A*
122	BovineHD1600007781	16	27,843,498	rs136736277	*16_781*	29%	0.3026	C	C*

#	Illumina name [1]	Chr	Position	Location	SNP	MAF [2]	P_{HWdev} [2]	MA (A1)	TENDER
123	**BovineHD2900013183**	29	44,067,207	rs17872099	*29_183*	46%	0.7765	C	C*
124	**BovineHD2900013184**	29	44,067,968	rs17871986	*29_184*	34%	0.1966	A	G*
125	**CAPN1_1**	29	44,069,063	rs17872000	*29_316*	20%	0.1192	C	C*
126	**BovineHD2900013185**	29	44,070,926	rs110104039	*29_185*	28%	0.0005	A	A*
127	**BovineHD2900013186**	29	44,076,213	rs137225062	*29_186*	38%	0.0099	A	C*
128	**BovineHD2900013187**	29	44,081,056	rs135658374	*29_187*	37%	0.0013	C	T*
129	**UA-IFASA-1370**	29	44,085,769	rs17871058	*29_370*	23%	0.2932	A	A*
130	**CAPN1_2**	29	44,087,629	rs17872050	*29_4751*	49%	0.0027	T	C*
131	**BovineHD2900013188**	29	44,088,897	rs17870847	*29_188*	41%	0.0184	G	A*
132	**BovineHD2900013189**	29	44,093,671	rs134827338	*29_189*	37%	0.0314	C	T*
133	**BovineHD2900013190**	29	44,097,970	rs135736399	*29_190*	40%	0.0006	A	G*
134	BovineHD2900013191	29	44,101,417	rs133641190	*29_191*	32%	0.0435	C	T*

[1] Coloured blocks represent haplotype blocks determined using default settings in Haploview 4.2 (Gabriel *et al.*, 2002; Barrett *et al.*, 2005);
[2] SNPs that failed quality control for MAF and P_{HWdev} were highlighted in red.

Chr – chromosome number; MA(A1) – The minor allele as determined from pooled data for all breeds; MAF – minor allelic frequency; P_{HWdev} – *p*-value for Hardy-Weinberg exact test; Position – position in UMD_3.1.1 (NCBI, 2021); SNP – shortened name of SNP; TENDER – The allele favourable for tenderness determined from means of WBSF, MFL, calpastatin and calpain protease activities.

Table S3. *Number of bulls per haplotype block, identified in the genes of the calpain-calpastatin system in five South African beef breeds, genotyped with the Illumina® BovineHD SNP BeadChip (777K).*

		Pooled (n = 166)	Angus (n = 27)	Bonsmara (n = 35)	Brahman (n = 35)	Charolais (n = 34)	Nguni (n = 35)
				cast			
Block 1	**H1** [1]	61 (37%)	18 (67%)	13 (37%)	10 (29%)	9 (26%)	11 (31%)
	H2	20 (12%)	5 (19%)	4 (11%)	3 (9%)	5 (15%)	3 (9%)
	H3	20 (12%)	1 (4%)	11 (31%)	–	3 (9%)	5 (14%)
Block 2	**H1** [1]	72 (43%)	18 (67%)	13 (37%)	18 (51%)	12 (35%)	11 (31%)
	H2	38 (23%)	7 (26%)	15 (43%)	4 (11%)	6 (18%)	6 (17%)
Block 3	**H1** [1]	104 (63%)	19 (70%)	28 (80%)	20 (57%)	16 (47%)	21 (60%)
	H2	45 (27%)	7 (26%)	7 (20%)	6 (17%)	12 (35%)	13 (37%)
Block 4	**H1** [1]	102 (61%)	19 (70%)	28 (80%)	18 (51%)	15 (44%)	22 (63%)
	H2	15 (9%)	–	–	2 (6%)	2 (6%)	11 (31%)
	H3	17 (10%)	2 (7%)	5 (14%)	4 (11%)	4 (12%)	2 (6%)
Block 5	**H1** [1]	87 (52%)	10 (37%)	28 (80%)	13 (37%)	14 (41%)	22 (63%)
	H2	34 (20%)	1 (4%)	5 (14%)	10 (29%)	5 (15%)	13 (37%)
				capn3			
Block 1	**H1** [1]	112 (67%)	16 (59%)	25 (71%)	22 (63%)	20 (59%)	29 (83%)
	H2	19 (11%)	–	3 (9%)	9 (26%)	2 (6%)	5 (14%)
	H3	27 (16%)	10 (37%)	6 (17%)	–	10 (29%)	1 (3%)
Block 2	**H1**	20 (12%)	–	12 (34%)	2 (6%)	2 (6%)	4 (11%)
	H2	41 (25%)	6 (22%)	13 (37%)	–	4 (12%)	18 (51%)
	H3	25 (15%)	6 (22%)	3 (9%)	–	10 (29%)	6 (17%)
	H4	18 (11%)	–	–	16 (46%)	2 (6%)	–
Block 3	**H1** [1]	34 (20%)	10 (37%)	10 (29%)	6 (17%)	3 (9%)	5 (14%)
	H2	51 (31%)	9 (33%)	6 (17%)	16 (46%)	7 (21%)	13 (37%)
	H3	26 (16%)	3 (11%)	10 (29%)	1 (3%)	9 (26%)	3 (9%)
	H4	25 (15%)	3 (11%)	1 (3%)	11 (31%)	4 (12%)	6 (17%)
	H5	26 (16%)	2 (7%)	6 (17%)	1 (3%)	10 (29%)	7 (20%)
Block 4	**H1** [1]	120 (72%)	16 (59%)	25 (71%)	33 (94%)	26 (76%)	20 (57%)
	H2	38 (23%)	10 (37%)	9 (26%)	2 (6%)	7 (21%)	10 (29%)
				capn2			
Block 1	**H1**	25 (15%)	–	8 (23%)	14 (40%)	1 (3%)	2 (6%)
	H2	45 (27%)	5 (19%)	15 (43%)	13 (37%)	6 (18%)	6 (17%)
	H3	48 (29%)	13 (48%)	5 (14%)	5 (14%)	12 (35%)	13 (37%)
	H4	14 (8%)	3 (11%)	1 (3%)	1 (3%)	1 (3%)	8 (23%)
Block 2	**H1**	24 (14%)	6 (22%)	4 (11%)	4 (11%)	–	10 (29%)
	H2	19 (11%)	7 (26%)	1 (3%)	1 (3%)	7 (21%)	3 (9%)
	H3	31 (19%)	6 (22%)	12 (34%)	3 (9%)	4 (12%)	6 (17%)
Block 3	**H1**	45 (27%)	8 (30%)	20 (57%)	3 (9%)	5 (15%)	9 (26%)
	H2	74 (45%)	19 (70%)	10 (29%)	7 (20%)	14 (41%)	24 (69%)
				capn1			
Block 1	**H1**	19 (11%)	6 (22%)	3 (9%)	–	2 (6%)	8 (23%)
	H2	26 (16%)	9 (33%)	6 (17%)	–	11 (32%)	–
	H3	46 (28%)	6 (22%)	2 (6%)	18 (51%)	15 (44%)	5 (14%)
	H4	23 (14%)	–	2 (6%)	17 (49%)	2 (6%)	2 (6%)
Block 2	**H1**	16 (10%)	–	–	7 (20%)	3 (9%)	6 (17%)
	H2	37 (22%)	13 (48%)	15 (43%)	–	8 (24%)	1 (3%)
Block 3	**H1** [1]	69 (42%)	13 (48%)	20 (57%)	–	19 (56%)	17 (49%)
	H2	47 (28%)	9 (33%)	12 (34%)	5 (14%)	11 (32%)	10 (29%)
	H3	36 (22%)	2 (7%)	2 (6%)	24 (69%)	4 (12%)	3 (9%)

Number in parenthesis represents the percentage of bulls from the pooled data from all breeds, or from each individual breed with the specific haplotype. Block 1 – Block 5 – haplotype blocks representing at least 10% of bulls identified from pooled data using the default settings of pairwise analysis in Haploview 4.2 (Gabriel *et al.*, 2002; Barrett *et al.*, 2005).

[1] All bulls were homozygous for the favourable alleles for tenderness at all loci.

Table S4. *Ranking of the SNP,* 29_184 *(rs17871986) for data analyses in GAPIT (in R) for tenderness traits in five South African beef breeds for electrical stimulation and control, over four ageing periods up to d 20 post-mortem.*

		Ranking NS			Ranking ES		
Trait	**Ageing**	**BLINK**	**MLM**	**MLMM**	**BLINK**	**MLM**	**MLMM**
MFL	**d 3**	52	53	27	2	2	2
	d 9	3	2	3	4	5	5
	d 14	4	15	4	2	9	4
	d 21	84	53	92	4	3	8
WBSF	**d 3**	5	1	6	1	1	7
	d 9	54	8	46	12	4	10
	d 14	54	9	54	91	24	91
	d 21	25	8	25	125	47	125

Ranking – position of the SNP when probability values were sorted from lowest to highest (regardless of statistical significance), for the 134 SNPs within the calpain system genes using the Illumina® BovineHD SNP BeadChip (777K).

GAPIT software packages in R software (Wang & Zhang, 2021): BLINK – Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway (Huang *et al.*, 2018); MLM – Mixed Linear Model (Yu *et al.*, 2006); MLMM – Multi-Locus Mixed Model (Segura *et al.*, 2012).

ES – electrically stimulated or treatment; MFL – myofibril fragment length (µm); NS – non-electrically stimulated or control; WBSF – Warner-Bratzler shear force (kg).

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