Evaluation of genetic and physiological parameters associated with meat tenderness in South African feedlot cattle

By

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I declare that this thesis for the degree M.Sc. (Agric) Production Physiology at the University of Pretoria has not been submitted by me for a degree at any other University.

The beginning of wisdom is found in doubting, by doubting we come to the question, and by seeking we may come upon the truth.

Pierre Abelard (1079-1142)

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1 Corinthians 10:31

LIST OF ABBREVIATIONS

A	-	Adenine
AA	-	Amino acid
A_{260}	-	Absorption at 260 nm
A_{280}	-	Absorption at 280 nm
ANOVA	-	Analysis of variance
ATP	-	Adenosine triphosphate
ARC	-	Agricultural Research Council
ARC-API	-	Agricultural Research Council - Animal Production Institute
Bh	-	Brahman-crosses
Brahman-X	-	Brahman-crosses
BTA	-	Bovine autosomes
C	-	Cytosine
˚C	-	Celsius degrees
CA	-	Calpstatin activity
CAPN1	-	Micro molar calcium-activated neutral protease
CAST	-	Calpastatin gene
CSIRO	-	Commonwealth Scientific and Research Organization
CUT	-	Estimated cutability
CWT	-	Carcass weight
1D	-	One-dimensional
DAG	-	Dystrophin associated glycans
DAP	-	Dystrophin associated protein
dH_2O	-	Distilled water
DM	-	Dry matter
DMW	-	Dry matter weight
DNA	-	Deoxyribo nucleic acid
DFD	-	Dark, firm and dry
d.p.m.	-	Days *post mortem*
EBV	-	Estimated breeding values
EDTA	-	Ethylenediaminetetra-acetic acid
e.g.	-	For example
EPD	-	Expected progeny difference
ES	-	Electrical stimulation
FAT	-	Fat depth
G	-	Guanine
g	-	Gram
g/kg	-	Gram per kilogram
h	-	Hour
HCl	-	Hydrochloric acid
H_2O_2	-	Hydrogen peroxide
HRP	-	Horseradish peroxidase
i.e.	-	For example
IgG	-	Immunoglobulin G
IMT	-	Intra-muscular fat

IUB	-	International Union of Biochemistry
kb	-	Kilo base
kg	-	Kilogram
kDa	-	Kilo Dalton
KOH	-	Potassium hydroxide
L	-	Leader
LL	-	*M. longissimus dorsi* (L1-L6)
LSD	-	Least significant difference
LT	-	*M. longissimus thoracis*
M	-	Molar
MARB	-	Marbling score or percentage of intra muscular fat
MARC	-	Meat Animal Research Centre
MAS	-	Marker-assisted selection
$MgCl_2$	-	Magnesium chloride
MFI	-	Myofibrillar fragmentation index
MFL	-	Myofibrillar fragment length
MJ/kg	-	Mega joules per kilogram
ml	-	Millilitre
mm	-	Millimetre
mM	-	Milli molar
MM	-	Molecular mass
M_r	-	Molecular weight
MW	-	Molecular weight
n	-	Number
N	-	Avogadro's number
NaCl	-	Sodium chloride
Na_2HPO_4	-	Disodium hydrogen orthophosphate
NaH_2PO_4	-	Sodium dihydrogen orthophosphate
NaN_3	-	Sodium azide
Ng	-	Nguni-crosses
Nguni-X	-	Nguni-crosses
ng/µl	-	Nanogram per micro litre
nm	-	Nanometer
No	-	Number
NRF	-	National Research Foundation
NS	-	Not electrical stimulated
PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate buffer saline
PCR	-	Polymerase chain reaction
PSE	-	Pale, soft and exudative
Pr	-	*Pre-Rigor*
QTL	-	Quantitative trait loci
r	-	Correlation
REA	-	Longissimus muscle area
Rm	-	*Rigor mortis*
RMRDT	-	Red Meat Research and Development Trust
rpm	-	Resolution per minute
S	-	Skelemins

SA	-	South Africa
SACCS	-	South African Carcass Classification System
SAFA	-	South African Feedlot Association
SDS	-	Sodium dodecyl sulphate
SDS-PAGE	-	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	-	Standard errors of means
SL	-	Sarcomere length
Sm	-	Simmentaler-crosses
Simmentaler-X	-	Simmentaler-X
SNP	-	Single nucleotide polymorphism
T	-	Thiamine
TEND	-	Taste panel tenderness score
TRIS	-	Tris(hydroxymethyl)amino methane
UP	-	University of Pretoria
USA	-	United States of America
UTR	-	Untranslated region
UV	-	Ultraviolet
vol/vol	-	Volume per volume
WBC	-	Water binding capacity
WBSF	-	Warner-Bratzler shear force
wt/vol	-	Weight per volume
α	-	Alfa
β	-	Beta
γ	-	Gama
θ	-	Cross sectional
µ	-	Micro
µl	-	Micro litre
µm	-	Micrometer
µM	-	Micro molar
%	-	Percentage
>	-	Higher than
<	-	Lower than
~	-	Approximately
3'	-	Three prime
5'	-	Five prime

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ABSTRACT

The objective of this study was to compare prediction of meat tenderness by means of gene technologies (markers) with established physical estimates of meat tenderness. Weaned, young bulls (n = 60) were selected on phenotype from various commercial producers to represent a Brahman (*Bos indicus*; n = 20), Simmental (continental *Bos Taurus*; n = 20) and Nguni (*Sanga*; n = 20) crossbred group. After being raised under intensive feedlot conditions the animals were slaughtered according to normal South African slaughter procedures at an A-age (10 - 12 months) with a fatness class of two or three (lean-medium fatness). At slaughter the carcasses were not electrical stimulated because electrical stimulation influences the processes of meat tenderness, and the emphasis was on the expression of the inherent tenderness characteristics without external *post mortem* influences. Carcasses were halved, chilled at 4 ˚C within 2 hours *post mortem.* The *M. longissimus thoracis et lumboram* (LT and LL) of the right and left sides were removed from the third last rib to the last lumbar vertebra and sub sampled for shear force evaluations, SDS-PAGE, Western-blotting, myofibril fragmentation (MFL), sarcomere length (SL), calpain, calpastatin, total collagen, % collagen solubility and marker analysis. The position of sampling for each test was consistent and the different samples were either frozen immediately at -20 ˚C or -80 ˚C or vacuum packed and aged (2 ± 2 ˚C) for 7 or 14 days *post mortem.*

Two single nucleotide polymorphism (SNP) markers were employed in this study for the bovine *CAPN1* gene, which is found or situated on bovine chromosome 29, namely a SNP marker which is situated on exon nine (*CAPN1-316*) and the other on intron 17 (*CAPN1-4751*). The inhibitor, calpastatin (*CAST*) found on chromosome seven was also analysed in this study. Both the *CAST* markers (*CAST* and *CAST-Brahman*) lie in the three prime untranslated regions (3' UTR) of the *CAST* gene. Genotype data of two-markers were used to determine the two-marker haplotypes.

The results of the study showed that differences exist in meat quality of the different crossbreds. Brahman- and Nguni-crosses had lower shear force values (more tender) than that of the Simmentaler-crosses under these specific experimental conditions. The pH decline did not differ significantly ($p > 0.001$) between the crossbreds. The carcass temperature and temperature decline rate although similar between the Brahman- and the Simmentaler-crosses for three hours and up to eight hours *post mortem* differed significantly from that of the Nguni-crosses ($p < 0.006$). The sarcomere lengths were mostly under 1.7 µm, which indicate that shortening (caused by rapid chilling) can not be eliminated. No significant differences were found between the different crossbreds for the calpastatin levels but significant differences were found for the µ-calpain activity and µ-calpain / calpastatin activity ratios. The Brahman-crosses had longer myofibril fragment lengths on average, indicating lower proteolysis / myofibrillar fragmentation compared to the other crossbreds. Myofibrillar protein degradation (titin, nebulin, desmin) and myofibrillar protein formation (30 kDa) during *post mortem* ageing was examined as a confirmation for the myofibril fragment length results. Significant differences between the crossbreds were found for titin degradation. Nguni-cross

animals had significantly ($p < 0.033$) more titin present than the other crossbreds. Nebulin degradation showed a significant ($p < 0.038$) breed effect at 7 days *post mortem* and a significant degradation rate difference for breed types between 1 day and 7 days *post mortem* ($p < 0.03$) and 1 day and 14 days *post mortem* ($p < 0.034$). Desmin degradation evaluated with SDS-PAGE and Western-blotting indicated that the Simmentaler-cross animals had significantly ($p < 0.018$; $p < 0.024$, respectively) lower desmin levels compared to the other crossbreds. For the 30 kDa proteins there were no significant ($p > 0.001$) differences in data evaluated at 1 day, 7 and 14 days *post mortem* as well as for the formation rate.

The results indicates that Brahman-crosses had the highest frequency for haplotypes that are associated with increased shear force, and thus tougher meat compared to the Simmentaler-crosses that had the highest frequency for haplotyes that are associated with lower shear force, and thus more tender meat, while Nguni-crosses were intermediate. A multiplex marker system incorporating both markers (316 and 4751) and indexes for the markers at *CAST* and *CAPN1* genes were evaluated in this study. Considering the average index for the interactions between the *CAST* and *CAPN1* genes, it can be concluded that the Nguni-cross was overall the breed with the highest potential for inherently tender meat. In general, the animals in this study had the tendency for tougher meat. The genetic markers (*CAST* and *CAPN1*) showed no association with Warner-Bratzler shear force (WBSF) ($p > 0.05$), which suggest that various mechanisms and environmental factors may be involved and give another outcome compared to the genetic make up. Simple correlation coefficients were generated between the different characteristics measured.

If the group of animals in this study is indeed a typical representation of South African feedlot finished crossbred animals, the relatively high WBSF values emphasise the challenge to manipulate their intrinsic tenderness potential by making use of various *pre-* and *post-slaughter* techniques and procedures.

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CHAPTER 1

INTRODUCTION

The beef industry at present is faced with a major problem, which has been indicated in numerous international consumer surveys namely the inconsistency in meat tenderness (Koohmaraie, 1992a; Morgan *et al*., 1991; Ouali, 1991; Warkup *et al*., 1995). A significant portion of commercially available beef is regarded as unacceptable, due to toughness (Jeremiah, 1996). Meat tenderness is therefore considered as one of the most important meat qualities attributes (Lawrie, 1985; Jiang, 1998). Despite many changes in consumer attitude towards red meat consumption, one expectation has remained constant namely that meat should be consistently tender. Tenderness can be defined as: 1) Ease of penetration by teeth, 2) Ease with which meat breaks into fragments, and 3) The amount of residue remaining after chewing (Jeremiah and Phillips, 2000). The concept of meat tenderness is regarded as a multi-factorial process as it is biologically dependant on a combination of many genetic and physiological factors. To understand the complex processes involved with meat tenderness, it is important to know what tenderness is and to identify and study the mechanisms involved with meat tenderness and tenderisation.

The quality of meat with specific reference to meat tenderness has received much attention in South Africa (SA) over the past decade. From the producers' point of view, much money is at stake in supplying consistent meat products to the market, because consumers influence the market through the purchase and repurchase of products. The South African meat consumer in general, fined red meat or beef acceptable and popular. Red meat is rather a luxury product in SA, as most meat-eating consumers fall into the low-income group rather than in the high-income group. These consumers are also more concerned whether they will have food to eat, rather than whether food is safe or of high quality. A survey done by the South African Feedlot Association (SAFA) during 2003 indicated that consumers that fall in the high-income group are concerned about health. As a matter of fact beef produced in SA and internationally is regarded as a lean end product. Lean beef also receives the best prices according to the South African Carcass Classification System (SACCS), thus the South African beef industry attempts to ensure the production of consistent quality lean meat. The SACCS and the Namibian Carcass Classification systems are basically the same, except that the SACCS categorises the carcass of 2-tooth animals in a separate age-class (AB), than the 3- to 6-tooth animals (B) and 0-tooth (A). Both systems classify carcasses from 7- to 8-tooth animals in an age-class (C). The United States meat industry grades beef according to Prime, Choice and Select. The quality grade factors that are considered include marbling and maturity, while inconsistent meat tenderness is considered as a top priority. Since the 1990's, the United States of America (USA) beef industry has adopted new technologies based on a genotypic approach to meet consumer expectations (Koohmaraie *et al*., 2003). A segment of consumers worldwide are always willing to pay some level of premium for guaranteed tenderness (Boleman *et al.,* 1997; Wheeler *et al*., 2002; Rhee *et al*., 2004). With this in mind, the beef

industry had changed from being production driven to being more concerned with the demands of the consumer (Harrington, 1994; Dransfield, 2003).

In SA the beef industry is currently moving towards the selection of economically relevant beef carcass traits (i.e. tenderness). For many years beef breeders in SA have focussed primarily on production traits in their selection programmes. Traits associated with reproduction (i.e. calving ease) and growth (i.e. weaning weight) received by far the most attention compared to selection for carcass characteristics (Gertenbach and Kars, 1999). In the past decade various factors contributed to highlighting quality traits, with specific interest in tenderness and marbling. The development of molecular theory provides new strategies for improvement of meat tenderness through a genetic approach (Koohmaraie *et al*., 2003) that is a non-invasive method and can be applied while the animal is still alive. GeneStar® technology is a commercial deoxyribo nucleic acid (DNA) based test that was developed from gene research in Australia and now available to producers worldwide (Australia, New Zealand, North America, Europe, Asia and SA) to test for tenderness and marbling (Alison, 2006; Gao *et al.,* 2007). GeneStar®-tenderness test for variants of the bovine calpastatin gene located on chromosome seven and the estimated contribution of this gene on meat tenderness is 10 - 12% (www.agribsa.co.za, 21 January 2006). The gene for marbling is less important in SA since it is not regarded in the classification system. However, marbling is positively associated with juiciness of meat (www.agribsa.co.za, 21 January 2006). The use of gene technology from a quantitative and molecular point of view to predict meat tenderness provides a useful aid to beef producers. It can give an indication of which animal has the potential to produce more tender meat.

Certain breeds are genetically dispositional to produce meat that is tougher. Many researchers have focused their studies on the difference in tenderness between *Bos indicus* and *Bos taurus* breeds (Gregory *et al*., 1994; O'Connor *et al*., 1997; Sherbeck *et al*., 1995; Campo *et al*., 1999). It is well documented that tenderness decreases as the percentage *Bos indicus* increases in a crossbred animal (Crouse *et al*., 1989; De Bruyn, 1991; Shackelford *et al*., 1991; Shackelford *et al*., 1994; Whipple *et al*., 1990a; Koohmaraie, 1996). It has been shown that differences occur between *Bos indicus* and *Bos taurus* due to variation in the properties of the muscle proteolytic calpain enzyme system, (Whipple *et al*., 1990a; Shackelford *et al*., 1991; Koohmaraie, 1996) and the related effects on the myofibrillar properties of the muscle.

This study is based on two important factors namely the importance of the red meat industry in SA with specific emphasis on the increasing awareness of meat tenderness. The second factor is the availability of new molecular technologies for the assessment of meat tenderness. According to SAFA, a large proportion of weaners finished in South African feedlots for beef production include Brahman- and Simmentaler-crosses. Therefore these types were included as experimental groups in this study representing medium and large frame breeds. Nguni-crosses were also included to represent a small frame breed. The Nguni that is an indigenous (*Sanga*) breed-type is also a popular breed in SA (See Addendum A for the migration of the breed-types to SA). The objective of this study was therefore to compare prediction of meat tenderness by means of gene technologies (markers) with established physical estimates of meat tenderness.

CHAPTER 2

LITERATURE OVERVIEW

The exact mechanisms involved in the *post mortem* meat tenderisation process and the nature of changes associated with improvement of meat tenderness is complex and not fully understood (Ouali, 1991, Fritz and Greaser, 1991; Koohmaraie, 1994; Odeh, 2003). Meat quality is becoming increasingly important to meat processors and consumers (Beattie *et al*., 1999). Solving the problem of inconsistent meat tenderness has become a top priority of the beef industry due to consumer demands and preferences. Consumers have widely diverging expectations of the specific product of which, their own conception of "value" and "quality" is the most important parameter (Breidenstein *et al.,* 1983; Naudé, 1985; Huffman *et al.,* 1996; Miller *et al.,* 2001). Production, processing, value adding and the cooking method to prepare the meat for consumption are important factors determining the end result of meat tenderness (Lee, 1986; Thompson, 2002).

Tenderness has been defined as "the composite of those properties which arise from structural elements, and the manner in which it registers with the physiological senses". This definition recognises three essential elements: tenderness is the result of the structure; it is a composite of several properties and a sensory quality (Lawrie, 1985; Scheepers, 1999). Since tenderness is a major palatability trait that determines consumer acceptability, it is important to understand what causes meat to be tender or less tender (tough). Factors affecting muscle and meat tenderness have been extensively research over the past decade as illustrated in Figure 2.1.

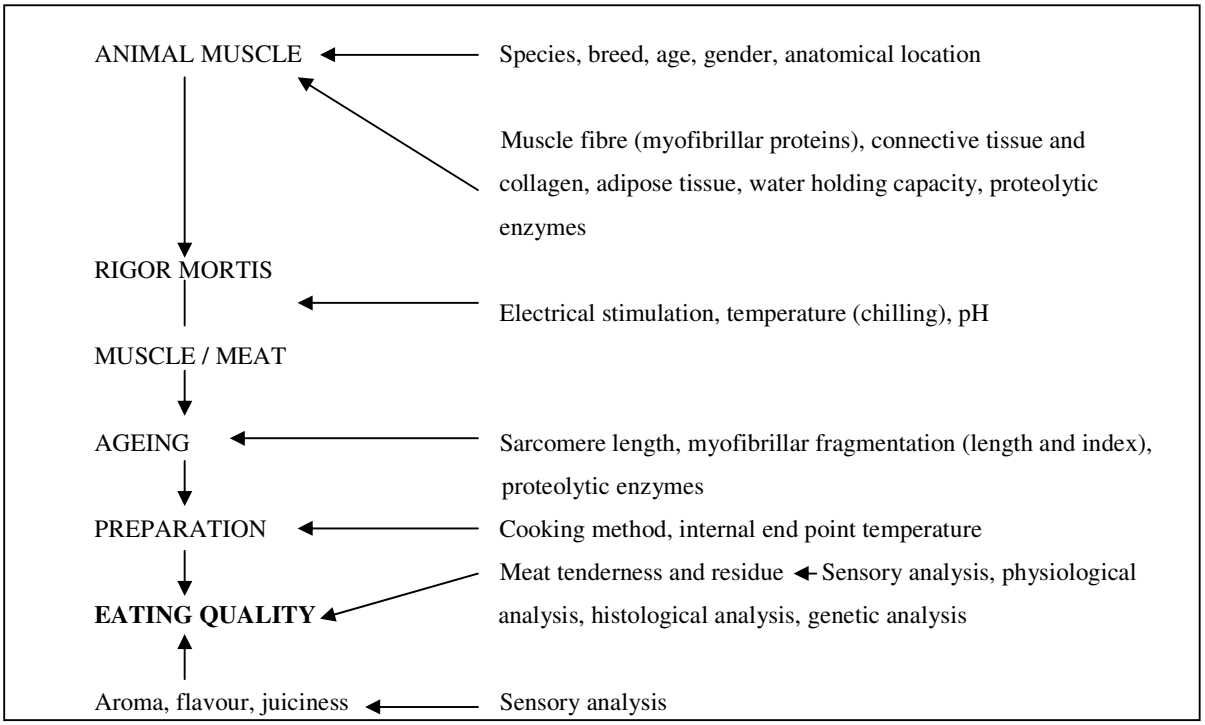

Figure 2.1: Factors determining meat tenderness (adapted from Scheepers, 1999).

Although meat tenderness is highly dependent on *pre-* and *post-slaughter* factors, physiological factors and measurable genotype differences (genetic factors) make a contribution to the total variation in tenderness. It is essential to gain an understanding of the contribution made by these factors (genetic and physiological), in order to develop parameters for them to aid in the assessment of meat tenderness.

2.1 Role of physiological factors on meat tenderness

The concept of meat tenderness is dependant on various physiological factors namely muscle structure and connective tissue characteristics; meat ageing and tenderisation by means of proteolytic degradation of cyto-skeletal proteins (proteolytic calpain system and myofibrillar fragmentation); the energy status of the muscle (muscle glycolysis, sarcomere length and change in muscle pH:temperature ratio decline), which influence the extent of muscle contraction (Morton *et al*., 1999; Monin, 1998; Webb *et al.,* 1964). All of these physiological factors contribute to the degree of meat tenderness of the final product.

2.1.1 Muscle structure

Muscle tissue is composed of long cylindrical cells termed myofibres that are surrounded by collagen fibres in the extra cellular space (Figure 2.2).

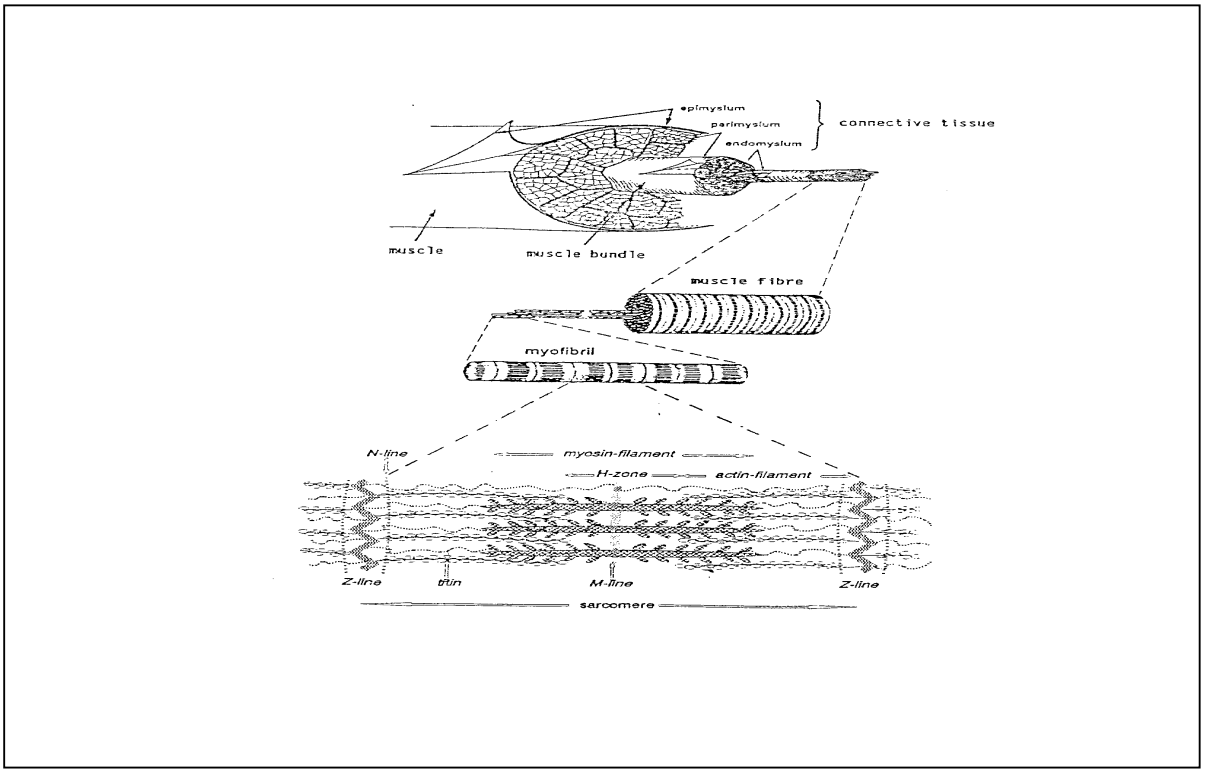

Figure 2.2: Schematic overview of the muscle structure (Geesink, 1993).

Muscle cells are packed with smaller cylindrical organelles called myofibrils that occupy over 80% of the cell volume. There may be as many as 1000 of these 1-2 µm diameter myofibrils in a cross section of a muscle fibre. Observation of these organelles in a phase contrast microscope reveals alternating light and dark bands. Electron microscopy shows that the bands arise because of the presence of two major filaments: thick filaments in the A-band and thin filaments in the I-band. A dense line bisects the I-band perpendicular to the myofibril's long axis and is termed the Z-line. An M-line is located in the middle of the A-band. The filaments are composed of proteins, with myosin being the major constituent of the thick filaments while actin, tropomyosin and troponin make up most of the thin filaments. Costameres (Figure 2.3) link myofibrils to the sarcolemma, and N_2-lines have been reported to be areas where titin and nebulin filaments, form a cytoskeletal network linking thick and thin filaments, respectively, to the Z-disk (Craig and Pardo, 1983; Geesink, 1993).

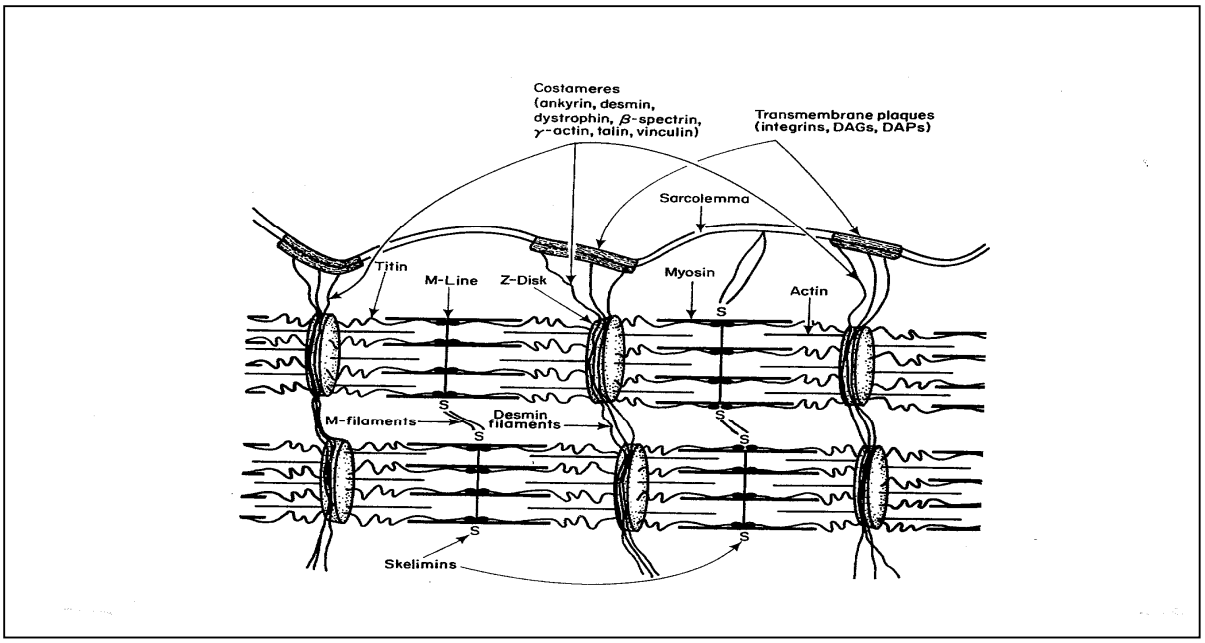

Figure 2.3: Schematic diagram showing the structure and protein composition of costameres in striated muscle relative to Z-disks and the myofibrillar lattice (Adopted from Taylor *et al*., 1995).

The probable position of titin filaments is shown in this diagram, but nebulin filaments have been omitted to simplify the diagram. The N-terminal end of the large titin molecule is anchored in the Z-disk. Desmin, vinculin, and ankyrin, three of the protein constituents of costameres, extend into the muscle cell where they encircle myofibrils at the Z-disk and run from myofibril to myofibril to link adjacent Z-disks laterally. DAPs = dystrophin associated proteins; DAG = dystrophin associated glycans; S = skelemins.

Two phases can be distinguished in the conversion of muscle to meat (Figure 2.4). During the first phase, *pre-rigor* (Pr), energy-rich compounds, including adenosine triphosphate (ATP), creatine phosphate and glycogen, are almost completely depleted (Greaser, 1986). Due to the anaerobic conversion of glycogen to lactate the muscle pH decreases from 7.2 to about 5.5. At extremely low concentrations of ATP, myosin

filaments in the myofibril form bonds with the overlapping actin filaments and, as a consequence, the muscle becomes inextensible and rigid: *rigor mortis* (Rm) sets in. At this point *post mortem* muscle reaches maximum toughness. Goll *et al.* (1995a) postulated that the increase in toughness observed during the first 24 - 36 hours *post mortem* is caused by a change in the actin / myosin interaction from a weak-binding state to a strong-binding state. The decrease in toughness that occurs after 24 - 36 hours *post mortem* is caused by two factors: a) a weakening of actin / myosin interaction; and b) calpain-induced proteolytic degradation of costameres, of intermediate filaments that constitute the inter-myofibrillar linkages in muscle fibres, and of the attachments of titin and nebulin to the Z-disk (Figure 2.3). Thus, meat tenderness has been generally resolved into at least two different components referred to as "background toughness" and "myofibrillar toughness" (Marsh and Leet, 1966; Ouali, 1991, Valin, 1995) (Figure 2.4).

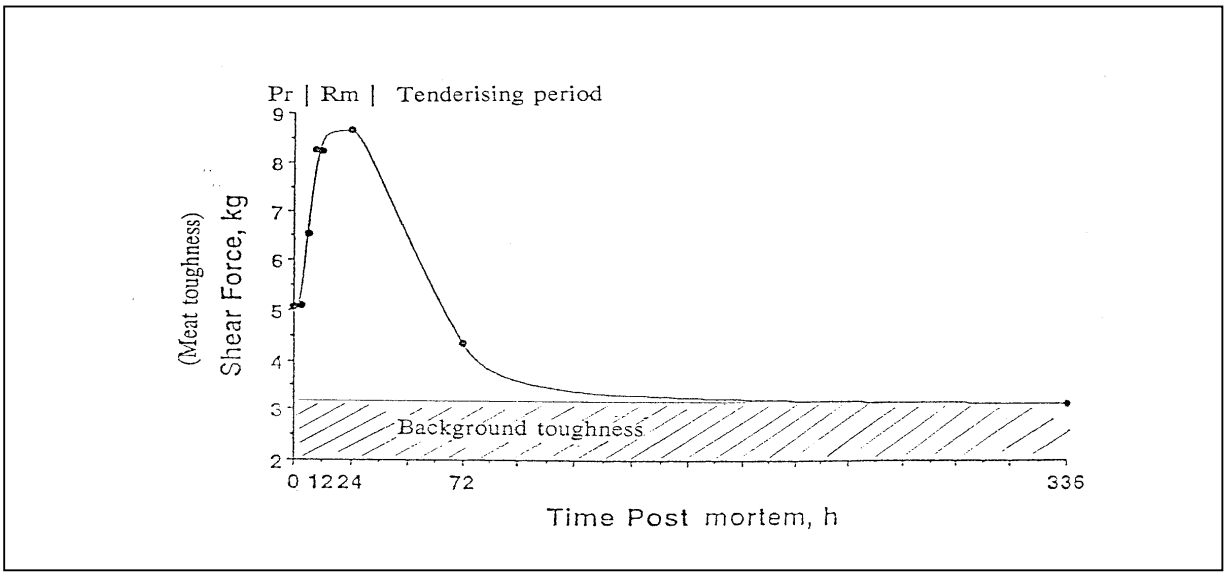

Figure 2.4: Changes in Warner-Bratzler shear force of lamb *M. longissimus thoracic et lumborum* muscle during *post mortem* storage (Wheeler and Koohmaraie, 1994) (Pr = *Pre-rigor*; Rm = *Rigor mortis*).

The toughening phase (background toughness and myofibrillar toughness) is similar in all carcasses under similar processing conditions. The tenderising period is highly variable. There is a large variation in both the rate and extent of *post mortem* tenderisation of meat, and this result in the inconsistency of meat tenderness found at the consumer level (Koohmaraie and Geesink, 2006).

2.1.2 Meat tenderisation

The physiological and biochemical mechanisms of meat tenderness and the meat tenderisation process are based on theory (Jiang, 1998). Tenderisation is a variable process determined by / or depending on a number of biological factors i.e. age, sex-type, muscle type and species. This is further affected by the rate of glycolysis, rate of pH decline, osmolarity of muscle cells, temperature, and genetic factors inherent to the

animal, amongst others (Geesink, 1993). Tenderness of meat originates in the biochemical and structural properties of skeletal muscle fibres (myofibrils, intermediate filaments, intramuscular connective tissue, the endomysium and the perimysium, which are composed of collagen fibrils and fibres) (Strandine *et al.,* 1949; Bailey, 1972; Takahashi, 1996). The connective tissue content and its properties are responsible for approximately 20% of variation in meat tenderness (Dransfield, 1995). Greaser and Fritz (1995) concluded that the weakening myofibril structure primarily control meat texture. The mechanisms underlying myofibrillar fragmentation or disintegration of the myofibril structure are, the result of proteolytic action of various enzyme systems (Ouali, 1990; Roncales *et al*., 1995) from which the calpain proteolytic system seems to play a major role.

The calpain proteolytic system has been identified as being most important in the tenderness of meat (Koohmaraie *et al*., 1995; Koohmaraie, 1996; Boehm *et al*., 1998; Koohmaraie and Geesink, 2006). According to Goll (1991) 90% or more of the tenderisation that occurs during *post mortem* storage (2 - 4 °C) can be contributed to the action of calpains. Calpains are calcium-activated proteases with an optimum activity at a neutral pH (Koohmaraie and Geesink, 2006). The calpain system contains four known proteins (For review see Koohmaraie and Geesink, 2006):

1) μ-calpain (mu-calpain) (calpain I), a proteinase that requires 5 to 50 μM Ca^{2+} for half maximal activity;
2) m-calpain (calpain II), a proteinase that requires 300 to 1000 μM Ca^{2+} for half maximal activity (Dayton, 1982; Nagainis *et al,* 1983; Suzuki *et al,* 1981);
3) a third proteinase (p94 or calpain-3) identified in 1989 and still poorly characterised; it evidently requires 3000 to 4000 μM Ca^{2+} for half maximal activity (Wolfe *et al*., 1989); and
4) calpastatin a polypeptide that is specific for inhibiting the proteolytic activity of μ-calpain and m-calpain (Maki *et al,* 1988; Goll, 1991; Goll *et al*., 1995b)

Theoretically the calpain system contains at least one more protein; an "activator" that is able in response to physiological demand to alter Ca^{2+} concentration required for activity of μ-calpain or m-calpain. The nature of this "activator" is unknown; it probably responds to Ca^{2+} fluxes in the nanomolar concentration range and may be a kinase, a phosphatase or a calmodulin-like molecule (Goll, 1991). μ-Calpain and m-calpain consist of a large 80 kDa subunit and a small 30 kDa subunit (Dayton *et al.,* 1976a; Dayton *et al.,* 1976b; Dayton *et al.,* 1981; Emori *et al.,* 1986), both of which can be readily truncated at their N-termini, thereby modifying their membrane binding properties and calcium requirement (Suzuki *et al*., 1995). The 80 kDa catalytic subunits of μ-calpain and m-calpain are different polypeptides but share 50% to 60% amino acid sequence homology; the 30 kDa regulatory subunits are identical.

Experimental evidence so far points to μ-calpain as the primary enzyme of *post mortem* proteolysis. The enzyme is thought to be the first to be activated *post mortem* as the pH declines to 6.02 and below, and intracellular calcium concentration rises from 0.1 - 0.2 μM to over 100 μM (Vidalence *et al.*, 1983; Jeacocke, 1993; Dransfield, 1993). Dransfield (1994) suggested that the most tenderisation is caused by μ-calpain (Figure 2.5) and that about 50% of the tenderisation occurs before 24 hours, after which tenderisation

continues approximately exponentially with time. Under normal muscle *post mortem* conditions (pH_u 5.5 to 5.8 and 5 °C) the enzyme retains 20% to 38% of at-death activity by 24 hours *post mortem* (Koohmaraie *et al.*, 1986; Boehm *et al.*, 1998).

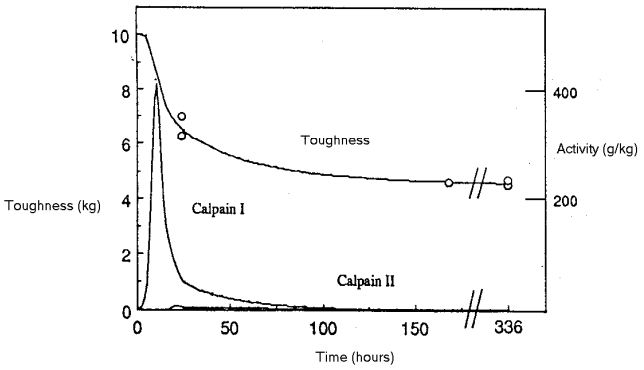

Figure 2.5: Tenderisation of bovine longissimus dorsi muscle by calpains - relationship between the activities of μ-calpain, m-calpain and *post mortem* tenderisation (Dransfield, 1994). Curves were calculated using a calpain – activity model from measured levels of calpains, rigor development, temperature and shear force determined experimentally and show the sharp rise in activity of μ-calpain and the much smaller contribution of m-calpain to tenderisation and ageing of beef longissimus dorsi.

In general there is a suggestion that m-calpain is responsible for tenderisation that occurs beyond 24 hours *post mortem* (Dransfield, 1994) but other scientists doubt its contribution because its concentration remains largely unchanged during *post mortem* ageing (Vidalence *et al.*, 1983; Geesink and Koohmaraie, 1999; Koohmaraie and Geesink, 2006).

Calpain 3 is a single polypeptide of 94 kDa with sequence homology to the large subunits of μ-calpain and m-calpain (Sorimachi *et al.,* 1989). Purification and characterisation of calpain 3 has been extremely difficult for several reasons. Unlike μ-calpain and m-calpain, calpain 3 cannot be easily extracted from skeletal muscle due to its association with the myofibrillar protein, titin (Sorimachi *et al.,* 1995; Koohmaraie and Geesink, 2006).

Calpastain is the endogenous specific inhibitor of μ-calpain and m-calpain (Maki *et al.,* 1988; Koohmaraie and Geesink, 2006) with a predicted molecular weight of 77 kDa. Several isoforms of this protein exist (Lee *et al.,* 1992), but the predominant form contains five-domains (Figure 2.6). Of the five domains, the N-terminal leader (L) domain does not appear to have any inhibitory activity, but may be

involved in targeting or intracellular localisation. The other domains (I – IV) are highly homologous and are each capable of inhibiting calpain.

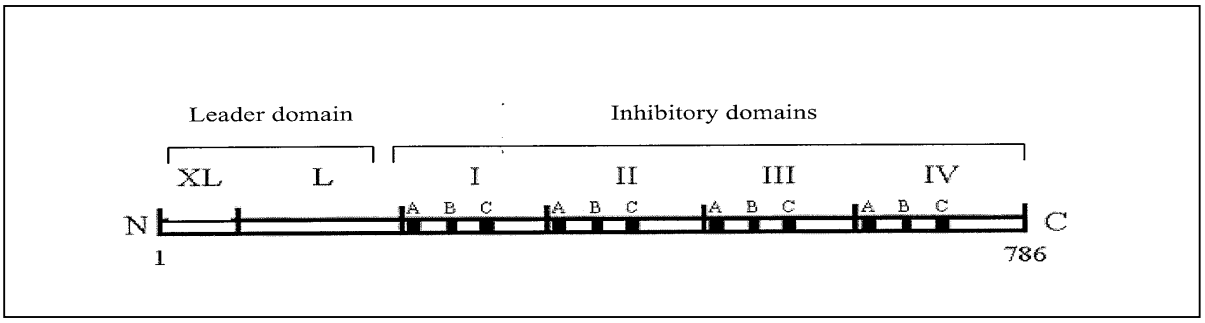

Figure 2.6: The five domain inhibitory protein, calpastatin (Odeh, 2003). It includes an N-termini leader (XL and L) domain and four inhibitory domains (I-IV). The inhibitory domains of calpastatin contain three conserved regions, A, B, and C.

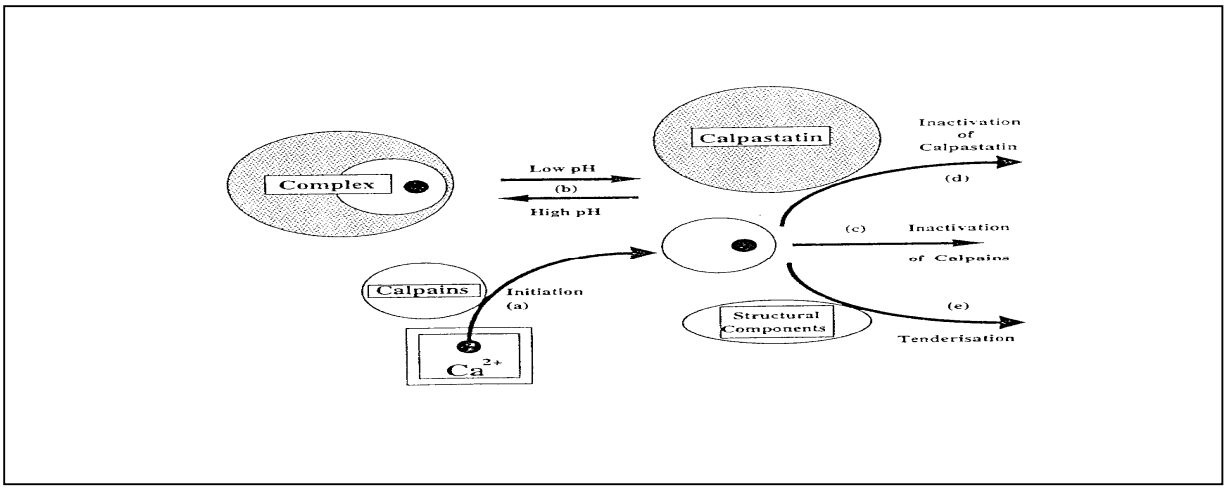

Figure 2.7: Model of activation of calpains and muscle tenderisation (Dransfield, 1993). (a) Initiation. The inert calpains are activated by the rise in calcium ion concentration and enter into the tenderisation system. (b) Binding. The equilibrium of the binding of calpains to calpastatin determines the level of free activated-calpains, which increase as the pH declines. (c) Inactivation of free activated-calpains. Decay of free activated-calpains by autolysis. (d) Inactivation of calpastatin (it should be noted that the model makes no distinction between proteolysis by calpains of the complexed and free calpastatin, but, for clarity, inactivation is shown only to free calpastatin). (e) Tenderisation. Proteolysis of structural components by calpains causes tenderisation.

Calpastatin in combination with Ca^{2+} is a major regulator of the calpain system in *post mortem* muscle (Figure 2.7) (Dransfield, 1993). There is a strong negative correlation between calpastatin and the rate of tenderisation (Doumit and Koohmaraie, 1999; Morgan *et al.*, 1993). A high calpastatin activity results in decreased calpain activity and thus, decreased tenderness (Boehm *et al.*, 1998; Sazili *et al.*, 2003).

The calpain system is closely linked to the proteolytic breakdown of myofibrillar proteins (Goll *et al.,* 1991; Koohmaraie, 1994; Huff-Lonergan *et al*., 1996a). Myofibrillar proteins can be subdivided into contractile, regulatory and cytoskeletal proteins. Myosin and actin are the main contractile proteins and are directly involved in muscle contraction and relaxation; these proteins account for 43% and 22% of total myofibrillar protein, respectively. Regulatory proteins are associated with the actin and myosin filaments and play an indirect role in the contraction-relaxation cycle of muscles by controlling the interaction between actin and myosin.

The main determinant of ultimate tenderness appears to be the extent of proteolysis of key proteins within muscle fibres (Taylor *et al*., 1995). These proteins are involved in both inter- (e.g. desmin) and intra-myofibrils (e.g. titin, nebulin and possibly, troponin-T) linkages and the function of these proteins are to maintain the structural integrity of myofibrils (Price, 1991; Robson *et al.,* 1991; Jiang, 1998), (See Addendum B, Table 1 for the major characteristics, potential roles and importance of desmin, titin and nebulin in muscle).

Desmin (55 kDa) is a member of the type III group of intermediate filament proteins and is localized at the periphery of the myofibrillar Z-disk in skeletal muscle. Desmin filaments probably play an important role in connecting adjacent myofibrils and in linking myofibrils to sub cellular organelles and the sarcolemma in the costamere region. Desmin is a rather insoluble protein although it is not as insoluble as intact titin, or nebulin (Robson *et al*., 1991).

Titin is a large structural protein that span the distance from the Z-line to nearly the M-line (half of the skeletal sarcomere muscle), thus titin may serve to aid in maintaining the overall structural integrity of the myofibril. It comprises approximately 8% to 10% of total myofibrillar protein in skeletal muscle. It is the third most abundant myofibrillar / cytoskeletal protein in these cells (Furst *et al*., 1988; Robson *et al*., 1991).

Nebulin is large structural cytoskeletal protein that comprises 5% of the total myofibrillar proteins. It spans the distance from the Z-line to near the free end of the thin filament. It is highly insoluble, and has a very high molecular weight (MW) (M_r = 6 x 10^5 to 9 x 10^5). Nebulin has also recently been shown to be capable of linking actin and myosin, thus nebulin may also have a regulatory function in skeletal muscle contraction. The *post mortem* degradation of nebulin may alter actin-myosin interactions (Wang and Wright, 1988; Robson *et al*., 1991).

The degradation of structural elements that connect the major components (i.e. the myofibrils) of a muscle cell together (resulting in gaps between myofibrils in *post mortem* muscle), as well as the peripheral layer of myofibrils to the cell membrane, could affect the development of tenderness (Taylor *et al*., 1995; Koohmaraie, 1996) (Figure 2.8). A number of studies have suggested that Z-disk degradation is a major factor contributing to *post mortem* tenderisation (Davey and Gilbert, 1969). These conclusions seem to have been based largely on experimental findings showing that the calpain system has a major role in *post mortem* tenderisation. It has been shown that when incubated with myofibrils or muscle strips, purified calpain tend to removes Z-disks.

Approximately 65 - 80% of all *post mortem* tenderisation occurs during the first 3 to 4 days *post mortem*, and there is little or no ultra structurally detectable Z-disk degradation during this period (Figure 2.8 B-E; Taylor *et al.,* 1995). Electron microscope studies showed that, during the first 3 to 4 days of *post mortem* storage at 4 °C, both costameres and N_2-lines are degraded. Costameres link myofibrils to the sarcolemma, and N_2-lines have been reported to be areas where titin and nebulin filaments, which form a cytoskeletal network linking thick and thin filaments, respectively, to the Z-disk. Filamentous structures linking adjacent myofibrils laterally at the level of each Z-disk are also degraded during the first 3 or 4 days of *post mortem* storage at 4 °C, resulting in gaps between myofibrils in *post mortem* muscle. Degradation of these structures would have important effects on tenderness. The proteins constituting these structures, nebulin and titin (N_2-lines); vinculin, desmin, and dystrophin (three of the six to eight proteins constituting costameres); and desmin (filaments linking adjacent myofibrils) are all excellent substrates for the calpains, and nebulin, titin, vinculin, and desmin are largely degraded within 3 days *post mortem* in the *M. semimembranosus* muscle. Electron micrographs of myofibrils used in the myofibril fragmentation index (MFI) (i.e. the turbidity of a myofibrillar suspension, with a fixed protein concentration, at 540 nm) assay show that these myofibrils, which have been assumed to be broken at their Z-disks, in fact have intact Z-disk and are broken in their I-bands as shown by Taylor *et al*. (1995) in Figure 2.8 F. Other structural changes, which have been reported, include loss of transversal alignment of the sarcomeres (Rowe, 1977; Ouali, 1990) and longitudinal splitting of the myofibres (Gann and Merkel, 1978).

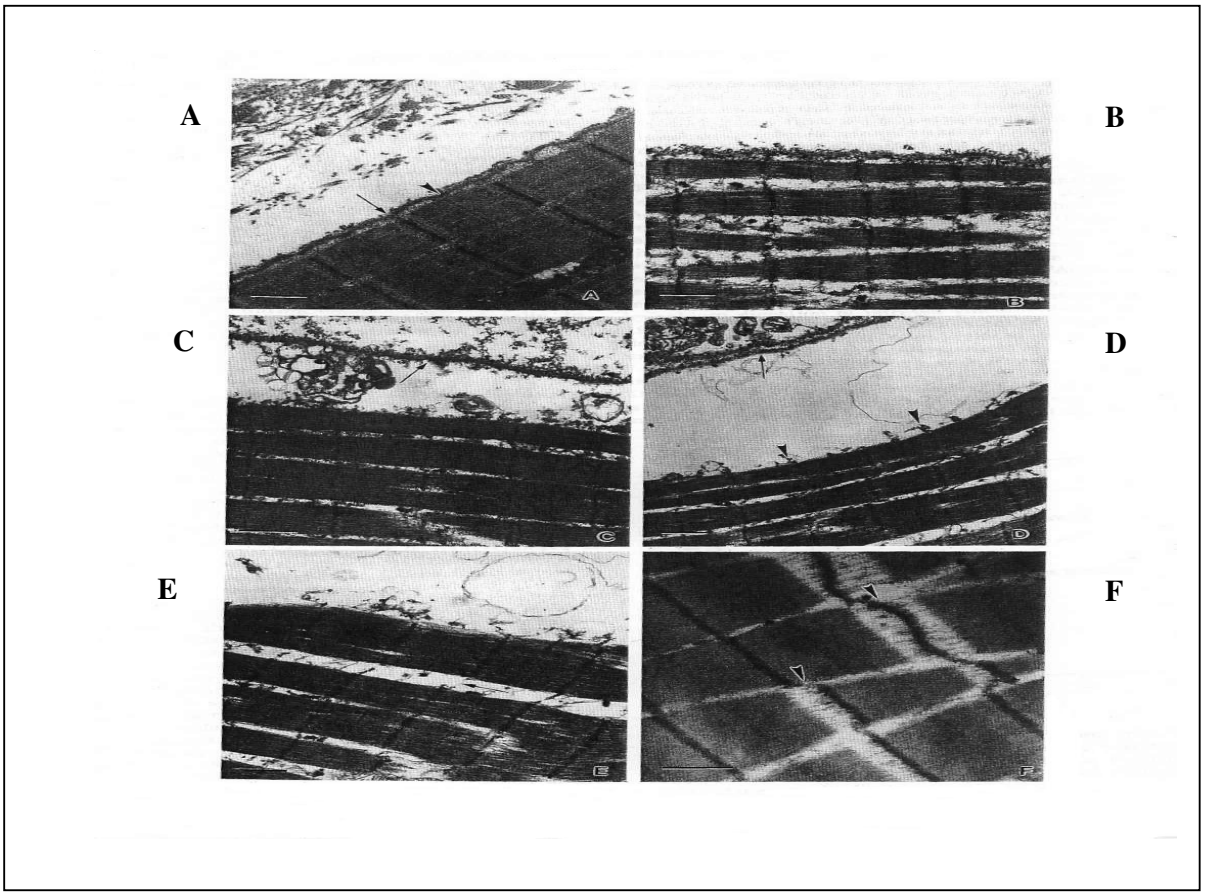

Figure 2.8: Electro-micrographs of sections of bovine biceps femoris muscle samples after different times of *post mortem* storage at 4 °C (Taylor *et al*., 1995). (A) Electro micrograph of bovine skeletal muscle sampled within 45 minutes after exsanguinations. Arrow and arrowhead indicate areas of density (costameres) where the sarcolemma is attached to the Z-disk and M-line, respectively. (B and C) Bovine biceps femoris muscle sampled 24 hours after death. Both these micrographs show structural disintegration and broadening of the sarcolemma compared with the structure of at-death sarcolemma. The sarcolemma is clearly detached and has been displaced from the myofibril in C. This detachment is representative of approximately 50% of the structures observed after 24 hours *post mortem*. (D and E) Bovine biceps femoris muscle after 3 days of *post mortem* storage. Arrowheads (D) point to patches of densely staining material at the level of the Z-disk. These densely staining areas may be remnants of the transmembrane patches containing integrins, DAGs, and DAPs. The arrow in D shows the detached membrane that has now been pulled away a considerable distance from the myofibril. E shows examples of widening of the distance between adjacent myofibrils in *post mortem* muscle and loss of material that seems to connect Z-disks from adjacent myofibrils (arrow). This material probably contains desmin and filamin. (F) Bovine biceps femoris muscle after 16 days *post mortem*. The Z-disk structure is well preserved even after 16 days *post mortem*. Arrowheads point to gaps in the I-band adjacent to the intact Z-disk. These gaps were observed in muscle that has been stored for 4 days or longer at 4 °C.

2.1.3 Effects of the calpain system mechanism on the macro level

Most of the quantitative changes described above have been at the ultra structural level - structures that are too small to relate to the sensorial perception of tough and tender meat. These structural changes have been related to tenderness by temporal correlation, association with specific degradation of proteins and relationships to shear force measures (Ho *et al.*, 1997; Taylor *et al.*, 1995). Taylor and Frylinck (2003) demonstrated that at the light microscopic level the evident changes included fibre contraction, fibre attachment, and partial and full breaks in fibres. Therefore, structures that relate to tenderness perception are approximately the size of two fibres. Brady (1937) and Ramsbottom *et al.* (1945) published erudite manuscripts, which described the relationship of muscle fibres and connective tissue to sensory quality and mechanical shear force measures. Both concluded that the amount of connective tissue and its organisation are important parameters, which contribute to meat quality in different muscle types. Studies of tenderness variation within a muscle type were advanced significantly when Locker and Hagyard (1963) demonstrated that fibre contraction is one of the major factors causing tough meat, and Herring *et al.* (1965) showed that fibre diameter is highly correlated with shear force. Taylor and Frylinck (2003) examined changes in whole myofibre using optical microscopy, especially fibre breaks and fibre-to-fibre attachment. Figure 2.9 shows examples of the changes observed and quantified.

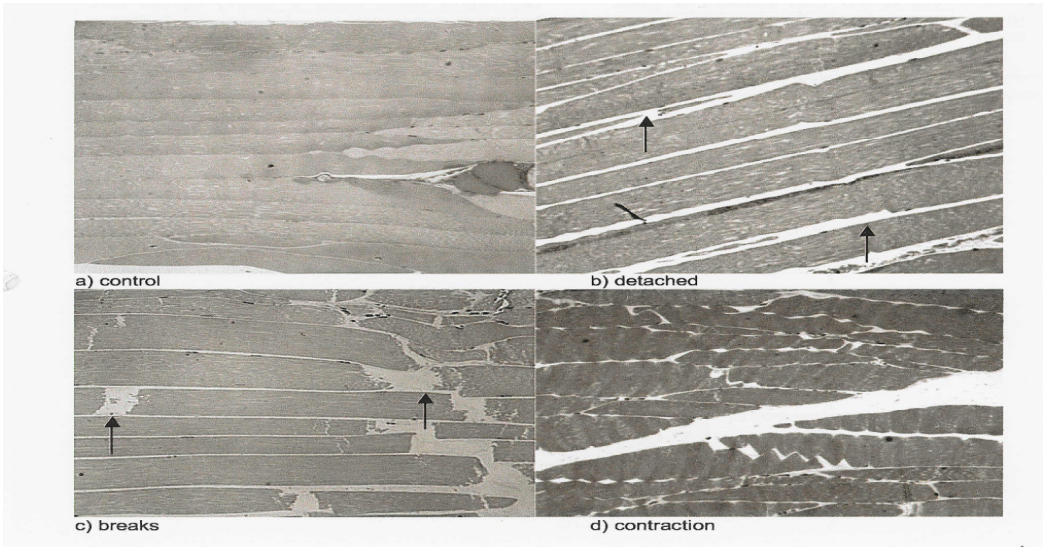

Figure 2.9: Examples of changes observed in meat samples at the microscopic level (Taylor and Frylinck, 2003).

Fibre breaks across the entire width of the fibre (*a* shows normal fibres and *c* shows breaks marked by arrows) were only observed at long *post mortem* storage times and contribute significantly to meat texture.

Fibre contraction is evident as waves and contraction bands as shown in *d*. Detachment of fibres from the endomysium is shown in *b* (arrows indicate detachment).

It was concluded that fibre detachment might be a factor, which contributes primarily to early tenderness of meat. Fibre detachment also occurs at time of slaughter and is maximal after 1 day of storage. In fact some reports indicate that as much as 50% of tenderness can occur within 24 hours of storage (Koohmaraie *et al.*, 1988). Since fibre-to-fibre adhesion changes at this time, but not fibre breaks. Thus the ultimate sensory perception of tenderness of meat is due to fibre detachment in the first 24 hours, with fibre contraction as a second factor associated with changes in tenderness.

2.1.4 Extent of muscle contraction

Myofibrillar contribution to meat tenderness depends on the extent of shortening during rigor development and proteolysis during conditioning (Warriss, 2000). Thus it is determined by the conditions during rigor development and *post mortem* tenderisation. As muscle ATP concentration decreases during *post mortem* storage, myofibrils attempt to contract, initiating the transition from weak to a strong actin / myosin cross bridge stage. The weakening of the actin / myosin interaction is directly responsible for most of the decrease in toughness between 24 hours and 72 hours *post mortem* and pH-related differences in sarcomere length can be associated with the toughness of meat (Watanabe and Devine, 1996). Hwang *et al*. (2004) suggest that sarcomere shortening may have an impact on the toughness of meat via various mechanisms. Sarcomere length is related to tenderness, especially in cases of severe shortening (Whipple *et al.,* 1990b). The apparent increase in sarcomere length with ageing, such as the increase from 1.76 µm at 24 hours to 1.90 µm at 336 hours *post mortem* that was reported by Wheeler and Koohmaraie (1994) suggests that these interactions are slackened during conditioning. The rate of *post mortem* glycolysis is a significant factor affecting the relationship between sarcomere length and muscle toughness - increased toughness is associated with shortened sarcomeres (shortening) (Smulders *et al.*, 1990; Goll *et al.*, 1995a). As the degree of overlap between the thick and thin filaments increase during sarcomere shortening, the “denser” myofilament lattice resulting from this shortening increase muscle toughness.

2.1.5 Other factors influencing meat tenderness

Muscle is a composite structure of contractile fibres, which are attached to each other and organized by connective tissue. The connective tissue role in meat quality is both quantity and organisation. The amount, spatial distribution and composition of the connective tissue within muscle vary with muscle positions in the carcass and with animal age (Purslow, 2005). Connective tissue toughness is often referred to as background toughness because the tissue hardly changes during the standard lengths of meat storage *post mortem* (McCormick, 1994). This has long been recognised to influence the tenderness of cooked meat.

The connective tissue content is responsible for some of this variation (~20%), but the virtual lack of change in this component during *post mortem* storage while considerable tenderisation occurs, has led to the conclusion that the proteins in the muscle myofibril primarily control meat texture (Greaser and Fritz, 1995). Collagen is the predominant protein of perimysial and endomysial connective tissues, constituting some 1.6 to 14.1% of the dry matter weight (DMW) of muscle (Purslow, 1999). Collagen characteristics, mainly the content and solubility, are thus the basis for the determination of connective tissue contribution to meat toughness.

2.2 The genetic basis of meat tenderness

Meat tenderness is dominated by both physiological and environmental influences. More recent research indicates that genetics of the animal play a significant role (Sellier, 1994; Smith *et al*., 2000). Various researchers suggested that the tenderisation process and the extent of muscle contraction occur unequally in different breeds (Whipple *et al*., 1990b; Shackelford *et al*., 1991; Shackelford *et al*., 1994; Frylinck and Heinze, 2003; Koohmaraie *et al*., 2003; Riley *et al*., 2003). It has been shown that differences occur between *Bos indicus* and *Bos taurus* due to differences in the properties of the proteolytic enzyme systems (calpain system) and their effects on the myofibrillar properties of the muscle. It is well documented that tenderness decreases as the percentage *Bos indicus* increases (Crouse *et al.,* 1989; De Bruyn, 1991; O'Connor *et al.,* 1997) (Figure 2.10), thus meat from *Bos indicus* tend to age slower (O'Connor *et al.,* 1997) than meat from *Bos taurus* due to the inhibiting effect of calpastatin on the ageing process (Shackelford *et al.,* 1991; De Bruyn, 1991; Frylinck and Heinze, 2003).

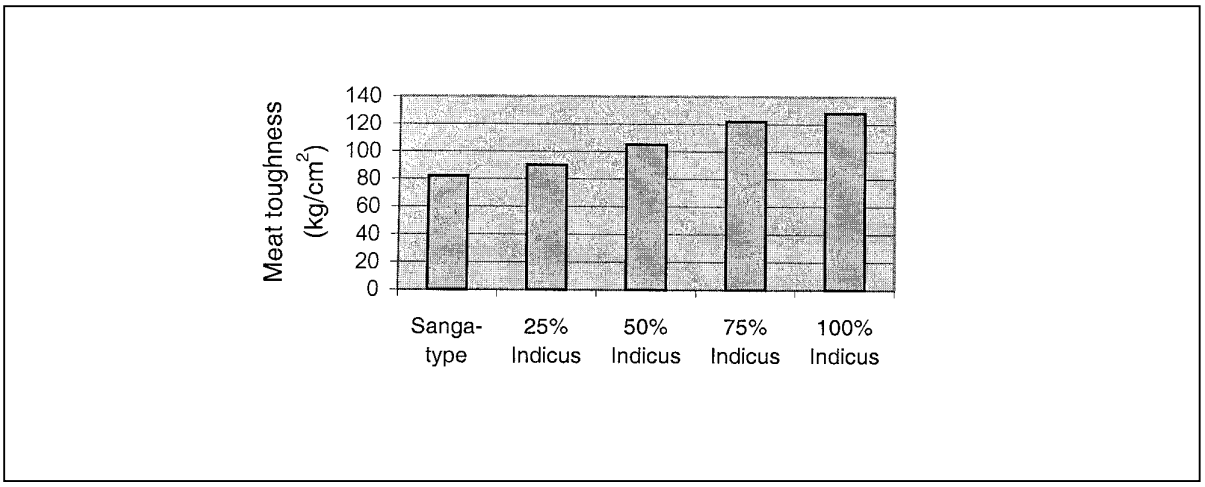

Figure 2.10: Effect of *Bos indicus* blood on muscle tenderness (De Bruyn, 1991).

De Bruyn (1991) found that the Brahman produced significant tougher meat compared to the *Bos taurus* genotypes for example the Hereford and Charolais. The indigenous Afrikaner and the Bonsmara also compared favourable with the tenderness of the *Bos taurus* genotypes. Lombard (1960) and Naudé and

Boccard (1973) also observed favourable meat tenderising characteristics in the Afrikaner as compared to the Hereford. Swanepoel (1988) showed that this positive observation in the Afrikaner is a general characteristic of the *Sanga* genotypes. The tenderness in the Afrikaner corresponds with that in the Pedi and Nguni. In SA the indigenous breeds (*Sanga* types) are also well adapted to a sub-tropical climate and applied in the same farm systems as *Bos indicus* breeds. Although it is assumed that they share similar characteristics, the indigenous South African breeds produce more tender meat compared to the *Bos indicus* breed types (De Bruyn, 1991). More information is still required to explain these differences. Once it is known how the calpain system functions within the indigenous South African breeds, probes could be developed to facilitate the identification of animals or breeds with the superior trait.

2.2.1 Quantitative approach

It has been suggested that genetics can solve the beef industry's problem with tenderness (Sellier, 1994; Eggen and Hocquette, 2003). It is clear that genetic composition makes a significant contribution to the total variation in tenderness as tenderness varies among and within breeds (Shackelford *et al*., 1995). It is well documented that tenderness decreases as the percentage *Bos indicus* inheritance increase (Crouse *et al*., 1989; Shackelford *et al*., 1991, De Bruyn, 1991; O'Connor *et al.,*1997) it is therefore found that *Bos taurus* genotypes produce more tender meat. These differences occur due to differences in the properties of the muscle enzyme system (calpastatin activity) and their effects on the myofibrillar properties of the muscle. To make progress within a breed requires identifying superior sires and dams producing progeny with an improvement in meat tenderness, either through progeny testing or some direct measurement on the sire and dam to predict the tenderness of their progeny.

Animal breeders use selection to increase the frequency of desirable alleles for a desirable trait within a population. Selection response is very high for qualitative traits because the phenotypic variation associated with the trait can be completely explained by a few genes or loci. Selection for complex or quantitative traits is more difficult because the genetic component is controlled by many loci, which may only explain a small portion of the phenotypic variation (Sellier, 1994). Improvements in quantitative trait selection for traits of economic importance have been realised with use of performance records (animal recording) for estimating the genetic value of each animal. Estimated breeding values (EBV) are based on phenotypic information of the individual and / or relatives and their pedigrees (Kappes, 1996). In SA various breed associations make use of EBV's to select their animals for the desired traits. In the USA the industry make use of expected progeny difference (EPD). The procedure also eliminates the effects of the environment, which allows comparison of animals born in different years and under entirely different conditions. Bertrand *et al*. (2001) suggest that the industry movement toward alliances and grid pricing has led to increased interest in EPD for carcass traits in the USA. Carcass EPD's are becoming common in this era and added to the economically important traits in national cattle evaluation programs. In general, the rate

of genetic improvement in a given trait is a function of the heritability of the trait, the generation interval, and the selection differential. Most estimates indicate that, within a breed, additive gene effects control 30% of the variation in beef tenderness (Wheeler *et al.,* 1995). This 30% represents the heritability of tenderness within a breed (Koch *et al.,* 1982). This implies that within a breed, 70% of the variation is explained by, environmental and non-additive gene effects (dominance and epistasis) (Odeh, 2003). Thus, high heritability estimate would decrease the time required to make an improvement in tenderness through quantitative selection. Heritability estimates as discussed by Bertrand *et al.* (2001) indicates that carcass traits will respond well to selection (Table 2.1).

Table 2.1: Heritability estimates for selected carcass traits (As cited in Bertrand *et al*., 2001).

CWT[a]	REA	FAT	MARB	% CUT	WBSF	TEND	CA
0.48	0.40	0.52	0.47	0.49			
0.43	0.56	0.41	0.40	0.63	0.31		
	0.60		0.45		0.09	0.10	
			0.23	0.18			
0.38	0.51		0.31				
0.31	0.32	0.26	0.26				
			0.93	0.45	0.53		0.65
0.23	0.22	0.25	0.48	0.47	0.12	0.22	
			0.40		0.04	0.10	
0.37	0.38	0.35	0.40				
0.15	0.65	0.56	0.73		0.37	0.50	
0.34			0.35	0.26			
	0.51	0.34	0.79		0.18	0.47	0.42
0.59	0.39	0.27					
0.60	0.97	0.46	0.88				
		0.29	0.15	0.49	0.04		
0.30	0.27	0.23	0.36	0.23			
0.39[c]	**0.47[c]**	**0.34[c]**	**0.46[c]**	**0.41[c]**	**0.22[c]**	**0.28[c]**	**0.54[c]**

CWT[a] = carcass weight, REA = longissimus muscle area, FAT = fat depth, MARB = marbling score or percentage of intramuscular fat, % CUT = estimated cutability or percentage of retail cuts, WBSF = Warner-Bratzler shear force, TEND = taste panel tenderness score, and CA = calpastatin activity. [b]First row of estimates for Robinson *et al*., (1998) is for tropical breeds; second row is for temperate breeds. [c]Average.

In SA the Angus breed association makes use of EBV's for carcass traits that is calculated by the Agricultural Research Council - Animal Production Institute (ARC-API). The main reason for limited

application of EBV's on carcass traits in SA is the difficulty in measuring the different carcass traits during production.

2.2.2 Molecular approach

Traditionally, the genetic improvement of beef cattle (meat tenderness included) has been based on phenotypic selection alone. For many years beef breeders in SA focused primarily on production traits in their selection programmes, associated with reproduction (i.e. calving ease) and growth (i.e. weaning weight). These traits received by far the most attention compared to selection for carcass characteristics (Gertenbach and Kars, 1999). Now, like in the rest of the world, they developed a growing concern towards the selection of economically relevant beef carcass quality traits such as tenderness.

Advanced bio-technologies including the use of marker-assisted selection, artificial insemination, cloning, transcriptional and translation assays, and gene transfer are becoming more available and may lead to commercial applications with the potential to change the way we identify superior animals and the dissemination of favourable genes to the beef production. In farm animals, molecular markers have several important applications including the use of polymerase chain reaction (PCR) based techniques for studying the genome, comparative genome mapping, identification of a candidate gene(s) for particular quantitative trait loci (QTL's), and for the investigation of animal biodiversity (Gustavo and Gresshoff, 1997; Odeh, 2003).

The use of DNA-markers to define the genotype and predict the performance of an animal is a powerful aid to animal breeding (Cockett *et al.,* 1995; Palmer *et al.,* 1999; Beuzen *et al.,* 2000). It enables scientists to study the genetic composition of the bovine at the molecular level, identify the regions of the genome that cause genetic variation and suggests strategies for improvement of meat tenderness through a molecular approach (Kappes, 1996; Koohmaraie *et al*., 2003).

The bovine genome consists of 30 chromosomes (Figure 2.11), 1558 genes, 4357 loci, 4764 PCR (polymerase chain reaction) markers and 2402 micro satellite markers (INRA bovmap database, http://locus.jouy.inra.fr, 12 December 2006). These markers also find application in QTL studies. A study by Casas *et al.* (2003), reported two QTL's for meat tenderness. One of these is on chromosome 20; affecting Warner-Bratzler shear force (WBSF) at 3 and 14 days *post mortem* and the second QTL are on chromosome 29 for WBSF at 14 days *post mortem*. Casas *et al.* (2003) suggested that carcass composition and meat quality traits are among those that would benefit most from the use of genetic marker information. The identification and confirmation of QTL is complex, time-consuming and costly process, but promises profitable commercial returns (Van Marle-Köster and Nel, 2003). QTL can lead to the potential use of marker-assisted selection (MAS). MAS can increase genetic response because it is a more accurate selection procedure and selection can be practiced at an earlier age (Kappes, 1996). MAS has potential to generate change in carcass traits such as meat quality (tenderness), for which selection has been historically difficult,

due to the fact that tenderness (genetically) is a multi-factorial trait (more than one factor involved in tenderness) (Frylinck, 2001; Gao *et al.,* 2007).

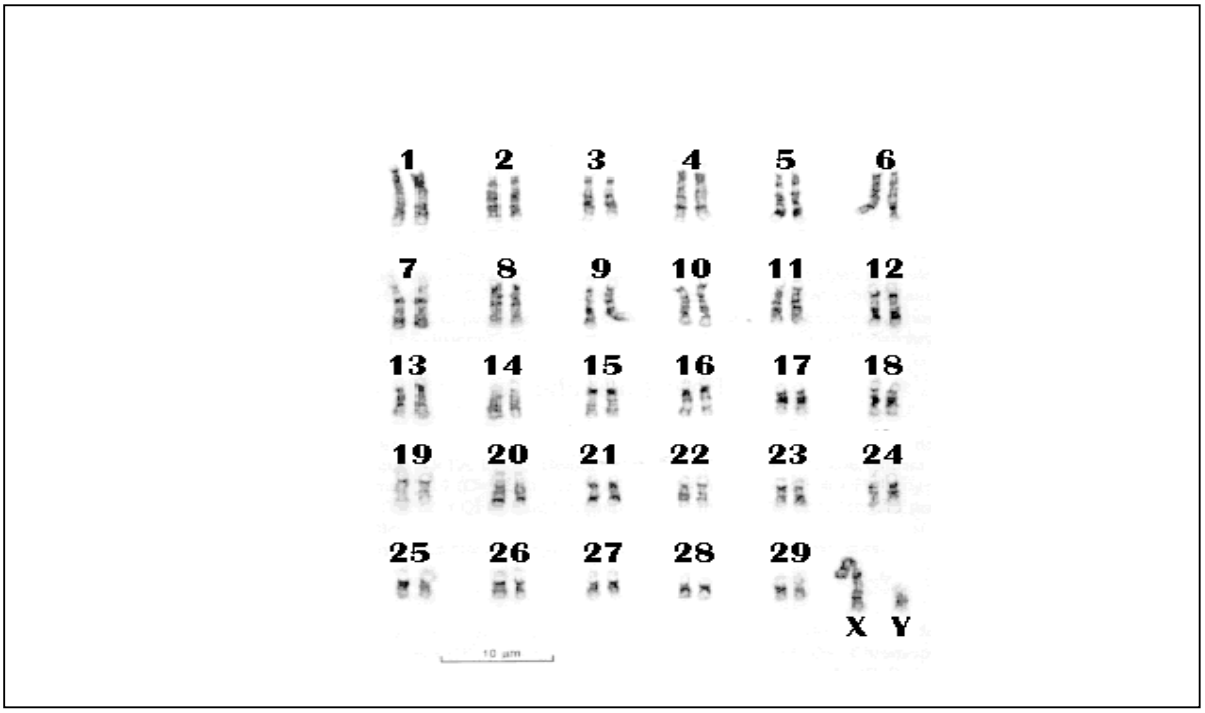

Figure 2.11: An integrated cytogenetic and meiotic map of the bovine genome, adapted from (Eggen and Fries, 1994).

Two main research groups in the world, namely USDA Meat Animal Research Centre in collaboration with the Texas A&M University and AgResearch, New Zealand and CSIRO Livestock Industries and Meat and Livestock, Australia made the greatest advances on the identification of genetic markers for factors that influence beef quality, including tenderness. The Australian consortium patented their developed gene marker tests, and not much is published in peer-reviewed articles. Information on the content of the tests is only available in the technical notes published on the web site: www.geneticsolutions.com.au. Information is available on the MARC gene markers, as it is not their policy to patent research, but rather publishes their research in pear-reviewed articles and shares their work with the scientific community.

Therefore, research on markers indicating a muscle's inherent ability to become tender has become of major importance. One such marker is the activity of calpastatin (*CAST*) (Casas *et al*., 2006; Schenkel *et al*., 2006), the specific inhibitor of calpain (Shackelford *et al.,* 1994). Estimates of the relationship between calpastatin activity and meat tenderness vary, but up to 40% of the variation in beef tenderness is explained by calpastatin activity at 24 hours *post mortem* (Koohmaraie, 1994). A high genetic relationship exists between calpastatin activity and beef tenderness with a genetic correlation reported in the literature between calpastatin activity and taste panel tenderness of -0.95 (Bertrand *et al.,* 2001). Such a high degree of

association justifies using calpastatin as a candidate gene for predicting meat tenderness, but the candidate gene approach only allows for examination of one gene at a time.

The drawback of this analysis is that the factors affecting the expression of the gene of interest (e.g. calpastatin) could be separated by large non-coding sequences (e.g. located on a different chromosome) (Koohmaraie *et al.,* 2003).

Several markers have been developed at the *CAST* gene and three SNP-markers (marker 316, marker 530 and marker 4751) have been developed at the *CAPN1* gene (Figure 2.12) (Casas *et al*., 2006). The micro molar calcium-activated neutral protease gene, *CAPN1* (White *et al*., 2005) on BTA29 (Smith *et al*., 2000) encodes a cysteine protease, μ-calpain, which is thought to be one of the most important enzymes involved in beef tenderness (Page *et al*., 2002).

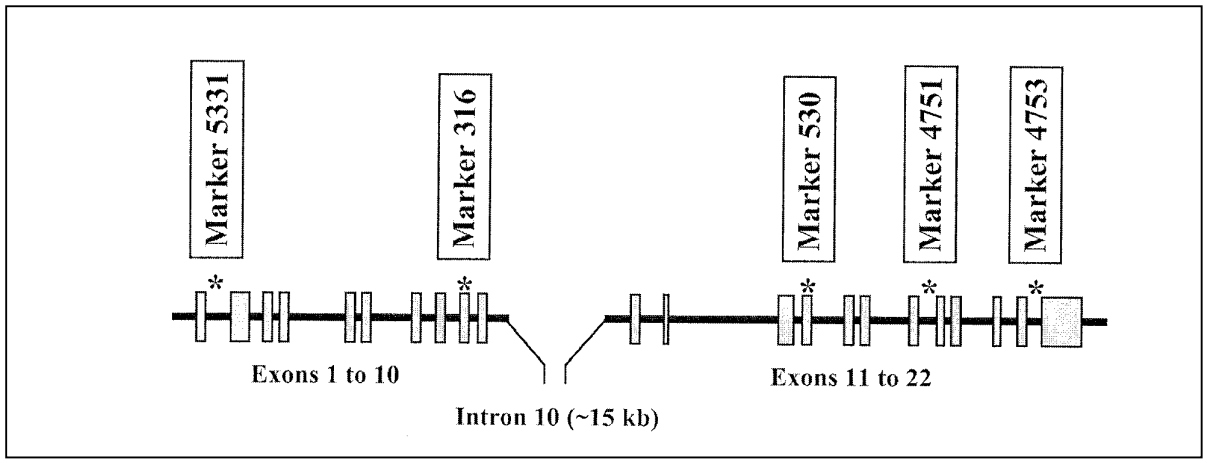

Figure 2.12: Genomic locations of SNP markers in *CAPN1* gene (White *et al.,* 2005).

Single nucleotide polymorphism (SNP) is found where different nucleotides occur at the same position in the DNA sequence (i.e. the substitution of one nucleotide for another or the addition or deletion of one or a few nucleotides). There is an increasing interest in the use of SNP's as markers for genetic analysis because these markers are found in coding and non-coding regions of the genome. In the coding regions SNP can be directly associated with the protein function and as the inheritance pattern is more stable, and they are more suitable markers for selection over time (Beuzen *et al*., 2000; Van Marle-Köster and Nel, 2003).

The SNP developed at the *CAST* gene is a transition from a guanine to an adenine at the 3' untranslated region of the gene (Casas *et al*., 2006). The SNP's in the *CAPN1* gene lies on exon 9 (marker-316) and exon 14 (marker-530) (Figure 2.12), both predict amino acid (AA) sequence changes. At AA number 316 (marker-316), a guanine (G-allele) to cytosine (C-allele) transverse predicts either glycine or alanine, and at number 530 (marker-530), an adenine (A-allele) to guanine (G-allele) transition predict either isoleucine or valine (Page *et al*., 2004).

These markers (markers 316 and 530) can be used to guide selection in *Bos taurus* cattle, but do not segregate at high frequencies in Brahman cattle (*Bos indicus*). Beef cattle of *Bos indicus* descent are widely

used for their heat tolerance and disease resistance, but tenderness has been problematic in many of these breeds. The reputation for less tender meat in these cattle presents an opportunity for improvement by use of genetic markers. White *et al*. (2005) extended the tenderness maker test to include cattle of *Bos indicus, Bos taurus* and crossbred descent. Marker 4751, which is equivalent to position 6545 (C/T) of GenBank accession number, AF248054 fulfilled this role. It was concluded that maker 316 may continue to be useful in a variety of populations with a high percentage *Bos taurus* background, and that a multi marker system (incorporating both markers 316 and 4751 for *CAPN1*) provides an optimal solution in cattle of all subspecies backgrounds.

A commercial test that was developed from gene marker research in Australia is based on DNA-testing namely: GeneStar® (Alison, 2006; Gao *et al.,* 2007). Producers worldwide (Australia and America) and in South Africa make use of this commercial test. GeneStar®-tenderness is a DNA based test for the variants of the bovine calpastatin gene located on chromosome seven. The test detects two different forms of the gene. The first form is associated with tenderness and the other with toughness. The concept of how favourable and unfavourable form of the markers work together to predict a certain favourable or unfavourable outcome of meat tenderness can be intricate for the layman. For simplicity, GeneStar® reports the result for each marker as being either 0, 1 or 2 stars. Stars represent the favourable form of the markers associated with the trait (tenderness). The more stars, the better. For a single DNA marker:

1. A zero star result means the animal carries neither of the favourable alleles for that marker. This animal did not inherit a favourable allele from either parent.
2. A one star result means the animal carries one favourable allele for that marker. This animal inherited a star from one parent and an unfavourable allele from the other parent.
3. A two star result means the animal carries both favourable alleles for that marker. This animal inherited one star allele from each parent for this marker.

At the moment GeneStar® tests for four tenderness DNA markers that are identified as T1, T2, T3 and T4 (Alison, 2006; Gao *et al.,* 2007). Not much is known about them except that results for each trait are claimed to be additive (Technical update October 2006, www.geneticsolutions.com.au, 7 May 2007). Thus an animal can score from 0 up to 8 stars for tenderness. According to their technical note (Technical update October 2006, www.geneticsolutions.com.au, 7 May 2007), they DNA tested and analysed large sets of carcass results with measurements of meat tenderness (i.e. mechanical shear force) as the objective measure, where lower shear force is more desirable. Their results showed that the four markers are independent and that the effects on tenderness are additive. The implication is that individual results for each marker can be added together to assess the potential impact 0 - 8 star results. Their results show that the toughness decrease as the number of stars increases in the total data set over 3000 carcasses from seven breeds.

A diagnostic test for a major gene for marbling in beef cattle, GeneStar®-marbling is also commercially available (Gao *et al.,* 2007). Marbling is the fine evenly distributed flecks of fat found through

the muscle and is often referred to as intra-muscular fat (IMT). GeneStar®-marbling indicates if the animal has the favourable genotype with either one desirable allele (1-STAR) or two desirable alleles (2-STAR) (www.agribsa.co.za, 21 January 2006). Consequently, marbling accounts for less than 10% of the variation in meat tenderness according to Dransfield (1994).

Gene marker tests have application in the seed stock and commercial level by selecting for a higher frequency of genes influencing meat tenderness. Under Australian conditions the estimated contribution of this gene on meat tenderness was found to vary between 10 - 12%. Taking into account the many other environmental and non-environmental conditions, that plays a role in meat tenderness and the elimination of this variable could have a significant effect on the consistency of meat tenderness.

2.3 Measurement of meat tenderness

Numerous methods have been developed to measure the tenderness of meat, but the oldest method is the consumption of meat samples (i.e. sensory analysis) (Boccard, 1973). An important part of sensory analysis is to show not only the attributes that consumers like or dislike but also the most important characteristics determining the overall acceptability. Therefore, sensory analysis enjoys recognition for its importance in new product development, basic research, ingredient and process modification, cost reduction, quality maintenance, and product optimisation (Lawless and Heymann, 1998; Scheepers, 1999).

Assessment of using instrumentation (shear force resistance measurement using Warner-Bratzler shear force attachment) has remained the most popular and accurate instrumental measure of meat tenderness (Culioli, 1995). WBSF based on the force needed to shear muscles fibres measures the tenderness of meat. Thus, the more force needed, the tougher the meat is and the units of measurements are kilograms of force needed to shear a one cubic centimetre muscle sample (Honikel, 1998). Factors that may affect the accuracy of these measurements include the doneness of the cooked meat, uniformity of cylindrical sample size, direction of the muscle fibre, amount of connective tissue and fat deposits present, temperature of the sample and the speed at which the sample is sheared. According to Cross *et al*. (1986) the results usually correlate well with the scores obtained from the sensory evaluation.

Sarcomere length (SL), myofibril fragmentation index (MFI) and myofibrillar fragment length measurements (MFL), as part of histological traits has been used by many researchers to predict the tenderness of meat (Thornberg, 1996). Sarcomere length is related to tenderness, especially in cases of severe shortening (Whipple *et al*., 1990b). Frylinck and Heinze (2003) concluded that myofibril fragment length correlates with sensory tenderness and WBSF, which support the statement that proteolysis of myofibrillar proteins leads to increased fragmentation of myofibrils and decreased shear force during *post mortem* storage (i.e. higher tenderness scores for shorter myofibril fragments). Thus, the MFI and changes in myofibril fragmentation during *post mortem* storage is related to tenderness and can be used widely as a method of estimating meat tenderness (Taylor *et al*., 1995).

It is well established that calpain-mediated degradation of myofibrillar proteins is responsible for *post mortem* tenderisation of meat (Frylinck and Heinze, 2003). Apart from regular tests used to measure the amount of protein in meat, an evaluation of their solubility is performed using electrophoresis, SDS-PAGE (i.e. sodium dodecyl sulphate polyacrylamide gel electrophoresis) (Pospiech *et al*., 2003). With this technique, proteins are separated on the basis of their molecular weight (Figure 2.13), thus evaluating the proteolysis process. After staining of the gels with protein-binding dye, several bands can be observed corresponding to proteins with a specific molecular weight. Degradation of a protein during meat ageing, results in a decrease in the intensity of the band corresponding to this protein. Simultaneously, new bands appear which correspond to protein fragments of the native protein.

The major contractile proteins, myosin and actin, are hardly or not at all degraded during meat ageing at refrigeration temperatures (Penny, 1980; Goll *et al*., 1983; Koohmaraie *et al*., 1988). However, during ageing of bovine muscles at high temperatures (23 - 37°C), substantial degradation of myosin heavy chains takes place (Geesink, 1993). The most reported change in the myofibrillar proteins during ageing is the gradual disappearance of troponin-T, with simultaneous appearance of protein fragments with a molecular weight of 27 - 30 kDa (Figure 2.13). The breakdown of troponin-T and the appearance of a 30 kDa peptide have been proposed as a measure of *post mortem* proteolysis and meat tenderness (Geesink, 1993). Although the degradation of troponin-T parallels meat tenderisation, it is unlikely that this degradation is directly responsible for *post mortem* tenderisation; troponin-T is located on the actin filaments and not at or near the Z-line, where myofibrillar fragmentation takes place. Degradation of titin, during ageing of meat at various temperatures (2 - 37 °C), has been reported for various muscles (Geesink, 1993). Although a great deal of experimentation is yet needed to demonstrate the role of titin in meat quality, the degree of titin degradation seems to parallel measures of fresh meat quality, such as tenderness (Huff *et al*., 1991). Nebulin is rapidly degraded in *post mortem* muscle, even faster than in titin (Penny *et al*., 1984). This degradation may trigger subsequent *post mortem* alterations in the myofibril. Due to nebulin that is a long, fibrous, structural protein of the sarcomere, and may anchor thin filaments to Z-lines, nebulin's demise *post mortem* may decrease overall cytoskeletal integrity of the myofibrils, muscle cells and muscle tissue (Robson *et al*., 1991). The desmin intermediate filaments are the only cytoskeletal elements discovered that link myofibrils together and the myofibrils to other structural entities in the muscle cell. It was also demonstrated that purified desmin is an excellent substrate for calpain. Desmin associated with isolated myofibrils or muscle preparations *in situ* is also quickly degraded by the calpains. Desmin is degraded during *post mortem* storage of muscle at about the same rate as troponin-T, which is degraded as one of the better substrates of calpain. It is well known that there are distinct alterations and weakening of the inter-myofibrillar links at the Z-line levels in *post mortem* muscle. Regarding the location of desmin at the periphery of the Z-lines, degradation of this protein is probably responsible for loss of transversal alignment of sarcomeres during ageing (Robson *et al*., 1991).

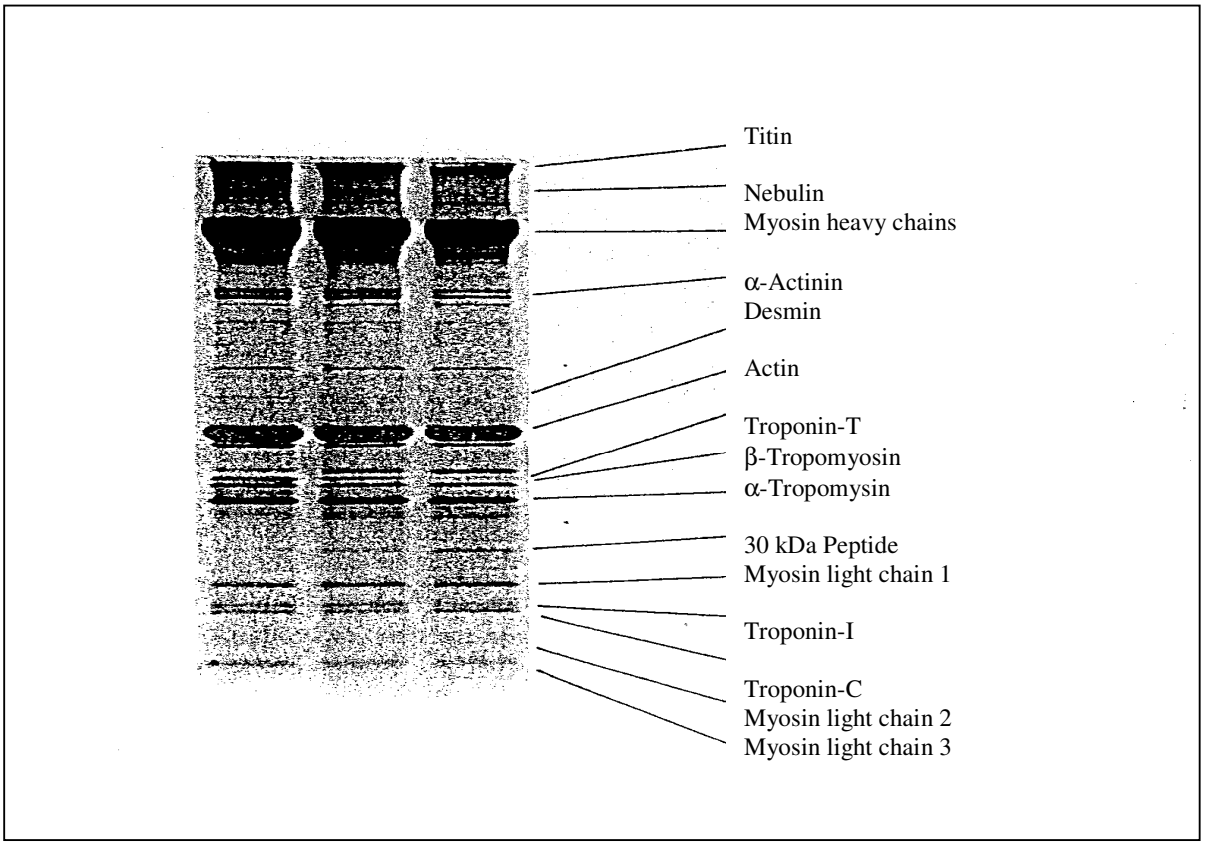

Figure 2.13: Sodium dodecyl sulphate polyacrylamide gel electrophoresis indicating protein separation base on molecular weight (Adapted from Geesink, 1993).

The technique, Western-blotting could be used to determine whether these proteins are degraded during *post mortem* storage and whether this degradation correlates to the loss of muscle structures (i.e. costameres) (Taylor *et al*., 1995). Thus, protein evaluation is often performed simultaneously with an estimation of proteolytic enzyme activity, changes of other compounds in muscles, especially those associated with glycolysis.

Assessing tenderness is even further extended to a molecular level, where advances (i.e. genetic markers) provide a potential for renewed methods in the beef industry. The extend to which meat tenderness can or will be controlled or measured from this level, the future only will show (Pospiech *et al*., 2003).

2.4 Future developments

The beef industry is constantly undergoing changes and these changes are forced by the increasing concern from the consumers for quality, safety, and trace ability. One of the main challenges in the future will be to solve the problems posed by the analysis, interpretation and the access to large amount of data that will become available from structural (QTL, SNP) and functional (protein levels) genomics. Beuzen *et al*. (2000)

predicted that the next century would likely see the combination of both quantitative and molecular genetics dominating the theory and practice in animal breeding. Thus, the challenges for the future can be overcome by meat research but it appears that it is necessary, to focus on basic research more than ever, using integrated multidisciplinary approaches.

CHAPTER 3

MATERIALS AND METHODS

MATERIALS

3.1 Animal management and harvest

The beef cattle used in this study were representatives of the crossbreds mostly responsible for meat production in South Africa (according to Dave Ford of the Feedlot Association of South Africa and committee member of the Red Meat Research and Development Trust) (RMRDT). The sixty beef animals were classified according to the three breed types (*Bos indicus* and *Sanga, Bos taurus*) namely:

- Brahman-crosses (Bh) (n = 20) (*Bos indicus*)
- Nguni-crosses (Ng) (n = 20) (*Sanga*)
- Simmentaler-crosses (Sm) (n = 20) (*Bos taurus*)

Weaned, young bulls (7 - 8 months, and average weight of 158 kg) were transported to the Agricultural Research Council - Animal Production Institute (ARC-API: Irene), where they were placed under feedlot conditions for a period of about 100 to 120 days until slaughter. The animals were housed in small pens holding ten animals each, with each animal having 10 cm^2 and 50 cm of feed-bunk space. Clean, fresh water was available at all times. A standard type of high concentrate diet was supplied to the animals (12 MJ/kg DM, 13,5% protein) to which they were allowed to adapt during a three-week period, from high levels of hay (15%) to low levels of hay (6%). The animals within the breed groups were blocked by weight and allocated to their respective pens. The animals were weighed at two weekly intervals and daily health observations (animal morbidity, consistent breathing and manure consistency) were made. All these actions were necessary to comply with the rules of the Ethics Committee of the ARC. The animals were slaughtered when at an A age (10 - 12 months) with a fatness class of two or three (lean-medium fatness) according to the current South African Beef Classification System (Government Gazette No. 5092, 1993). The average final *pre-slaughter* weight of the crossbreds in the feedlot was:

- Brahman-crosses (Bh) (final *pre-slaughter* weight in feedlot: +/- 430 kg)
- Nguni-crosses (Ng) (final *pre-slaught*er weight in feedlot: +/- 400 kg)
- Simmentaler-crosses (Sm) (final *pre-slaughter* weight in feedlot: +/- 370 kg)

3.2 The slaughtering process, *post mortem* sampling and storage

At slaughter the carcasses were not electrical stimulated (NS) because electrical stimulation (ES) influences the processes of meat tenderness, and we were interested in the expression of the inherent tenderness characteristics without external *post mortem* influences. After exsanguination the carcasses were dressed, halved and chilled at 4 °C within 2 hours of slaughter. The *M. longissimus thoracis et lumboram* (LT and LL) of the right and left sides were removed from the third last rib to the last lumbar vertebra and sub sampled for shear force evaluations, SDS-PAGE, Western-blotting, myofibril fragmentation (MFL), sarcomere length (SL), calpain, calpastatin, total collagen, % collagen solubility and marker analysis (Figure 3.1). The position of sampling for each test was consistent and the different samples were either frozen immediately at -20 ˚C or -80 ˚C or vacuum packed and aged (2 ± 2 ˚C) for 7 or 14 days *post mortem.*

The muscle pH and temperature decline were measured at the second last lumbar vertebrae (Figure 3.1) at 1 hour, 2, 3, 6, 8 and 20 hours, after slaughter to diagnose pale, soft and exudative (PSE) type (PSE is only found in pork, but a similar phenomenon exist in beef where a high drip loss takes place after a quick pH decline and a slow temperature decline) or dark, firm and dry (DFD) phenomena at slaughter. This phenomenon can influence the ageing process of the meat. The pH and temperature were determined with a digital handheld meat pH meter (Unitemp) fitted with a polypropylene spear type gel electrode. Muscle samples for other procedures were removed from *M. longissimus dorsi* (L1-L6) (LL) at specified positions and preserved at either -25 °C or -80 °C after ageing at 1 day, 7 and 14 days *post mortem* (Addendum C, Figure 1) (samples were aged in vacuum packaging at ± 2 °C) depending on method specification.

METHODS

3.3 Warner-Bratzler shear force measurements (WBSF)

Frozen *M. longissimus dorsi* (L1-L6) cuts aged for 1 day, 7 and 14 days were processed into 30 mm steaks by means of a band saw before being thawed at 4 °C for 24 hours, and prepared according to an oven-broiling method using direct radiant heat (AMSA, 1978). The steaks were broiled at 260 °C (pre-set) to 70 °C internal temperature. Each cooked steak was allowed to cool for 10 minutes and then wrapped in tin foil and stored overnight at 4 °C for WBSF resistance measurements. WBSF was measured after the meat had been removed from the chillers, and left to room temperature (± 22 °C) before being cored. Eight cylindrical samples with a core diameter of 12.5 mm were removed parallel to the grain of the meat and sheared perpendicular to the fibre direction using Warner-Bratzler shear device mounted on an Instron Universal Testing Machine, Model 4301, Series IX Automated Materials Testing System Version 5 (Instron, 1990). Shear force was measured as the peak force (kg) average for eight cores per sample. The shear force

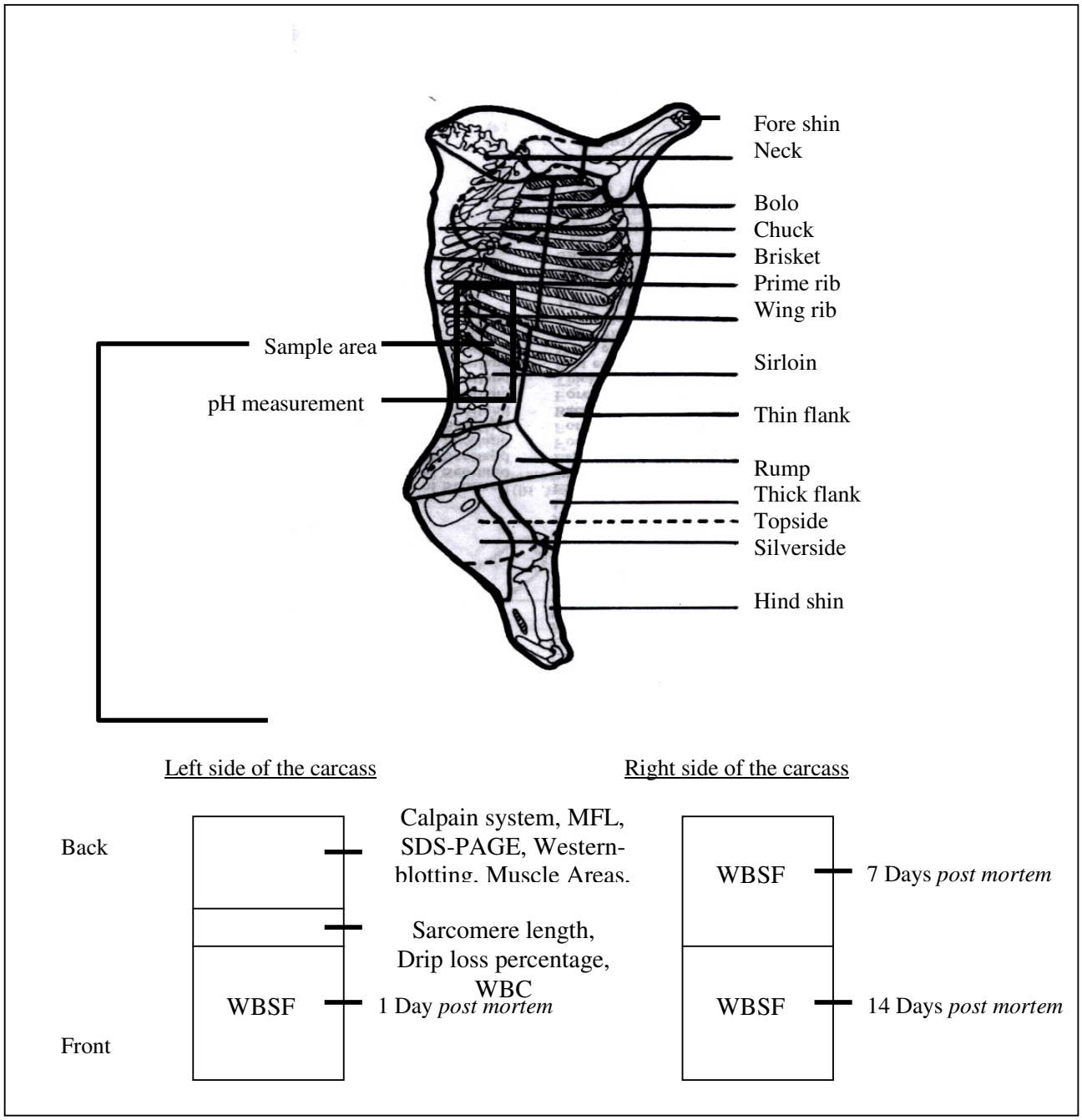

Figure 3.1: The cuts of a beef carcass (Hofmeyer, 1981) and a schematic representation of where the various samples were taken for the various analyses. MFL = Myofibril fragment lengths, WBC = Water binding capacity.

of each steak (1 day, 7 and 14 days) was measured as the maximum force (kg, kilograms) required to shear a cylindrical core cooked steak perpendicular to the grain, at a cross head speed of 400 mm per second.

3.4 Myofibrillar fragmentation length (MFL) determination

Myofibril fragment length (MFL) is a useful indicator of the extent of myofibrillar protein degradation and was used in the place of MFI determinations eliminating the drawbacks of background noise inevitable in the MFI measuring technique. The MFL changes as a result of ageing, which is used as an indicator of the weakening of the myofibrillar structure as a result of *post mortem* proteolysis. The extraction procedure was conducted as described by Culler *et al*. (1978) and adapted by Heinze & Bruggemann (1994). From a frozen *M. longissimus dorsi* (L1-L6) sample slices were cut with a knife for the MFL determination. Days 1, 7 and 14 samples were used for MFL determination. Any visible fat and connective tissue were removed. The sample was then finely minced by means of scissors. About 3 grams (g) of the minced sample was weighed into 50 ml Bűhler glass, and 30 ml of the MFL extraction buffer (0,02 M potassium phosphate buffer containing: 100 mM KCL, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM NaN_3, pH 7) (4 °C) was added. After allowing the sample to thaw for 60 seconds, the sample was homogenised for exactly 30 seconds in a Bűhler HO_4 homogeniser while the sample was chilled with ice water, at 20,000 rpm with the blade turned around in order to fragment the myofibrils with the blunt side of the blade rather than to cut them. The samples were subsequently transferred into centrifuged tubes and centrifuge at 4 °C, 3000 rpm for 15 minutes. The supernatant was discarded, and the pellet was suspended in 30 ml MFL extraction buffer. The sample was again centrifuge at 3000 rpm for 15 minutes, at 4 °C. The supernatant was discarded, and the pellet was again suspended in 10 ml MFL extraction buffer. The suspension was then filtered under vacuum through a 1000 µm polyethylene strainer. An additional 5 ml MFL extraction buffer was used to facilitate the passage of the myofibrils through the strainer. The filtrate was subsequently filtered under vacuum, through a 250 µm polyethylene strainer. The samples were transferred on to a slide and covered with a slip. The excess water is dried and the slide was cleaned. 100 Single myofibril fragments were measured by means of a Video Image Analyser (Kontron, Germany).

3.5 Sarcomere length (SL) determination

The sarcomere lengths (the length of the contractile unit of a muscle) were measured by using a Video Image Analyser (Kontron, Germany) after preparation of a fresh sample, according to the method of Hegarty and Naudé (1970) by using distilled water (dH_20) instead of Ringer Locke solution (Dreyer *et al*., 1979). Sarcomere lengths were determined on the *M. longissimus* muscle at 1 day, 7 and 14 days *post mortem* where a meat sample is scratched along the grain of the fibres. The sample is homogenised with an Ultra Turrax. After homogenising the sample is transferred on to a slide and covered with a cover slip. The excess water is dried off and slide is cleaned. Five sarcomere lengths are measure at a time, from the bottom of the first sarcomere to the top of the fifth sarcomere. The averages of 100 SL readings were calculated.

3.6 Calpain and calpastatin analysis

Calpastatin in combination with μ-calpain and m-calpain were extracted from 5 g of the *M. longissimus* frozen samples as described by (Dransfield, 1996) and separated by means of the two-step gradient ion-exchange chromatography-method according to Geesink and Koohmaraie (1999b). μ-Calpain activity in eluates containing both μ-calpain and calpastatin is estimated from calpastatin measurements before and after heating the eluates. Calpain assays were done using azo-casein as substrate according to Dransfield (1996). The use of azo-casein eliminates the problem of background absorbance of non-specific proteins in the extracts. One unit of calpain activity was defined as an increase in absorbance at 366 nm of 1.0 per hour, at 25 °C. One unit of calpastatin activity was defined as the amount that inhibited one unit of m-calpain activity. Data were expressed as units per gram of muscle or units per milligram of extractable protein (specific activity). Protein concentration of the *M. longissimus* frozen samples was determined by the buiret method of Cornall *et al*. (1949) as sited by Bailey (1967).

3.7 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blotting analysis

The separation of macromolecules in an electric field is called electrophoresis. A very common method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium, and sodium dodecyl sulphate (SDS) to denature the proteins. The method is called sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 3.2).

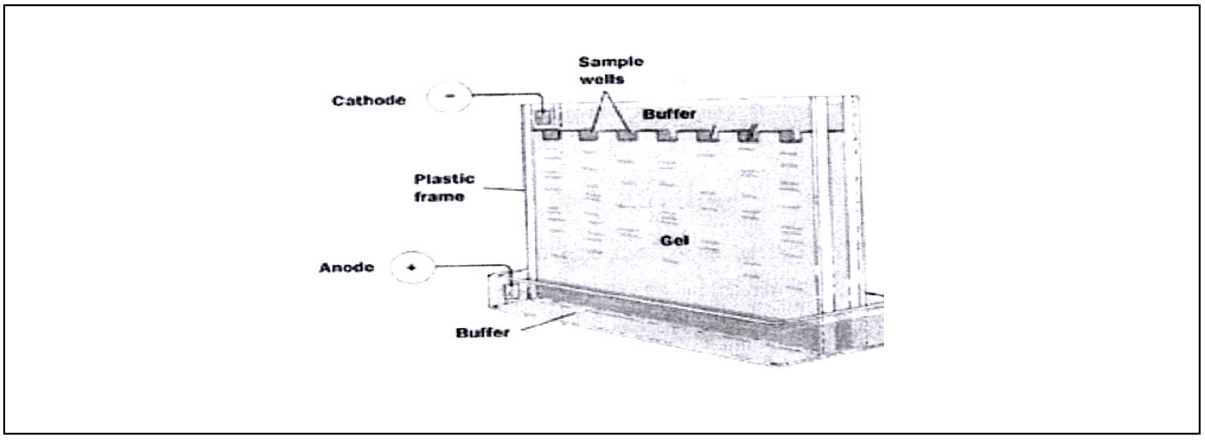

Figure 3.2: Illustration of the set up of a PAGE apparatus and concept of using electric charge to drive protein separation (Campbell, 1995).

To study the degradation of titin, nebulin, desmin and the appearance of the 30 kDa degradation products as a result of tenderisation. 0.25 g of 1day, 7 and 14 days *post mortem M. longissimus* samples were extracted according to the methods of Laemmli (1970). The protein concentration of the samples was determined by means of the biuret method as sited by Bailey (1967). A standard curve was prepared from which the protein concentration of the samples was calculated with the spectrophotometer (Beckman Spectrophotometer DU-7500). For the SDS-PAGE and Western-blotting (specialised technique to detect gene products and to find similarities) analysis the *M. longissimus* proteins were separated by means of 30% T, 0.5% C separation gels with 12% T, 12,5% C stacking gels (Fritz *et al*., 1989). A wide molecular weight range SigmaMarker™ (M 4038) containing thirteen proteins from 6 - 205 kDa were used as the standard (Table 3.1).

Table 3.1: Molecular weight distributions for proteins in the wide molecular weight range (SigmaMarker™).

Proteins	Molecular Weight (MW)
Myosin, rabbit muscle	205,000
B-Galactosidase, *E.Coli*	116,000
Phosphorylase b, rabbit muscle	97,000
Fructose-6-phosphate kinase, rabbit muscle	84,000
Albumin, bovine serum	66,000
Glutamic Dehydrogenase, bovine liver	55,000
Ovalbumin, chicken egg	45,000
Glyceraldehyde-3-phosphate Dehydrogenase, rabbit muscle	36,000
Carbonic Anhydrase, bovine erythrocytes	29,000
Trypsinogen, bovine pancreas	24,000
Trypsin Inhibitor, soybean	20,000
Lactalbumin, bovine milk	14,200
Aprotinin, bovine lung	6,500

After the gels were stained (Coomassie Blue staining solution) and destained, it was vacuum packed and stored at 4 °C until all SDS-PAGE analysis were completed. To further study the degradation of the protein desmin that is regarded as a key structural protein (Huff-Lonergan *et al*., 1996a; Ho *et al*., 1996) and is related to tenderisation, Western-blotting analysis was applied after the SDS-PAGE procedure. The gels used for Western-blotting was not stained and destained after electrophoresis but were equilibrated for 15 minutes at 4 °C in a transfer buffer containing 25 mM Tris, 192 mM glycine, 2 mM EDTA, 15% (vol/vol) methanol, and 0.1% (wt/vol) SDS. The transfer conditions were as described by Huff-Lonergan *et al*., (1996b) and a Mighty Small Transphor Tank Transfer Unit (TE 22, Amersham Pharmacia Biotech) (Addendum C, Figure 2) was used to transfer the proteins from the gels on to a nitrocellulose membrane (RPN 303D, Hybond ECL, AEC-Amersham). After transferring, the membranes were incubated in a

blocking solution (80 mM Na_2HPO_4, anhydrous, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1% [vol/vol] polyoxyethylene sorbitan monolaurate [Tween-20], 5% [wt/vol] non-fat dry milk) prepared as described by the manufacturer of the chemiluninescent Western-blotting detection system (RPN 2125, AEC-Amersham). The primary antibody used in the Western-blotting procedure included mono-clonal anti-desmin (Clone DE-U-10, Sigma Aldrich SA) diluted 1:25,000 in PBS-Tween (vol/vol). PBS-Tween solution is identical to the blocking solution except non-fat dry milk was not added. The blots were incubated with the primary antibody overnight at 4 °C. After incubation with the primary antibody, the blots were washed three times in PBS-Tween, one time 15 minutes and two times 10 minutes per wash. The bound primary antibodies were labelled with a secondary antibody (goat-anti-mouse IgG horseradish peroxidase, (A2554, Sigma Aldrich South Africa) as described by Huff-Lonergan *et al*. (1996b).

A chemiluminescent detection system (Figure 3.3) was used for detecting the labelled protein bands as described by the supplier (AEC-Amersham).

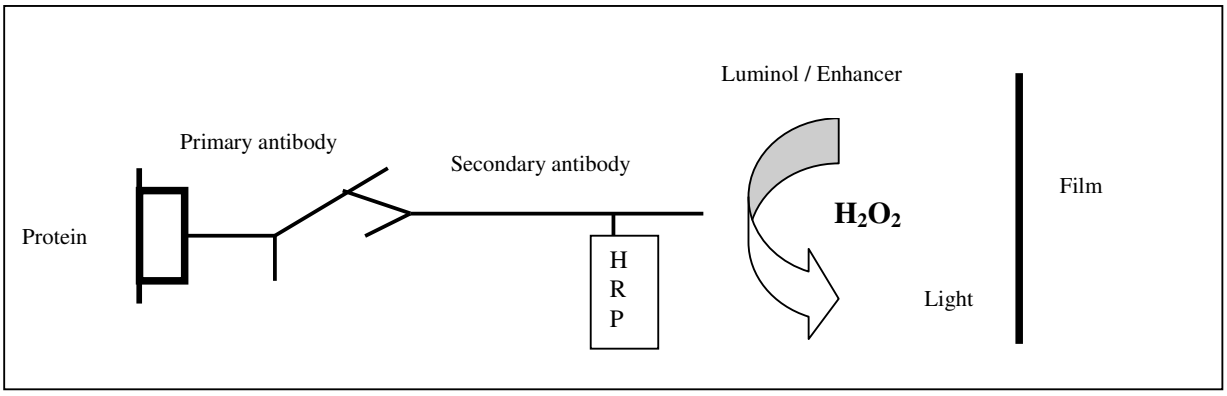

Figure 3.3: Principles of protein detection procedures (AEC-Amersham, 2002).

The principle of a chemiluminescent detection system is, that in the presence of hydrogen peroxide (H_2O_2), Horseradish peroxidase (HRP) catalyses the oxidation of cyclic diacylhydrazides, such as luminol. Immediately following oxidation, the luminol is in an excited state (intermediate reaction product), which decays to the ground state by emitting light. Enhancers, such as phenolic compounds, produce strong enhancement of the light emission. Using this method, it is possible to detect membrane immobilised specific proteins labelled directly with HRP or indirectly with HRP-labelled antibodies. The separated muscle protein bands by means of SDS-PAGE electrophoresis and the Western immuno-blot patterns (the developed film) were analysed densitometrically with the ImageMaster 1D-Software (Amersham Pharmacia Biotech).

3.8 Total collagen and percentage collagen solubility

The total collagen content of the *M. longissimus* muscle (total hydroxy-proline nitrogen content) was estimated by determining the hydroxy-proline content in hydrolysed samples. 30 ml 6N HCl was added to 0.5 g pulverized freeze dried sample and hydrolysed at 110 °C for 16 hours, followed by the addition of active carbon, filtered and diluted with distilled water to 100 ml. The total nitrogen content in muscle was determined after the samples had been digested in a micro Kjeldahl system.

The solubility of the intramuscular collagen (hydroxy-proline nitrogen content of soluble collagen) was determined according to the method of Hill (1966) with some modifications. Freeze dried meat samples (1 g) were pulverized and added to 12 ml of a 1% NaCl solution and heated in a water bath for 60 minutes at 78 °C. After centrifugation at 1,000 rpm for 30 minutes, 30 ml 6 N HCl was added to the supernatants and then hydrolysed for 16 hours at 110°C. Active carbon was added to the hydrolysed samples, then filtered and diluted with distilled water to 100 ml.

Hydroxy-proline were calorimetrically determined by neutralising the acid in the samples with 10% KOH, and then oxidized with Chloramine-T for 20 minutes. Ehrlich's reagent was then added and the samples were placed in a water bath for 15 minutes at 60 °C. The absorbance of the pink colour was measured at 558 nm in a 1-cm^3 cuvette, as described by Bergman and Loxley (1963). All determinations were performed in triplicate. Collagen solubility was calculated by expressing hydroxy-proline in the filtrate as a percentage of the total hydroxy-proline in the filtrate plus residue.

Collagen content was expressed as hydroxy-proline nitrogen per total protein nitrogen (Hypro N x 10^3 / total protein N) by calculating hydroxy-proline nitrogen from hydroxy-proline MM 131.13 and nitrogen atom number 14.0067.

3.9 DNA extraction and marker analyses

DNA extractions were performed at the Department of Animal and Wildlife Science, University of Pretoria (UP), South Africa, with a commercial kit, DNeasy Tissue (QIAGEN). Samples (Addendum C, Figure 1) from the *M. longissimus* that were stored at -20 °C were used for the DNA extraction as described in the protocol: "Purification of total DNA from total animal tissues".

The standard protocol of the DNeasy Tissue kit was used with the following adaptations: 150-170 g tissues was cut up in to small pieces, and placed in a 2 ml micro centrifuge tube. 600 µl ATL buffer and 60 µl Proteinase K was added and incubated at 55 °C until the tissue was completely lysed. After incubation, 620 µl AL buffer was added to the sample and incubated at 70 °C for 10 minutes. After the second incubation, 620 µl ethanol (96 - 100%) was added to the sample and was centrifuge at ≥ 8000 rpm for four minutes. The washing steps with buffers AW1 and AW2 were repeated twice.

After incubation and elution the DNA yield and purity were determined with a nanodrop (ND-1000) spectrophotometer, (Addendum C, Figure 3). DNA yield was determined by measuring the concentration of DNA in the eluant by means of its absorbance at 260 nm. The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of DNA with respect to contaminants that absorb UV, such as protein. The following estimates were set as a standard:

- Estimate of DNA yield, Absorbance (A_{260}): 0.1 - 1.0 ng/µl
- Purity of the DNA, Ratio (A_{260}/A_{230}): 0.8 - 1.8 ng/µl
- Estimate of the purity of DNA with respect to contaminants that absorb ultra violet (UV), such as protein, Ratio (A_{260}/A_{280}): 1.8 - 2.0 ng/µl

After determining the purity and yield of the DNA, 40 µl of the DNA was stored at -40 °C at the Department of Animal and Wildlife Science, University of Pretoria (UP), South Africa. 60 µl DNA was allocated into tubes with screw tops and labelled clearly. Samples were kept at -4 °C and were transported on ice with a courier services to the U.S. Meat Animal Research Centre (MARC) for further analyses.

At MARC, animal sequencing and single nucleotide polymorphism (SNP) identification were used to determine the following markers: the micro molar activated neutral protease (*CAPN1*) and its inhibitor calpastatin (*CAST*). Sequencing was performed with BigDye Terminator as recommended by the manufacturer (Applied Biosystems, Inc.) and was analysed on an ABI 3700 sequencer. Sequence data was entered into the database, base calls were made using Phrep, and the sequences were aligned using Phrap (Ewing and Green, 1998). A viewer (Consed) was used to identify and tag single nucleotide polymorphisms, according to the method proposed by Gordon *et al*. (1998) as described by Heaton *et al.* (2001).

Genotyping was performed using a primer extension method (Table 3.2) with mass spectrometry-based analysis of the extension products on a Mass Array system as suggested by the manufacturer (Sequenom, Inc., San Diego, CA) and described by Stone *et al*. (2002). A universal mass tag sequence was added to the 5' end of each gene-specific amplification primer sequence as recommended by the manufacturer.

Table 3.2: Genotyping primers for *CAPN1* markers (White *et al*., 2005)

Marker	Forward primer [a]	Reverse primer [a]	Probe
316	GAGCTGGCCCTCATAAGATAA	CCCATCCTCCATCTTGACC	CCTCGGAGTGGAACG
4751	AAGGGACAGATGTGGACAGG	GAGGGGTGTTCTCTGAGTGC	ACACAGCCCTGCGCCTC

[a] Amplification primers are shown minus standard mass tag

Genotypes for each animal were analysed, and the automated calls were checked by manual visualisation of the spectrographs to minimize errors. Thus, the methods for determining the *CAPN1*-316 and *CAPN1*-4751 were based on the methods described by Page *et al*. (2002) and White *et al*. (2005), where the determination of *CAST* was based on the method of Casas *et al*. (2006).

These genetic markers can be associated with meat tenderness and they expand the possibilities to improve meat tenderness in many commercial herds, especially those including cattle of Brahman and / or crossbred descent.

3.10 Statistical analysis

The data of WBSF, MFL, SL, calpains- and calpastatin levels, SDS-PAGE, Western-blotting, total collagen, collagen solubility and genetic markers were subjected to analysis of variance for a split-plot design (GenStat® 7, 2003) with the three crossbreds (Brahman, Nguni and Simmentaler) as whole plots and the three ageing periods (1 day, 7 and 14 days *post mortem*) as sub-plots. Means for the interactions between the sub-plot and whole plot were separated using Fisher's protected t-test least significant difference (LSD) at the 1% and 5% level of probability (Snedecor and Cochran, 1980). ANOVA was used to analyse the variation caused by breed effect for all other data collected. Allele frequencies were estimated by direct count. Simple correlation coefficients were generated between the different characteristics measured.

CHAPTER 4

RESULTS

4.1 Animal characteristics

The sixty beef carcasses studied were representative of three cattle genotypes and were phenotypically classified according to the three breed groups (*Bos indicus, Sanga* and *Bos taurus*) namely:

- Brahman-crosses (*Bos indicus*) (n = 20; Bh)
- Nguni-crosses (*Sanga*) (n = 20; Ng)
- Simmentaler-crosses (*Bos taurus*) (n = 20; Sm)

The characteristics of the experimental animals are summarised in Table 4.1. The small-framed Nguni-crosses had a significantly ($p < 0.001$) lower live weight than the Brahman-crosses and Simmentaler-crosses. The Simmentaler-crosses and the Brahman-crosses had similar average carcass weight (hot and cold) that was significantly ($p < 0.001$) higher than the average carcass weight of the Nguni-crosses. The average percentage carcass weight loss from warm to cold differed significantly ($p < 0.002$) between the Nguni-crosses and the Brahman-crosses, with the Brahman-crosses being significantly lower than the Nguni-crosses (Table 4.1). Drip loss percentage differed significantly ($p < 0.003$) between the three genotypes. The Brahman-crosses had a significantly ($p < 0.003$) higher drip loss of 1.725%, followed by the Simmentaler-crosses (1.465%) and the Nguni-crosses with a drip loss percentage of 1.178. The Brahman-crosses differed significantly from the Nguni-crosses in terms of drip loss percentage. Water binding capacity (WBC) did not differ significantly ($p > 0.05$) between the crossbreds (Table 4.1).

The pH and temperature measurements at 1 hour, 3, 6, 8 and 24 hours *post slaughter* are reported in Table 4.1. The pH changed over time showed a normal profile to non-electrically stimulated carcasses and did not differ significantly ($p > 0.05$) between the crossbreds. Carcass temperature and rate of temperature decline rate were significantly similar between the Brahman-crosses and the Simmentaler-crosses for 3 hours and up to 8 hours *post mortem* (Table 4.1). Carcasses from Nguni-crosses cooled down quicker (smaller than the Brahman- and Simmentaler-crosses) (Figure 4.1), thus the lower carcass temperatures at 24 hours *post mortem* (Table 4.1).

Table 4.1: Least square means and standard errors of means (SEM) describing the carcass mass and percentage carcass weight loss characteristics, water binding capacity (WBC), the effect of genotype and *post mortem* metabolism on the temperature and pH decline of the *M. longissimus dorsi* (L1-L6) (non-electrically stimulated carcasses) in three crossbreds evaluated.

		Genotype			
Characteristic	**SEM**	**Brahman-X**	**Simmentaler-X**	**Nguni-X**	**p-Value**
Live animal weight at slaughter (kg)	5.10	485.2[b]	483.8[b]	366.2[a]	< 0.001
Hot carcass weight (kg)	3.20	282.4[b]	275.4[b]	206.9[a]	< 0.001
Cold carcass weight (kg)					
24 hours *post mortem*	3.13	277.3[b]	270.0[b]	202.6[a]	< 0.001
% Carcass loss	0.05	1.796[a]	1.935[ab]	2.067[b]	< 0.002
% Drip loss	0.11	1.725[b]	1.465[ab]	1.178[a]	< 0.003
WBC	0.01	0.448	0.448	0.463	0.329
Muscle pH					
1 hour *post mortem*	0.04	6.565	6.635	6.606	0.530
3 hours *post mortem*	0.05	6.209	6.317	6.288	0.283
6 hours *post mortem*	0.04	5.957	6.043	6.040	0.225
8 hours *post mortem*	0.04	5.780	5.879	5.897	0.106
24 hours *post mortem*	0.04	5.704	5.721	5.812	0.071
Muscle temperature (°C)					
1 hour *post mortem*	0.25	38.30	38.70	38.63	0.491
3 hours *post mortem*	0.50	28.32[b]	28.84[b]	25.88[a]	< 0.001
6 hours *post mortem*	0.49	18.34[b]	18.30[b]	15.93[a]	< 0.001
8 hours *post mortem*	0.53	15.27[b]	14.84[b]	12.92[a]	< 0.006
24 hours *post mortem*	0.40	4.04	3.90	3.73	0.858

[abc] – Means in row with different superscripts differ significantly based on the Fishers' means separation test;

Brahman-X: Brahman-crosses; Simmentaler-X: Simmentaler-crosses; Nguni-X: Nguni-crosses.

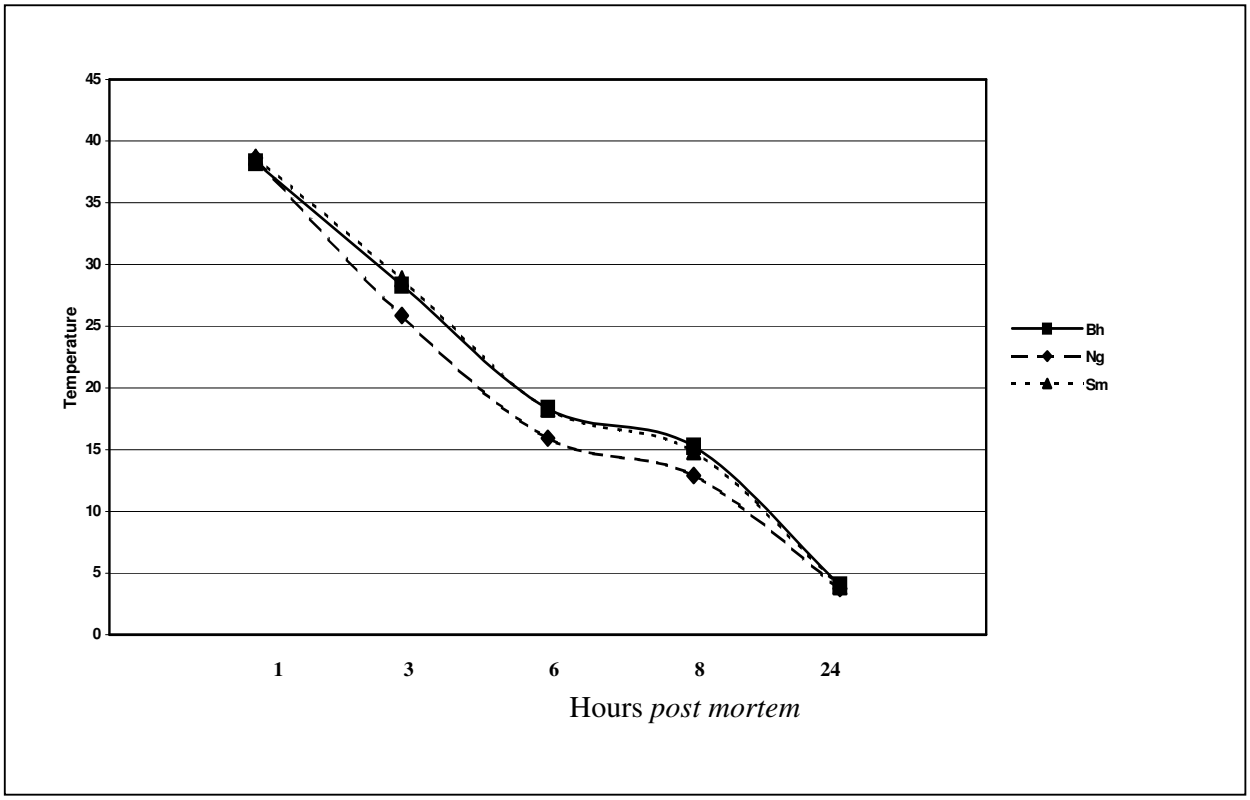

Figure 4.1: Temperature (°C) decline of the *M. longissimus dorsi* (L1-L6) (non-electrically stimulated carcasses) in the three crossbreds evaluated.

4.2 Tenderness of the loin samples

Accurate assessment of beef tenderness necessitates careful consideration of the methodologies employed (Davis *et al*., 1980). Least square means and standard errors for shear force measurements are presented in Table 4.2.

Significant differences were found ($p < 0.003$) in the overall Warner-Bratzler shear force (WBSF) values of the three different crossbreds for an ageing effect. From the results presented, the Brahman-crosses (*Bos indicus*) were found to have overall significant lower shear force values (more tender) and the Simmentaler-crosses (European *Bos taurus*) with the highest shear force values (less tender) with the Nguni-crosses (*Sanga*) in an intermediate position under these specific experimental conditions. The shear force measurements for 1 day, 7 and 14 days *post mortem* did not differ significantly ($p > 0.05$) between the crossbreds (Table 4.2).

Table 4.2: Least square means and standard errors of means for shear force measurements and the effect of ageing in the *M. longissimus dorsi* (L1-L6) (non-electrically stimulated carcasses) in the three crossbreds evaluated.

		Genotype			
Characteristic	**SEM**	**Brahman-X**	**Simmentaler-X**	**Nguni-X**	**p-Value**
Shear force (kg/12.5mm θ)					
Ageing effect average	0.21	6.33[a]	7.30[b]	6.53[a]	< 0.003
1 day *post mortem*	0.395	8.30	9.52	8.52	0.075
7 days *post mortem*	0.309	5.53	6.29	5.83	0.225
14 days *post mortem*	0.342	5.14	6.22	5.33	0.068

[abc] – Means in row with different superscripts differ significantly ($p < 0.003$) based on the Fishers' means separation test

The average pH measurements 3, 6 and 8 hours *post mortem* correlate positively with WBSF 1 day *post mortem* ($r = 0.4$, $r = 0.37$, $r = 0.35$, respectively; $p = 0.05$), and at 6 hours correlates positively with 14 days *post mortem* ($r = 0.3$, $p = 0.05$). The temperature measurements, hot carcass weight, cold carcass weight, percentage carcass loss and WBC did not correlate with WBSF 1 day, 7 and 14 days *post mortem*. The percentage drip loss correlated negatively ($r = -0.31$, $p = 0.05$) with WBSF 1 day *post mortem*. Meaning that when the percentage drip loss is higher the degree of tenderness will be less.

4.3 Genetic considerations and the expression of the calpain system

4.3.1 *CAPN1* effect

The genotypes observed in this study for the three crossbreds are presented in Table 4.3 for the *CAPN1* gene at positions 316 and 4751.

The relatively small number of animals of each crossbred ($n = 20$) used limits the ability to estimate allele frequency by breed cross; however, the data from Table 4.3 suggest that the C-allele of marker 316 and 4751 had the highest frequency in the Nguni-crosses (14.3%), and the T-allele of marker 4751 had the lowest frequency (33.3%) in this crossbreed. For the marker 316 the Nguni-crosses had a lower frequency for the G-allele compared to the Brahman- and Simmentaler-crosses. In the Nguni-crosses, the C-allele was actually the major allele, whereas the C-allele frequencies for the Brahman- and Simmentaler-crosses were 0%. At marker 4751 the Nguni-crosses C-allele frequency was 66.7%, whereas the other two crossbreds ranged from 7.1 - 7.7%.

The two-marker genotype data were used to determine two-marker haplotypes. Animals that were homozygous for at least one of the markers is GG and CT, then it must carry one G/C haplotype and one G/T haplotype.

Table 4.3: Allele and haplotype frequencies of the three crossbreds evaluated at the *CAPN1* gene.

Allele / Haplotype	Number of animals [1]	Brahman-X [2]	Simmentaler-X [2]	Nguni-X [2]
316 marker allele				
S (CG)	11	1	2	8
C	3 (6.4%)	0 (0%)	0 (0%)	3 (14.3%)
G	44 (93.6%)	19 (100%)	18 (100%)	18 (85.7%)
4751 marker allele				
Y (CT)	20	5	7	8
C	10 (25.6%)	1 (7.1%)	1 (7.7%)	8 (66.7%)
T	29 (74.4%)	13 (92.9%)	12 (92.3%)	4 (33.3%)
Haplotype (316/4751)				
C/C - desirable	1 (3.1%)	0 (0%)	0 (0%)	1 (16.7%)
C/T - intermediate	1 (3.1%)	0 (0%)	0 (0%)	1 (16.7%)
G/C - intermediate	5 (15.6%)	1 (7.1%)	1 (8.3%)	3 (50.0%)
G/T - undesirable	25 (78.1%)	13 (92.9%)	11 (91.7%)	1 (16.7%)

[1] Total number of animals studied and the frequency for an allele described as a percentage

[2] Total numbers of animals per crossbreed and the frequency for an allele describe as a percentage

The results in Table 4.3 show that the higher incidence of a C-allele at marker 316 and 4751 in the Nguni-crosses is reflected in an increased percentage of animals with the C/T and C/G haplotypes. The Nguni-crosses was the only crossbreed with and C/C haplotype (desirable) and C/T haplotype (intermediate, rarest). The Brahman- and Simmentaler-crosses had 0% occurrence for these two haplotypes (Table 4.3). The Nguni-crosses had more than six and seven times higher percentages of G/C haplotypes (intermediate) compared to the Simmentaler- and Brahman-crosses. For the G/T haplotype (undesirable) the Nguni-crosses had the lower frequency (16.7%), whereas the Simmentaler- and Brahman-crosses ranged between (91.7 - 92.9%). Five animals were double heterozygotes (i.e. CG and CT), and could not be definitely assigned haplotypes because they could result either from a combination of C/C and C/T haplotypes or G/C and G/T haplotypes. However, the haplotypes could be estimated based on observed frequency assigned to haplotypes among each group of animals. For example one Brahman-cross animal was identified as being double heterozygote. The haplotype that could possibly be assigned is G/T because the Brahman-crosses have the highest frequency (92.9%) for the G/T haplotype compared to the other possible haplotype combinations (Table 4.3). From the results presented in Table 4.3, it can be concluded that the G/C haplotype is more common in the Nguni-crosses, while the G/T haplotype is more common in the Brahman- and Simmentaler-crosses.

Table 4.4 shows the number of animals with the genotypes for the single nucleotide polymorphisms (SNP) used in the *CAPN1* marker at positions 361 and 4751 for the three crossbreds evaluated.

Table 4.4: Number of individuals inheriting the CC, CG and GG genotypes at position 316 (*CAPN1*316) and CC, CT and TT genotypes at position 4751 (*CAPN1*-4751).

	***CAPN1*-4751**											
	Brahman-X				**Simmentaler-X**				**Nguni-X**			
	CC	CT	TT	Total	CC	CT	TT	Total	CC	CT	TT	Total
***CAPN1*-316**												
CC	0	0	0	0	0	0	0	0	1	1	1	3
CG	0	1	0	1	0	1	1	2	3	3	2	8
GG	1	4	14	19	1	6	11	18	3	4	1	8
Total	1	5	14	20	1	7	12	20	7	8	4	19

The results from Table 4.4 indicate that for *CAPN1* there were 59 animals with both 316 and 4751 markers called. Table 4.4 indicates that the Nguni-crosses had one "no call" for the markers. A "no call" indicates that the assay did not produce a reliable genotype. Genotypes for the CC class at *CAPN1* were 0% for the Brahman- and Simmentaler-crosses. The CC genotypes at both markers (316 and 4751) represent animals that are more tender than any other animal or group compared to inheriting the GG and TT genotype (undesirable or tough) at both markers. When considering all the results presented (Table 4.3 and Table 4.4), it can be concluded that the Nguni-crosses have the higher frequency of more preferred alleles for tender meat compared to the other breeds. The Brahman-crosses had the most undesirable alleles followed by the Simmentaler-crosses.

The results from Table 4.3 and Table 4.4 can also be explained via indexes. Indexes for both markers were calculated with a total value of three for each marker, meaning that the higher the index value the tougher the meat compared to a lower value indicating tenderness. For both markers (316 and 4751) at the *CAPN1* gene the total value of the index adds up to six. Table 4.5 represents the average indexes for the three crossbreds evaluated in this study.

Table 4.5: The average indexes for the markers (316 and 4751) at the *CAPN1* gene for the three crossbreds evaluated.

Marker	Brahman-X	Simmentaler-X	Nguni-X
CAPN1-316	2.95	2.90	2.26
CAPN1-4751	2.63	2.55	1.80
CAPN1 (316 + 4751)	5.58	5.45	4.11

The results from Table 4.5 confirm that the Nguni-crosses had the higher frequency of more preferred alleles for tender meat compared to the other crossbreds. The Brahman- and Simmentaler-crosses have the tendency for tougher meat and differ with a range of 0.05 - 0.13.

4.3.2 *CAST* effect

The genotypes observed in the three crossbreds are presented in Table 4.6 for the *CAST* gene.

Table 4.6: Allele and haplotype frequencies of the three crossbreds evaluated at the *CAST* gene.

Allele / Haplotype	Number of animals [1]	Brahman-X [2]	Simmentaler-X	Nguni-X
***CAST* marker allele**				
Y (CT)	17	8	4	5
C	0 (0%)	0 (0%)	0 (0%)	0 (0%)
T	41 (100%)	11 (100%)	16 (100%)	14 (100%)
***CAST-Brahman* allele**				
W (AT)	0	0	0	0
A	3 (25.0%)	1 (33.3%)	2 (66.7%)	0 (0%)
T	9 (75.0%)	2 (66.7%)	1 (33.3%)	6 (100%)
Haplotype (*CAST*/*CAST-Bh*)				
C/A - desirable	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Y/A – intermediate, desirable	15 (30.6%)	7 (41.2%)	4 (22.2%)	4 (30.8%)
T/A – intermediate, undesirable	32 (65.3%)	10 (58.8%)	13 (72.2%)	9 (69.2%)
T/W – undesirable	2 (4.1%)	0 (0%)	2 (11.1%)	0 (0%)

[1] Total number of animals studied indicating the frequency for an allele described as a percentage

[2] Total numbers of animals per crossbred indicating the frequency for an allele describe as a percentage

The results from Table 4.6 suggest that the T-allele of *CAST* markers have a higher frequency in the Nguni-crosses, and the C-allele of *CAST* marker and the A-allele of *CAST-Brahman* marker have a lower frequency (0%) compared to the other crossbreds. At the *CAST* marker the Brahman-, Nguni- and Simmentaler-crosses had no occurrence (0%) of the C-allele. The Simmentaler-crosses (16 animals) indicated a higher frequency of the major allele at the *CAST* marker followed by the Nguni- (14 animals) and Brahman-crosses (11 animals). However, at the *CAST-Brahman* marker the Nguni-crosses has the higher frequency for the desirable allele followed by the Brahman- and Simmentaler-crosses (Table 4.6). For the A-allele at the *CAST-Brahman* marker, the Simmentaler-crosses has the highest frequency of occurrence (66.7%) compared to the other breeds with a range of (0 - 33.3%). This suggests strongly that some Simmentaler-cross animals could be crossed with the *Bos indicus* breed.

As stated previous, haplotypes of the two markers in relation with tenderness are C/A and Y/A, associated with increased shear force relative to the alternative haplotypes (T/A and T/W). The results from Table 4.6 indicate that the Brahman-crosses had the highest frequency for haplotypes that are associated with increased shear force, tougher meat compared to the Simmentaler-crosses that had the highest frequency for

haplotypes that are associated with lower shear force, tender meat. The Nguni-crosses were intermediate; the reason for this could be due to the fact that the *CAST* markers are more prominent in *Bos indicus*, than in *Bos taurus* or animals from a crossbred (*Bos Taurus* / *Bos indicus*) descent. Table 4.7 represents the average indexes for the three crossbreds evaluated in this study for the *CAST* gene.

Table 4.7: The average indexes for the markers (*CAST* and *CAST-Brahman*) at the *CAST* gene in the three crossbreds evaluated.

Marker	Brahman-X	Simmentaler-X	Nguni-X
CAST	1.42	1.20	1.26
CAST-Brahman	1.06	1.00	1.11
CAST+CAST-Brahman	2.50	2.32	2.31

According the indexes for the *CAST* marker, the Simmentaler-crosses should have more tender meat compared to the Nguni- and Brahman-crosses. The *CAST-Brahman* marker on the other hand indicated that the Nguni-crosses should have the toughest meat (Table 4.7). However, according to the total index for the *CAST* gene, the Nguni-crosses had more favourable genotypes compared to the Simmentaler- and Brahman-crosses, and this corresponds with the data for the *CAPN1* gene.

4.3.3 Interactions (*CAPN1* and *CAST*)

Table 4.8 shows the number of animals with genotypes for the SNP used in the *CAST* and *CAPN1* markers.

Table 4.8: Shows the number of animals with genotypes for the SNP used in the *CAST* and *CAPN1* markers.

	CAPN1											
	Brahman-X				**Simmentaler-X**				**Nguni-X**			
CAST	CC	CT	TT	Total	CC	CT	TT	Total	CC	CT	TT	Total
CC	0	0	1	1	0	0	1	1	0	1	6	7
CT	0	1	4	5	0	3	4	7	0	2	6	8
TT	0	6	6	12	0	1	11	12	0	1	3	4
Total	0	7	11	18	0	4	16	20	0	4	15	19

Genotypes for the CC class at the *CAPN1* marker had no frequency across the crossbreds. Thus, comparisons are only made between CT and TT genotypes. The low frequency of animals inheriting the CC genotype at the *CAST* marker generated a low frequency of allelic combinations with *CAPN1*. For example,

only eight animals inherited the CC *CAST* and the TT *CAPN1* genotype in the three crossbreds. According to Casas *et al*. (2006) animals inheriting the CC genotype at both markers were more tender. Animals inheriting the CC genotype for the *CAST* produced tougher meat when they inherited either the CT or the TT genotypes in *CAPN1*.

Table 4.9 indicates the average index for the interactions between the *CAST*- and *CAPN1* genes. The total index adds up to 10. This means that animals with a total index value of 10 have tough meat compared to animals with a lower index value (tender meat).

Table 4.9: The average indexes for the *CAST* (*CAST* + *CAST-Brahman*) and *CAPN1* (316 and 4751) gene in the three crossbreds evaluated.

Marker	Brahman-X	Simmentaler-X	Nguni-X
CAST + *CAPN1*	8.11	7.74	6.62

Considering all the marker data used (interaction between the genes), it can be concluded that overall the Nguni-crosses had the highest potential to inherit tender meat based on the total index value of 6.62 (Table 4.9). The results from Table 4.9 suggest that the Brahman-crosses (8.11), inherited the alleles for tougher meat and the Simmentaler-crosses (7.74) were intermediate. The animals in this study generally, had a tendency for tougher meat, because the total index values for the three crossbreds evaluated were more than the intermediate index value of five.

4.3.4 Minor Allele Frequencies

The minor allele frequency is the ratio of chromosomes in the population, carrying the less common variant to those with the more common variant. Table 4.10 shows the minor allele frequencies and the total number of animals with calls.

Table 4.10: Minor allele frequency within the three different crossbreds and the total number of animals with calls.

Marker	*CAPN1*-316	*CAPN1*-4751	*CAST*	*CAST-Brahman*
Minor allele frequency	C: 0.14	C: 0.34	C: 0.15	T: 0.03
Animals with calls	59	59	57	51

The minor allele frequency of marker 316 (C-allele), marker 4751 (C-allele), *CAST* marker (C-allele) and *CAST-Brahman* marker (T-allele) were 14%, 34%, 15% and 3% respectively. The frequency evaluated for

CAPN1-316 was in the same range as that found in the study of Page *et al.* (2004), which reported a minor allele frequency of 17% and 20% for two different populations.

4.4 Association of SNP markers with shear force values

4.4.1 *CAPN1* association with shear force

Table 4.11 shows the genotype contrast for WBSF values with the *CAPN1*-316 marker. The values overall are not significant ($p > 0.05$).

Table 4.11: Genotype contrast for shear force at 1 day, 7 and 14 days *post mortem* with *CAPN1*-316 marker in the animals evaluated.

Genotype	WBSF, Kg[a]	No.	p-Value
1 day *post mortem*			p = 0.368
CC	8.55 ± 7.40	3	
CG	8.13 ± 3.91	11	
GG	9.00 ± 3.14	45	
7 days *post mortem*			p = 0.505
CC	5.80 ± 0.72	3	
CG	5.35 ± 4.15	11	
GG	5.91 ± 1.60	45	
14 days *post mortem*			p = 0.197
CC	6.03 ± 1.82	3	
CG	4.79 ± 0.83	11	
GG	5.76 ± 3.10	45	

[a] Mean and variation in Warner-Bratzler shear force (WBSF)

At 1 day, 7 and 14 days *post mortem* the CC genotype was associated with higher shear force values than the CG genotypes and at 14 days *post mortem* it was also associated with higher shear force values than the GG genotypes that were unexpected. Previous results demonstrate that the C allele is associated with lower shear force values (increased tenderness) (Page *et al*., 2004). Thus, animals inheriting the CC and CG genotypes produce more tender meat when compared to animals inheriting the GG genotype (Casas *et al*., 2006).

Analysis of the *CAPN1*-316 marker with WBSF values in the crossbreds is presented in Table 4.12. The values in Table 4.12 are not significantly ($p > 0.05$) associated with WBSF. The animals homozygous for the C-allele at marker 316 at 1 day, 7 and 14 days *post mortem* for the Brahman- and Simmentaler-crosses were absent. However, the Nguni-crosses indicated that the CC genotype was present and that three animals had the combination for tender meat. The WBSF values for the CC genotype were still tougher than

expected, because the values were above 5 kg. From Table 4.11 it can be concluded that after ageing the WBSF values were less, meaning more tender meat with days *post mortem*. The reason for this is that at 1 day *post mortem* all the processes for tenderisation still need to be put into motion. The CG genotype unexpectedly had lower WBSF values than the CC genotype despite the fact that White *et al.* (2005) indicated that "C" is the more favourable allele.

Table 4.12: Genotype contrast for shear force at 1 day, 7 and 14 days *post mortem* with *CAPN1*-316 marker in the three crossbreds evaluated.

Genotype	Brahman-X		Simmentaler-X		Nguni-X	
	WBSF, Kg [a]	No.	WBSF, Kg [a]	No.	WBSF, Kg [a]	No.
1 day *post mortem*						
CC	-	-	-	-	9.01 ± 3.77	3
CG	6.88	1	6.85 ± 0.02	2	8.58 ± 5.40	7
GG	8.37 ± 2.02	19	9.82 ± 2.45	18	8.77 ± 4.06	9
7 days *post mortem*						
CC	-	-	-	-	5.75 ± 0.45	3
CG	4.08	1	6.02 ± 1.97	2	5.85 ± 5.77	7
GG	6.00 ± 3.23	19	6.55 ± 1.60	18	5.86 ± 1.89	9
14 days *post mortem*						
CC	-	-	-	-	5.63 ± 1.71	3
CG	4.18	1	5.16 ± 1.19	2	4.54 ± 0.78	7
GG	4.80 ± 1.68	19	6.10 ± 1.90	18	5.67 ± 1.59	9

[a] Mean and variation in Warner-Bratzler shear force (WBSF)

Only one Brahman-cross had the CG genotype. Table 4.12 indicates that the Brahman-crosses were tougher for this genotype compared to the Simmentaler-crosses and the Nguni-crosses with the lowest WBSF values for the CG genotype.

In Table 4.13 the genotype contrast for WBSF values with the *CAPN1*-4751 marker are presented. Overall, the differences in WBSF were not significant ($p > 0.05$). It is reported that the C-allele is associated with lower shear force (increase tenderness). Thus, animals inheriting the CC and CT genotypes produce more tender meat when compared to animals inheriting the TT genotype (Casas *et al*., 2006). The results of Table 4.13 correspond with the report of Casas *et al.* (2006), that the CC and CT genotypes are associated with lower WBSF values thus increase tenderness. In this study it is indicated that the CC genotype is associated with the tenderest meat (lowest shear force values), where the TT genotype is associated with high shear force values (tougher meat).

Table 4.13: Genotype contrast for shear force at 1 day, 7 and 14 days *post mortem* with *CAPN1*-4751 marker in the animals evaluated.

Genotype	WBSF, Kg[a]	No.	p-Value
1 day *post mortem*			p = 0.539
CC	8.35 ± 6.24	10	
CT	8.68 ± 4.12	20	
TT	9.07 ± 2.17	29	
7 days *post mortem*			p = 0.986
CC	5.75 ± 4.76	10	
CT	5.81 ± 2.01	20	
TT	5.84 ± 1.24	29	
14 days *post mortem*			p = 0.391
CC	5.01 ± 1.58	10	
CT	5.56 ± 1.49	20	
TT	5.84 ± 3.83	29	

[a] Mean and variation in Warner-Bratzler shear force (WBSF)

Table 4.14 indicates the association of the *CAPN1*-4751 marker with WBSF. The values in Table 4.14 were not significantly ($p > 0.05$) associated with the WBSF values. As with marker *CAPN1*-316 the Brahman-crosses again had the lower WBSF values associated with the *CAPN1*-4751 marker, indicating the tendency for more tender meat compared to the Simmentaler- and Nguni-crosses at 1 day *post mortem*. At 1 day and 14 days *post mortem* the CC genotype was associated with less tender meat (higher WBSF values) which was unexpected. However, at 7 days *post mortem* the CC genotype presented more favourable WBSF values as expected with the CT genotype in an intermediate position with the TT genotype presenting higher WBSF values thus less tender meat.

The haplotype contrast of *CAPN1*-316 and *CAPN1*-4751 are presented in Table 4.15. According to Table 4.15 the Brahman-crosses for the GG-CT haplotype was associated with lower shear force values at 1 day, 7 and 14 days *post mortem* compared to the Nguni- and Simmentaler-crosses.

Table 4.14: Genotype contrast for shear force at 1 day, 7 and 14 days *post mortem* with *CAPN1*-4751 marker in the three crossbreds evaluated.

Genotype	Brahman-X		Simmentaler-X		Nguni-X	
	WBSF, Kg [a]	No.	WBSF, Kg [a]	No.	WBSF, Kg [a]	No.
1 day *post mortem*						
CC	7.54	1	9.17	1	8.45 ± 6.86	8
CT	7.33 ± 0.09	5	10.03 ± 3.99	7	8.52 ± 3.55	8
TT	8.85 ± 2.22	13	9.25 ± 2.80	12	9.22 ± 0.80	4
7 days *post mortem*						
CC	4.38	1	5.38	1	6.13 ± 5.58	8
CT	4.86 ± 0.74	5	6.76 ± 1.60	7	5.68 ± 1.26	8
TT	6.47 ± 3.86	13	6.44 ± 1.66	12	5.66 ± 0.65	4
14 days *post mortem*						
CC	4.78	1	4.99	1	5.01 ± 2.16	8
CT	4.29 ± 0.57	5	6.34 ± 1.27	7	5.37 ± 1.39	8
TT	5.06 ± 2.02	13	5.91 ± 2.32	12	5.18 ± 0.76	4

[a] Mean and variation in Warner-Bratzler shear force (WBSF)

Table 4.15: Haplotype contrast of *CAPN1* marker (316 and 4751) for shear force at 1 day, 7 and 14 days *post mortem*.

Haplotype	Brahman-X		Simmentaler-X		Nguni-X	
	WBSF, Kg [a]	No.	WBSF, Kg [a]	No.	WBSF, Kg [a]	No.
1 day *post mortem*						
GG-CT (G/Y)	7.44 ± 0.03	4	10.03 ± 3.99	7	8.85 ± 2.69	5
7 days *post mortem*						
GG-CT (G/Y)	5.05 ± 0.73	4	6.76 ± 1.60	7	5.81 ± 1.75	5
14 days *post mortem*						
GG-CT (G/Y)	4.32 ± 0.76	4	6.34 ± 1.27	7	5.75 ± 1.79	5

[a] Mean and variation in Warner-Bratzler shear force (WBSF)

4.4.2 *CAST* association with shear force

In Table 4.16 the *CAST* marker genotypes were associated with WBSF. As in Table 4.11 and Table 4.13 the values in Table 4.16 were not significant ($p > 0.05$). A report by Casas *et al*. (2006) indicated that animals inheriting the CC and the CT genotypes (*CAST*) produce tougher meat when compared with animals that inherited the TT genotype. In this study the CC genotype was absent however the CT genotype at 7 days

post mortem corresponds with the results of Casas *et al.* (2006). At 1 day and 14 days *post mortem* the CT genotype were associated with lower shear force values (increased tenderness) compared to the TT genotype with higher shear force values (decreased tenderness) which was unexpected.

Table 4.16: Genotype contrast for shear force at 1 day, 7 and 14 days *post mortem* with *CAST* marker in the animals evaluated.

Genotype	WBSF, Kg[a]	No.	p-Value
1 day *post mortem*			p = 0.963
CC	-	0	
CT	8.83 ± 4.09	17	
TT	8.86 ± 3.23	41	
7 days *post mortem*			p = 0.598
CC	-	0	
CT	5.96 ± 2.53	17	
TT	5.75 ± 1.87	41	
14 days *post mortem*			p = 0.919
CC	-	0	
CT	5.59 ± 2.63	20	
TT	5.64 ± 2.76	29	

[a] Mean and variation in Warner-Bratzler shear force (WBSF)

The values in Table 4.17 were not significantly ($p > 0.05$) associated with the WBSF values. Table 4.17 indicated that no CC genotypes were present for the *CAST* marker. For the CT genotype at 1 day and 7 days *post mortem* the Nguni-crosses had the lowest WBSF value associated with the genotype combination compared to the Brahman- and Simmentaler-crosses. However, at 14 days *post mortem* the Brahman-crosses had the lowest WBSF association while the Simmentaler-crosses had the highest WBSF association. For the TT genotype the Brahman-crosses overall had the lowest WBSF values associated with the genotype for the *CAST* marker.

Table 4.17: Genotype contrast for shear force at 1 day, 7 and 14 days *post mortem* with *CAST* marker in the crossbreds evaluated.

Genotype	Brahman-X		Simmentaler-X		Nguni-X	
	WBSF, Kg [a]	No.	WBSF, Kg [a]	No.	WBSF, Kg [a]	No.
1 day *post mortem*						
CT	8.42 ± 1.55	8	10.63 ± 6.32	4	8.05 ± 4.59	5
TT	8.35 ± 2.52	11	9.24 ± 2.16	16	9.00 ± 4.27	13
7 days *post mortem*						
CT	6.38 ± 2.35	8	6.56 ± 2.67	4	5.31 ± 3.28	5
TT	5.62 ± 4.23	11	6.48 ± 1.44	16	6.09 ± 2.92	13
14 days *post mortem*						
CT	5.16 ± 1.74	8	6.26 ± 3.14	4	5.27 ± 3.24	5
TT	4.62 ± 1.51	11	5.95 ± 1.69	16	5.28 ± 1.10	13

[a] Mean and variation in Warner-Bratzler shear force (WBSF)

Table 4.18: Genotype contrast for shear force at 1 day, 7 and 14 days *post mortem* with *CAST-Brahman* marker in the three crossbreds.

Genotype	Brahman-X		Simmentaler-X		Nguni-X	
	WBSF, Kg [a]	No.	WBSF, Kg [a]	No.	WBSF, Kg [a]	No.
1 day *post mortem*						
AA	8.21 ± 1.90	18	9.46 ± 3.43	17	8.91 ± 3.96	13
AT	10.65	1	9.44 ± 1.23	2	-	-
7 days *post mortem*						
AA	5.73 ± 3.04	18	6.48 ± 1.81	17	6.10 ± 3.63	13
AT	8.97	1	6.70 ± 0.61	2	-	-
14 days *post mortem*						
AA	4.58 ± 1.20	18	6.07 ± 1.97	17	5.25 ± 1.50	13
AT	7.81	1	5.37 ± 2.48	2	-	-

[a] Mean and variation in Warner Bratzler shear force (WBSF)

The association between the *CAST-Brahman* marker and WBSF are presented in Table 4.18. The results in Table 4.18 did not exhibit significant ($p > 0.05$) values. Only the AA genotype at the *CAST-Brahman* marker can be used for discussion since the other two genotypes (AT and TT) are too few or absent for comparison between the crossbreds. In this study the AA genotype was associated with lower WBSF values at 14 days *post mortem*. At 7 and 14 days *post mortem* the Brahman-crosses had the lowest WBSF values thus more tender meat compared to the Nguni- and Simmentaler-crosses.

Table 4.19 presents haplotype contrasts for the *CAST* and *CAST-Brahman* markers. According to Barendse (2002) the haplotypes of the two markers in relation with tenderness are C/A and Y/A, associated with increased shear force relative to the alternative haplotypes (T/A and T/W). The results in Table 4.19 corresponds with Barendse (2002) that the C/A and Y/A are associated with increased shear force relative to the alternative haplotypes (T/A and T/W), except at 1 day *post mortem* for the Brahman- and Nguni-crosses. This may be due to the fact that the mechanism of tenderisation still needs to be completed. Table 4.19 indicates that the Brahman-crosses for Y/A and T/A were associated with lower shear force values compared to the other crosses. From this results it can be explained why the Brahman-cross animals from this study performed better than the Nguni-crosses that were unexpected.

Table 4.19: Haplotype contrast of *CAST* marker and *CAST-Brahman* marker for shear force at 1 day, 7 and 14 days *post mortem*.

Haplotype	Brahman-X		Simmentaler-X		Nguni-X	
	WBSF, Kg [a]	No.	WBSF, Kg [a]	No.	WBSF, Kg [a]	No.
1 day *post mortem*						
CT-AA (Y/A)	8.10 ± 0.86	7	10.63 ± 6.32	4	8.33 ± 5.63	4
TT-AA (T/A)	8.43 ± 2.72	10	9.10 ± 2.39	13	9.49 ± 3.05	8
7 days *post mortem*						
CT-AA (Y/A)	6.00 ± 1.45	7	6.56 ± 2.67	4	5.39 ± 4.34	4
TT-AA (T/A)	5.58 ± 4.67	10	6.46 ± 1.74	13	6.45 ± 3.92	8
14 days *post mortem*						
CT-AA (Y/A)	4.78 ± 0.69	7	6.26 ± 3.14	4	5.62 ± 3.53	4
TT-AA (T/A)	4.55 ± 1.63	10	6.01 ± 1.83	13	5.22 ± 0.78	8

[a] Mean and variation in Warner-Bratzler shear force (WBSF)

4.5 The expressed calpain system

Least square means and standard errors of means describing the calpain systym activities *post mortem* of the crossbreds are presented in Table 4.20.

The results indicate that the Simmentaler-crosses had the tendency for the highest calpastatin levels at 1 hour and 24 hours *post mortem*, the Brahman-crosses with intermediate levels and the Nguni-crosses with the lowest calpastatin levels (more tender).

On average, between 1 hour and 24 hours *post mortem* (Table 4.20) the Brahman-crosses had the lowest significant μ-calpain activity, which differed significantly from the other crossbreds evaluated. The Simmentaler-crosses and the Nguni-crosses were significantly similar with the Nguni-crosses being higher on average, favouring the proteolytic *post mortem* tenderising process. The m-calpain concentration was similar for all the crossbreds at 1 hour and 24 hours *post mortem*.

The calpastatin / μ-calpain ratio for the Brahman was significantly higher ($p < 0.001$) (Figure 4.2) than that for the other crossbreds. The Simmentaler- and Nguni-crosses were significantly similar (1 hour and 24 hours *post mortem*).

No significant ($p > 0.05$) correlations between the calpain system and sarcomere lengths (1 day and 3 days *post mortem*) were found in this study. The calpastatin activity (1 hour and 24 hours *post mortem*), calpastatin / μ-calpain ratio (24 hours *post mortem*) and calpastatin / μ+m-calpain ratio (1 hour and 24 hours *post mortem*) correlated with myofibrillar fragment lengths 7 days *post mortem*, r = 0.38, 0.42, 0.37, 0.38 and 0.44 respectively.

Table 4.20: Least square means and standard errors for μ-calpain, m-calpain and calpastatin activity levels measured in the *M. longissimus dorsi* (L1-L6) (non-electrically stimulated carcasses) in the three crossbreds evaluated at 1 hour and 24 hours *post mortem*.

		Genotype			
Characteristic	**SEM**	**Brahman-X**	**Simmentaler-X**	**Nguni-X**	**p-Value**
Calpastatin activity (U/g)					
1 hour *post mortem*	0.106	2.709	2.815	2.636	0.493
24 hours *post mortem*	0.109	2.655	2.675	2.582	0.821
μ-Calpain activity (U/g) [1]					
1 hour *post mortem*	0.128	1.681[a]	2.204[b]	2.219[b]	< 0.005
24 hours *post mortem*	0.104	1.481[a]	1.793[b]	1.908[b]	< 0.016
m-Calpain activity (U/g)					
1 hour *post mortem*	0.027	0.868	0.850	0.814	0.370
24 hours *post mortem*	0.032	1.092	1.077	1.115	0.695
Calpastatin / μ-Calpain ratio					
1 hour *post mortem*	0.083	1.715[b]	1.334[a]	1.276[a]	< 0.001
24 hours *post mortem*	0.106	2.255[b]	1.589[a]	1.417[a]	< 0.001
Calpastatin / μ-Calpain + m-Calpain ratio					
1 hour *post mortem*	0.042	1.081[b]	0.941[a]	0.893[a]	< 0.007
24 hours *post mortem*	0.040	1.057[b]	0.940[a]	0.863[a]	< 0.004

[abc] – Means in row with different superscripts differ significantly with the Fishers' means separation test

[1] One unit of calpastatin activity is defined as the amount that inhibited one unit of m-calpain activity

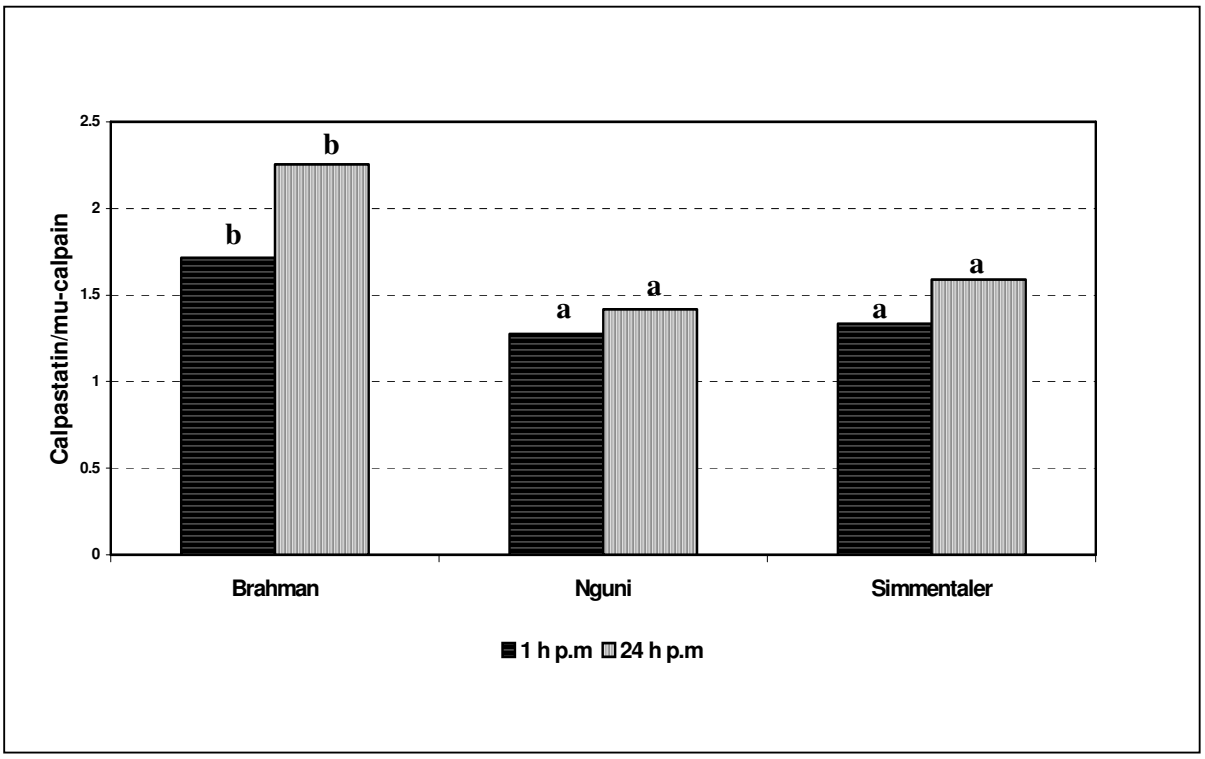

Figure 4.2: Calpastain / µ-calpain ratio of the *M. longissimus dorsi* (L1-L6) in the three crossbreds evaluated (means with different superscripts differ significantly, (p < 0.001). h = hours, p.m = *post mortem.*

4.6 Proteolytic degradation

Least square means and standard errors describing myofibrillar fragmentation lengths and proteolytic degradation profiles of the *M. longissimus dorsi* (L1-L6) (non-electrically stimulated carcasses) *post mortem* in the crossbreds are presented in Table 4.21.

Overall significant differences were found between the different crossbreds for myofibrillar fragment length ($p < 0.001$). The influence of ageing period on myofibril fragmentation was found to be different between days *post mortem.* The Brahman-crosses had the higher myofibril fragments (tougher) and the Nguni-crosses with the lowest myofibril fragments (more tender).

Significant differences ($p < 0.033$) between the crossbreds were found considering an ageing effect for titin degradation (Table 4.21). The Nguni-crosses indicated a lower degradation rate and were significantly similar to the Simmentaler-crosses, while the Brahman-crosses differed significantly from the other two crossbreds. Titin degradation 1 day, 7 and 14 days *post mortem* did not differ significantly ($p > 0.05$) between the crossbreds. On the other hand nebulin degradation showed no significant ageing effect, but at 7 days, 1-7 days and 1-14 days *post mortem* significant differences between the crossbreds were evaluated. At 7 days *post mortem* the Brahman-crosses were significantly different from the Simmentaler-

Table 4.21: Least square means and standard errors describing myofibrillar fragmentation lengths (MFL), proteolytic degradation profiles and an ageing effect in the *M. longissimus dorsi* (L1-L6) (non-electrically stimulated carcasses) *post mortem* of the three crossbreds.

		Genotype			
Characteristic	**SEM**	**Brahman-X**	**Simmentaler-X**	**Nguni-X**	**p-Value**
MFL (µm)					
Ageing effect average	1.102	48.46^{c}	31.89^{b}	25.43^{a}	< 0.001
1 day *post mortem*	1.427	43.28	42.30	41.61	0.710
7 days *post mortem*	1.259	33.35	31.72	30.07	0.193
14 days *post mortem*	0.703	25.50	24.94	24.82	0.767
Titin (~ 300 kDa)					
Ageing effect average	0.016	0.344^{ab}	0.308^{a}	0.365^{b}	< 0.033
1 day *post mortem*	0.034	0.447	0.383	0.466	0.203
7 days *post mortem*	0.025	0.327	0.312	0.368	0.264
14 days *post mortem*	0.021	0.259	0.231	0.264	0.504
1 – 7 days *post mortem*	0.017	0.120	0.071	0.098	0.138
1 – 14 days *post mortem*	0.020	0.187	0.149	0.202	0.150
Nebulin (~ 250 kDa)					
Ageing effect average	0.004	0.039	0.030	0.040	0.071
1 day *post mortem*	0.007	0.063	0.069	0.079	0.304
7 days *post mortem*	0.006	0.036^{b}	0.014^{a}	0.031^{ab}	< 0.038
14 days *post mortem*	0.004	0.019	0.005	0.009	0.078
1 – 7 days *post mortem*	0.007	0.028^{a}	0.055^{b}	0.047^{ab}	< 0.030
1 – 14 days *post mortem*	0.007	0.043^{a}	0.064^{b}	0.069^{b}	< 0.034
Desmin (~ 55 kDa)					
Ageing effect average	0.008	0.054^{b}	0.046^{a}	0.052^{b}	< 0.018
1 day *post mortem*	0.004	0.070	0.061	0.066	0.349
7 days *post mortem*	0.004	0.054	0.046	0.051	0.297
14 days *post mortem*	0.003	0.039	0.031	0.040	0.118
1 – 7 days *post mortem*	0.003	0.016	0.015	0.016	0.982
1 - 14 days *post mortem*	0.004	0.031	0.030	0.026	0.682
~ 32-29 kDa					
Ageing effect average	0.014	0.058	0.054	0.061	0.474
1 day *post mortem*	0.004	0.017	0.018	0.024	0.474
7 days *post mortem*	0.005	0.054	0.050	0.054	0.773
14 days *post mortem*	0.009	0.102	0.096	0.105	0.783
1 – 7 days *post mortem*	0.005	0.037	0.032	0.030	0.526
1 – 14 days *post mortem*	0.009	0.085	0.079	0.082	0.905

^{abc} – Means in row with different superscripts differ significantly with the Fishers' means separation test

crosses with a higher degradation rate and the Nguni-crosses with an intermediate rate. The degradation rate (1-7 days *post mortem*), the Brahman-crosses differed significantly from the Simmentaler-crosses.

The Simmentaler-crosses had the higher rate of nebulin degradation while the Brahman-crosses had the lowest rate. The Brahman-crosses were significantly different from the Simmentaler- and Nguni-crosses 1-14 days *post mortem* and had a lower degradation rate, while the Nguni-crosses had the lowest degradation rate.

Desmin degradation (Table 4.21) was significantly ($p < 0.018$) similar considering an ageing effect between the Brahman-crosses and the Nguni-crosses. The Simmentaler-crosses differed significantly from the other crossbreds with an intermediate degradation rate. Desmin and 30 kDa degradation 1 day, 7 and 14 days *post mortem* did not differ significantly ($p > 0.05$) between the crossbreds.

For the 30 kDa protein there was no significant values evaluated 1 day, 7 and 14 days *post mortem* as well as for the degradation rate (Table 4.21).

Least square means and standard errors of desmin degradation evaluated with Western-blotting are presented in Table 4.22.

Table 4.22: Least square means and standard errors of desmin degradation evaluated with Western-blotting and an ageing effect in the *M. longissimus dorsi* (L1-L6) (non-electrically stimulated carcasses) *post mortem* of the three crossbreds evaluated.

		Genotype			
Characteristic	**SEM**	**Brahman-X**	**Simmentaler-X**	**Nguni-X**	**p-Value**
Desmin (~55 kDa)					
Ageing effect average	0.164	8.31[ab]	7.90[a]	8.53[b]	< 0.024
1 day *post mortem*	0.305	9.33	8.96	9.26	0.657
7 days *post mortem*	0.270	8.31	7.97	8.48	0.404
14 days *post mortem*	0.284	7.30[ab]	6.79[a]	7.86[b]	< 0.030
1 - 7 days *post mortem*	0.184	1.03	0.99	0.78	0.600
1 – 14 days *post mortem*	0.250	2.03	2.17	1.40	0.074

[abc] – Means in row with different superscripts differ significantly with the Fishers' means separation test

The degradation of desmin evaluated by means of Western-blotting showed a significant ($p < 0.024$) ageing effect difference between the crossbreds and at 14 days *post mortem* ($p < 0.03$). The Simmentaler- and Nguni-crosses differed significantly, where the Brahman-crosses were significantly similar to the Simmentaler- and Nguni-crosses. The crossbreds did not differ significantly ($p > 0.05$) 1 day and 7 days *post mortem* and 1-14 days *post mortem*. The degradation of protein 1 day, 7 and 14 days *post mortem* was correlated with WBSF. Titin, nebulin and desmin did not correlate significantly ($p > 0.05$) with WBSF 1 day, 7 and 14 days *post mortem.* The 30 kDa proteins significantly correlated 1 day and 7 days *post mortem* ($r = -0.30$; $r = -0.59$ respectively) with WBSF 7 days *post mortem*. At 7 and 14 days *post mortem*

the 30 kDa proteins correlated with WBSF 14 days *post mortem* (r = -0.35; r = -0.31). The degradation of desmin determined with Western-blotting also correlated with WBSF (14 days *post mortem*) (r = -0.30).

A correlation-matrix showing how proteolytic degradation correlates with the calpain system are presented in Table 4.23.

Table 4.23: Correlation-matrix showing how proteolytic degradation correlates with the calpain system 24 hours *post mortem* of the *M. longissimus dorsi* (L1-L6) in the three crossbreds.

Protein	Calpastatin	µ-calpain	m-calpain	In/µ-calpain	In/µ+m-calpain
Titin (~ 300 kDa)					
1 day *post mortem*	0.08	0.18	-0.27	-0.07	-0.02
7 days *post mortem*	-0.08	0.25	-0.29	-0.19	-0.21
14 days *post mortem*	0.10	0.19	-0.24	-0.06	-0.03
Nebulin (~ 250 kDa)					
1 day *post mortem*	-0.01	-0.05	-0.16	0.03	0.07
7 days *post mortem*	0.03	-0.27	0.05	**0.35**	**0.30**
14 days *post mortem*	-0.04	-0.29	-0.02	**0.50**	0.27
Desmin (~55 kDa)					
1 day *post mortem*	-0.03	0.00	-0.12	0.16	0.05
7 days *post mortem*	0.01	-0.05	-0.23	0.11	0.14
14 days *post mortem*	-0.01	0.06	**-0.30**	0.01	0.04
Desmin – Western Blotting					
1 day *post mortem*	**0.44**	**0.42**	0.09	-0.20	0.00
7 days *post mortem*	**0.32**	**0.47**	-0.02	-0.26	-0.09
14 days *post mortem*	0.09	**0.39**	0.06	-0.28	-0.27
~ 32-29 kDa					
1 day *post mortem*	0.11	0.19	0.14	-0.07	-0.08
7 days *post mortem*	-0.08	0.23	0.10	-0.21	-0.29
14 days *post mortem*	-0.14	-0.01	0.21	-0.07	-0.16

Values in bold correlates significantly ($p < 0.05$), d.p.m – days *post mortem*, In/µ-calpain – Inhibitor / µ-calpain, In/µ+m-calpain – Inhibitor / µ+m-calpain

Titin and 30 kDa did not correlate significantly ($p > 0.05$) with the calpain system. Nebulin at 7 days *post mortem* correlated with both the inhibitor / µ-calpain and inhibitor / µ+m-calpain ratios, r = 0.35; 0.30 respectively. Desmin 14 days *post mortem* correlated with m-calpain, r = -0.30. Desmin degradation determined with Western-blotting 1 day, 7 and 14 days *post mortem* correlated with calpastatin and µ-calpain (Table 4.23).

4.7 Extent of muscle contraction

The extent of muscle contraction is reflected in the muscle histology (fibre area, fibre type and sarcomere length) presented in Table 4.24.

Table 4.24: Least square means and standard errors of means and ageing effect describing the histological characteristics of the *M. longissimus dorsi* (L1-L6) (non-electrically stimulated carcasses) in the three crossbreds evaluated.

		Genotype			
Characteristic	**SEM**	**Brahman-X**	**Simmentaler-X**	**Nguni-X**	**p-Value**
Muscle area (µm^2)	161.0	5940	6408	6096	0.122
Muscle fibre areas (µm^2)					
Red fibre	124.3	2323	2282	2160	0.632
Intermediate fibre	158.2	3302	3166	3072	0.590
White fibre	233.5	5709	5157	5219	0.196
Fibre type distribution (%)					
Red fibre	0.696	35.97	34.69	37.08	0.060
Intermediate fibre	0.805	27.43	28.50	27.52	0.584
White fibre	0.924	36.60	35.40	36.81	0.508
Sarcomere length (µm)					
1 day *post mortem*	0.023	1.697^b	1.657^b	1.611^a	< 0.035
3 days *post mortem*	0.022	1.698^b	1.681^b	1.624^a	< 0.049

^{abc} – Means in row with different superscripts differ significantly with the Fishers' means separation test

No significant ($p > 0.05$) differences for muscle area, fibre areas or fibre type distribution was found between the three different crossbreds (Table 4.24). However, the average fibre areas correspond to the study of Frylinck and Heinze (2003), indicating that the Brahman breed had larger fibre areas, as is the case in the current study and finding that larger areas are a characteristic of tougher meat. It was also shown by Frylinck and Heinze (2003) that the Simmentaler breed had significant higher percentages white fibres compared to the Brahman and Nguni. The percentage intermediate fibre values were similar between the breeds and the Simmentaler breed had the highest percentage red fibres compared to the other breeds.

In this study sarcomere lengths were significantly different between the crossbreds. The sarcomere length of the Brahman-crosses was significantly longer (more tender) than the sarcomere length of the Nguni-crosses but similar to the Simmentaler-crosses. This is not unexpected as the Brahman and Simmentaler carcasses are larger and thus less prone to muscle shortening.

4.8 Connective tissue

Least square means and standard errors (SEM) for connective tissue characteristics for the crossbreds are presented in Table 4.25. No significant ($p > 0.05$) differences for the connective tissue status in the three different crossbreds were found according to Fishers' means separation test.

Table 4.25: Least square means and standard errors for connective tissue characteristics of the *M. longissimus dorsi* (L1-L6) (non-electrically stimulated carcasses) in the three crossbreds evaluated.

		Genotype			
Characteristic	**SEM**	**Brahman-X**	**Simmentaler-X**	**Nguni-X**	**p-Value**
Connective status					
Total collagen[1]	0.095	1.522	1.607	1.722	0.333
Soluble collagen	0.027	0.281	0.304	0.329	0.463
Insoluble collagen	0.072	1.242	1.302	1.394	0.331
Collagen solubility (%)	0.798	18.27	18.38	18.76	0.902
Protein (%)	0.144	22.56	22.38	22.48	0.671

[1] Total collagen, soluble collagen and insoluble collagen were expressed in Hypro N x 10^3 / Total N

4.9 Correlation between muscle characteristics

The correlation-matrix for WBSF and various muscle characteristics are presented in Table 4.26. The calpastatin activity 1 hour and 24 hours *post mortem* did not correlate with WBSF 1 day, 7 and 14 days *post mortem*. The extractable calpastain / μ- and m-calpain activity 24 hours *post mortem* correlates ($r = 0.34$) significantly ($p < 0.05$) with WBSF tenderness 7 days *post mortem*. Sarcomere length 3 days *post mortem* and myofibril length 7 days *post mortem* correlated with WBSF 7 days *post mortem*, $r = -0.30$ and 0.33, respectively. The muscle fibre area distribution (%) of the intermediate fibres correlated ($r = 0.40$) with WBSF, the other fibre area characteristics and connective tissue characteristics did not correlate with WBSF (Table 4.26).

Table 4.26: Correlation-matrix showing simple correlation coefficients between tenderness (WBSF) and various muscle characteristics in the three crossbreds evaluated.

Characteristics	Warner-Bratzler shear force (Kg/12.5mm θ)		
	1 day *post mortem*	7 days *post mortem*	14 days *post mortem*
Calpastatin			
1 hour *post mortem*	-0.06	0.13	0.24
24 hours *post mortem*	-0.01	0.25	-0.02
μ-calpain			
1 hour *post mortem*	0.01	0.18	0.16
24 hours *post mortem*	-0.11	-0.11	-0.13
m-calpain			
1 hour *post mortem*	-0.13	-0.25	-0.14
24 hours *post mortem*	-0.18	-0.18	-0.24
Calpastatin / μ-calpain			
1 hour *post mortem*	-0.04	-0.19	-0.09
24 hours *post mortem*	0.04	0.17	0.15
Calpastatin / μ + m-calpain			
1 hour *post mortem*	-0.02	-0.04	0.09
24 hours *post mortem*	0.09	**0.34**	0.15
Sarcomere length			
1 day *post mortem*	-0.29	-0.25	-0.17
3 days *post mortem*	-0.28	**-0.30**	-0.14
MFL			
1 day *post mortem*	-0.08	0.03	0.05
7 days *post mortem*	0.08	**0.33**	0.28
14 days *post mortem*	0.28	0.22	0.28
Total collagen	0.03	0.00	-0.08
Insoluble collagen	0.04	0.04	-0.09
Soluble collagen	0.01	-0.09	-0.03
% Collagen solubility	-0.01	-0.16	0.05
% Protein	0.07	-0.11	0.14
Fibre areas			
Red fibre	-0.24	-0.04	-0.11
Intermediate fibre	-0.18	-0.03	0.00
White fibre	-0.10	-0.19	-0.05
% Red fibre	-0.06	-0.17	0.20
% Intermediate fibre	0.21	**0.42**	0.10

Values in bold correlates significantly ($p < 0.05$), MFL – Myofibrillar fragment length

CHAPTER 5
DISCUSSION

The tenderness of meat is influenced by various *ante mortem* and *post mortem* factors. The *ante mortem* factors include the species of the animal, breed or genotype, age of the animal, gender, anatomical location, size and type of muscle, rate of glycolysis, connective tissue and collagen solubility, adipose tissue, water-holding capacity and proteolytic enzymes (Koohmaraie, 1994).

It is well documented that tenderness decreases as the percentage *Bos indicus* increases (Crouse *et al*., 1989; De Bruyn, 1991; Shackelford *et al*., 1991; Shackelford *et al*., 1995; Whipple *et al*., 1990a; Koohmaraie, 1996; Riley *et al*., 2003). It has been shown that these differences (between *Bos indicus* and *Bos taurus*) occur due to differences in the properties of a specific muscle enzyme system (calpain system i.e. calpastatin / μ-calpain ratio) and their effects on the myofibrillar properties of the muscle. In this chapter the comparison of meat tenderness prediction by means of gene technologies (markers) with established physical estimates of meat tenderness will be discussed.

5.1 Animal characteristics

The characteristics of the animals at slaughter are summarised in Chapter 4, Table 4.1. Testosterone is involved in collagen synthesis, accumulation and maturation, which impact negatively on meat tenderness (Cross *et al.,* 1984; Seideman *et al.,* 1989). Muscles of bulls have higher levels of calpastatin with the consequent lower ability for tenderisation through the ageing process (Morgan *et al.,* 1993). These effects only come into effect after puberty; therefore bull meat from young animals (A-age) should not be affected. Water binding capacity did not differ significantly ($p > 0.05$) between the crossbreds (Table 4.1) and this corresponds with the study of Frylinck and Heinze (2003).

Silva *et al*. (1999) reported that meat with a high ultimate pH is dark and more susceptible to bacterial spoilage. Nevertheless, this meat is associated with a higher rate of tenderisation (Beltran *et al*., 1997). Carcasses from the Nguni-crosses cooled down quicker (smaller than the Brahman- and Simmentaler-crosses) (Figure 4.1), thus the lower carcass temperatures at 24 hours *post mortem* (Table 4.1). According to the study of Frylinck and Heinze (2003) the smaller carcasses (Afrikaner and Nguni) cooled down quicker resulting in significant lower carcass temperature at nine hours *post mortem* and 24 hours *post mortem* and more prone to cold shortening. To prevent cold shortening they hung the carcasses in a cool room at about 10 °C until 6 hours *post mortem,* after which the carcasses were shifted into the 4 °C cooler (Frylinck and Heinze, 2003). Another method to prevent cold shortening is electrical stimulation of the carcasses. None of these two methods were used in this study, because the emphasis was on the expression of the inherent tenderness characteristics without external *post mortem* influences. Thus cold shortening can be expected in the smaller carcasses (see discussions later). In the USA, typical carcasses are produced

bigger (heavier) compared to the carcasses of SA and thus the cool down period is longer, thus their carcasses are less susceptible to cold shortening. The reason for this is that their younger animals (steers and heifers) are considered until an age of 24 months to be graded in to the A and B maturity groups (best USA grades). The USA are more focussed on marbling, thus the marbling level is a primary determinant of the grade compared to South African conditions, where marbling are not considered (Lebert, 2000).

5.2 Tenderness of loin samples

The most commonly used instruments used to estimate meat tenderness are the Warner-Bratzler Shear device and the Instron apparatus. Tenderness is measured as the shear force (expressed as a value) required to cleave a standard cross-sectional area of cooked meat across the muscle cells or fibres (Davey, 1983). Factors that may affect the accuracy of these measurements include the doneness of the cooked meat, uniformity of cylindrical sample size, direction of the muscle fibre, amount of connective tissue and fat deposits present, temperature of the sample and the speed at which the sample is sheared. A lower shear force value means less force is required to shear through the sample and therefore the meat is tender.

The fact that the Simmentaler-crosses were significantly ($p < 0.003$) tougher (Table 4.2), than the other crossbreds was surprising, but agrees with the findings of De Bruyn (1991) and Frylinck and Heinze, (2003) on none electrical stimulated carcasses. Numerous studies have established that the *M. longissimus dorsi* from *Bos indicus* cattle is usually less tender than meat from *Bos taurus* breeds (Crouse *et al.,* 1989; De Bruyn, 1991; Koohmaraie, 1996; Shackelford *et al*., 1991; Shackelford *et al*., 1995; Whipple *et al*., 1990b). Herring *et al.* (1965) also reported significantly lower shear force values for the *Sanga*, compared to the *Bos indicus*, and were not found in this research. Although the breed-crosses differed phenotypically the animals represented in the breed-cross groups could be genotypically more similar (see Chapter 4, section 4.3), because the contribution of the different breeds represented in the crosses could not be established on phenotype (unknown). It can be concluded from the overall shear force data (Table 4.2), that the animals in this study had tough meat in general for the shear force values are above 5 kg, especially after 14 days *post mortem*. Tenderness of meat is influenced by various *ante mortem* and *post mortem* factors. *Ante mortem* factors include the species of the animal, breed or genotype, age of the animal, gender, anatomical location, size and type of muscle, rate of glycolysis, connective tissue and collagen solubility, adipose tissue, water holding capacity and the proteolytic enzymes. *Post mortem* factors include electrical stimulation of the carcass (which was not a factor in this study), ageing, chilling temperature, cooking method, internal end-point temperature of the meat, which influence the sarcomere length and the degradation of the myofibrillar proteins by the proteolytic enzymes (Koohmaraie, 1994). Although all these factors have an impact on tenderness, electrical stimulation and ageing could have improved the ultimate tenderness of the meat in this study. Ageing of meat at refrigeration temperature (1 - 5 ˚C) has long been recognised as resulting in a improvement of meat tenderness (Pearson, 1986). The effect of meat ageing is dependant on a number of

factors including age of the animal, gender, muscle type, electrical stimulation and the ageing period (Ouali, 1990).

Frylinck and Heinze (2003) found that only pH measured at 1 hour and 6 hours *post mortem* correlated with WBSF at 1day, 3, 7, 14 and 21 days *post mortem* (correlations = 0.300 - 0.403), and the carcass temperature at 24 hours *post mortem* showed a high correlation (r = 0.486) at 21 days *post mortem*.

5.3 Genetic considerations and the expression of the calpain system

In the past decade various factors contributed to highlighting quality traits, with specific interest in tenderness. It is necessary to consider the basic principles of genetics in order to gain an understanding of gene marker technology.

A gene is the physical unit of heredity, composed of a DNA sequence at a specific location on a chromosome. Gene marker tests are tests for DNA markers that form part of a gene. A DNA marker is a particular sequence of base pairs (represented as A (Adenine), T (Thiamine), C (Cytosine) and G (Guanine)) that indicate the genotype of an animal for that marker on the chromosome. Each marker located on a gene has two alleles, one inherited from each parent. Amongst others, the calpain proteolytic system has been identified as responsible for the *post mortem* meat tenderisation process. Two enzymes responsible for this process are the micro molar calcium-activated neutral protease μ-calpain (*CAPN1*), which is encoded by the *CAPN1* gene, and its inhibitor, calpastatin (*CAST*), which is encoded by the *CAST* gene (Koohmaraie, 1996). The hypothesis is that the effect on tenderness of an allele at one locus may depend on the allele at the other locus, as variation that influences the ability of *CAST* to inhibit *CAPN1*, could depend on the physical state or concentration of the enzyme (Casas *et al*., 2006). Identification of genetic markers for meat tenderness variation would provide some selection criteria to facilitate genetic improvement in this trait (Page *et al*., 2004).

5.3.1 Definition of markers, alleles and haplotypes

Two single nucleotide polymorphism (SNP) markers were employed in this study for the bovine *CAPN1* gene, which is found or situated on bovine chromosome 29. One SNP marker is situated on exon nine (*CAPN1*-316) and the other on intron 17 (*CAPN1*-4751). The inhibitor, calpastatin (*CAST*) found on chromosome seven was also employed in this study. Both the *CAST* markers (*CAST* and *CAST-Brahman*) lie in the three prime untranslated regions (3' UTR) of the *CAST* gene.

A report by Page *et al*. (2004) presents evidence that SNP determining amino acid variation of glycine or alanine at position 316 in the micro molar calcium-activated neutral protease gene, act as a marker for meat tenderness variation. There are two nucleotide alleles, C and G. The heterozygote genotype using standard IUB codes is S. The G-allele results in glycine at position 316 of the amino acid sequence; the

C-allele results in alanine. Previous results demonstrate that the C-allele is associated with lower shear force values (increased tenderness) (Page *et al*., 2004). Thus, animals inheriting the CC and CG genotypes produce more tender meat when compared to animals inheriting the GG genotype (Casas *et al*., 2006). *CAPN1*-316 does not segregate at appreciable frequencies in Brahman cattle (Casas *et al*., 2005).

White *et al*. (2005) developed and reported the marker developed at the *CAPN1* gene which is a transition from a cytosine (C) to a thymine (T). The marker is referred to as *CAPN1*-4751. The heterozygote genotype using standard IUB codes is Y. The C-allele is associated with lower shear force (increase tenderness). Thus, animals inheriting the CC and CT genotypes produce more tender meat when compared to animals inheriting the TT genotype (Casas *et al*., 2006). This marker is associated with tenderness in *Bos taurus* and *Bos indicus* cattle as well as in crossbred cattle (White *et al*., 2005). White *et al.* (2005) concluded that a multiplex marker system incorporating both markers (316 and 4751) provides an optimal solution in all populations (*Bos taurus, Bos indicus* and crossbreds) studied to date. The study of Casas *et al,* (2006) suggested that the markers developed at the *CAST* and *CAPN1* genes are suitable for the use in identifying animals with the genetic potential to produce meat that is more tender.

The *CAST* marker is a transition from a guanine to an adenine at the 3' UTR of the gene. *CAST* has alleles C and T. The heterozygote genotype using IUB codes is Y. *CAST-Brahman* has alleles A and T. The heterozygote genotype using IUB codes is W. A report by Casas *et al*. (2006) indicated that animals inheriting the CC and the CT genotypes (*CAST*) produce tougher meat when compared with animals that inherited the TT genotype. The study of Casas *et al.* (2006) suggested that animals inheriting the CC and the CT genotypes at the *CAST* gene produced tougher meat when compared to animals inheriting the TT genotype.

Haplotypes depend on the alleles of the two markers on individual chromosomes and are defined by the allele at a marker (i.e. *CAST*), presented first when discussing haplotype, followed by a slash and the allele at the other marker (i.e. *CAST-Brahman*). According to the markers patented by Barendse (2002) it specifies haplotypes of the two markers in relation with tenderness are C/A and Y/A, associated with increased shear force relative to the alternative haplotypes (T/A and T/W).

5.4 Association of SNP markers with shear force values

Markers 316 and 4751 (*CAPN1* gene) are generally preferred as a tool to guide selection, because they show association with tenderness in a wide variety of populations, compared to markers 530, 4753 and 5531 (*CAPN1* gene) (White *et al*., 2005).

In a study by Casas *et al*. (2006) on three defined populations containing about 1000 animals, they found the following association of SNP markers with shear force values: A SNP marker at the *CAST* gene had a significant ($p < 0.003$) effect on shear force and tenderness scores. Animals inheriting the TT genotype at the *CAST* had meat that was more tender than those inheriting the CC genotype. The 4751 marker at the *CAPN1* gene was also significant ($p < 0.03$) for tenderness score. Animals inheriting the CC

genotype at *CAPN1* had meat that was more tender than those inheriting the TT genotype. An interaction between *CAST* and *CAPN1* was detected ($p < 0.05$) for shear force on one of the populations that had *Bos taurus* influence. Animals inheriting the CC genotype for *CAST* produced tougher meat when they inherited either the CT or the TT genotypes in *CAPN1*. This report was the first evaluation of the calpastatin marker in scientific literature (the original finding was patented by Barendse, 2002) of the association of the *CAST* SNP with meat tenderness. The findings in the original work gave similar results, and these effects extend too many, but seemingly not all beef breeds. It seems that the present marker system is not adequately matched to functional alleles to be useful in *Bos taurus* populations (Casas *et al*., 2006).

Currently, the biochemical pathway with the most evidential support for involvement in *post mortem* tenderisation is that of the calpain family proteases. In combination with previous studies of crossbred populations, a report by Smith *et al.* (2000) provides the first evidence to support the possibility that genetic variation at the *CAPN1* locus could contribute to the heritable component of meat tenderness.

Most of the animals in the study presented the GG genotype at marker 316. This also corresponds with the WBSF values (Table 4.2) that indicated that overall the meat of the animals of this study is tough, since the WBSF values were above 5 kg. According to Table 4.12 the Brahman-crosses had the more favourable WBSF values associated with the *CAPN1*-316 marker indicating more tender meat compared to the Simmentaler- and Nguni-crosses and this correspond with Table 4.2. According to White *et al.* (2005) the *CAPN1*-4751 marker has a broad usefulness in cattle of *Bos taurus*, *Bos indicus* and crossbred descent.

However, it can be concluded that the data from Table 4.11, 4.12, 4.13, 4.14, 4.16 and 4.17 were not representative. The results were not significant ($p > 0.05$) and there were too few CC genotypes for comparison. For the *CAPN1* marker (Table 4.15) the only haplotype that was presented is the GG-CT combination since the other combinations were absent or too few for comparison. For the *CAST* haplotype (Table 4.19) only the YA and TA combinations were presented because the CA and TW combination were absent or too few for comparison. These results indicate that using genetic markers to improve meat tenderness in South African crossbreds are not that significant but it could be used as an additional aid when selecting for preferred traits i.e. tenderness.

5.5 The expressed calpain system

It is reported that the endogenous inhibitor of calpains, rather than calpain activity should be measured, because calpastatin activity *post mortem* is highly related to beef tenderness (Whipple *et al*., 1990b; Shackelford *et al*., 1991; Doumit *et al*., 1996). A high calpastatin activity results in decreased calpain activity and thus, decreased tenderness (Boehm *et al*., 1998). In some experiments where large metabolic differences are measured and calpastatin is expected to be the main component of the calpain system that is influenced, it is adequate to determine calpastatin activities and levels. In this study where the differences between crossbreds may not be very large, and where the enzyme activity could be influenced by genotypic

characteristics, it is necessary to determine all the factors of the calpain system (i.e. all the enzymes and inhibitors).

There was no significant difference ($p > 0.05$) between the different crossbreds for the calpastatin levels but significant differences were found for the μ-calpain activity and calpain / calpastatin ratios (Table 4.20). This can be an indication that μ-calpain is an essential factor for desired calpain system characteristics and corresponds with the findings of Koohmaraie and Geesink (2006) that μ-calpain activity is the primary source of variation in tenderness of muscles.

The intermediate level of the Brahman-crosses was unexpected, because higher levels of calpastatin activity have been associated with higher percentages of Brahman (*Bos indicus*) in cattle (Riley *et al*., 2003; Pringle *et al*., 1997). The consistent lower μ-calpain activities for the Brahman-crosses corresponded with the findings of Frylinck and Heinze (2003). The m-calpain concentration (Table 4.20) corresponds with that concluded from the literature that m-calpain does not play a significant role in the *post mortem* tenderising process (Koohmaraie, 1994; Doumit and Koohmaraie, 1999; Koohmaraie and Geesink, 2006).

The Simmentaler-crosses showed higher toughness ratings than the Brahman-crosses (Table 4.2), thus the calpain system results (Table 4.20) did not explain the differences in tenderness in this population tested. Other factors must have played a greater role (i.e. pH: temperature ratio). Table 4.1 indicates that carcasses of the Simmentaler-crosses compared to the Brahman-crosses cooled down faster between 3 and 6 hours *post mortem*. The muscle temperature during *rigor* development is critical in determining meat tenderness, primarily due to the sarcomere shortening produced by low temperatures (cold temperatures): cold shortening meat is tougher before ageing and does not tenderise even after prolonged storage when tested by shear force (Davey and Gilbert, 1975). Lower temperatures also inhibit the calpain enzyme system. This could also explain the higher WBSF measurements of the Simmentaler-crosses (Table 4.2) and the consequent tenderness. The calpain system activities seemed to support some of the meat tenderness similarities and differences of the crossbreds evaluated. On the other hand calpain system results of the Brahman-crosses partly explain the lower tender characteristics of this breed.

However, a large number of studies (for review see Koohmaraie and Geesink, 2006) have shown that μ-calpain is largely responsible for *post mortem* tenderisation. Koohmaraie and Geesink (2006) suggested that research efforts in this area should focus on elucidation of the regulation of μ-calpain activity in *post mortem* muscle, and that discovering the mechanisms of μ-calpain activity regulation and methods to promote μ-calpain activity should have a dramatic effect on the ability of researchers to develop reliable methods to predict meat tenderness (i.e. genetic markers) and on the meat industry to produce a consistently tender product.

The question could be raised that if all the animals in this study had favourable genes, the environmental factors still have the potential to influence the animals in a positive or negative way? For example, if the genetic marker data (see Chapter 4, Table 4.5) is considered, the Simmentaler-crosses had the potential to be more tender than the Brahman-crosses, but the calpain system did not support the tenderness outcome, thus environmental factors had a negative influence.

5.6 Proteolytic degradation

Myofibril fragmentation has been recognised as an important event during the ageing of meat. It is well established that proteolysis of myofibrillar proteins leads to increased fragmentation of myofibrils and decreased shear force during *post mortem* storage (for review see Koohmaraie, 1992a; Koohmaraie, 1992b and Koohmaraie, 1994). This fragmentation is the result of proteinase action (Ca^{2+} activated calpain enzymes). The shorter the myofibril fragment, the higher the proteinase action, resulting in a higher sensory panel tenderness rating and a lower WBSF measurement. The study of Scheepers (1999) indicated that the Brahman had the longest myofibrillar fragment length compared to the other breeds used, as was reported by Frylinck and Heinze (2003) and the current study (Table 4.21). Frylinck and Heinze (2003) concluded that the meat from the *Sanga* breeds broke down into shorter myofibril fragments at a much faster rate than those of *Bos taurus* and *Bos indicus* types.

Myofibrillar protein degradation in the *M. longissimus dorsi* (L1-L6) during *post mortem* ageing at the molecular level by means of SDS-PAGE (means to study calpain system substrate disappearance and product formation) separation of total soluble muscle proteins (sarcoplasmic and myofibrillar) was examined as a confirmation of the MFL results. Titin, nebulin, desmin and 30 kDa could be identified according to their molecular mass. Each sample consisted of at least 28 bands, some disappearing (nebulin and desmin) and some forming (30 kDa) with time *post slaughter.*

According to literature, titin a structural protein plays a partial role in the development of tenderness (Huff-Lonergan *et al*., 1996b; Huff-Lonergan *et al*., 1995; Taylor *et al*., 1995 and Robinson *et al*., 1991). The study of Huff-Lonergan *et al*. (1995) indicated that nebulin was degraded by 3 days *post mortem* in tender samples and was absent in all 7 days *post mortem* samples. In the current study there was still nebulin present at 3 and 7 days *post mortem.* Huff-Lonergan *et al*. (1995) suggested that titin and nebulin are degraded at faster rates in more tender beef samples and that the more tender samples (low shear force values) exhibited a more rapid *post mortem* disappearance than the less tender samples. The study of Huff-Lonergan *et al*. (1995) indicated that a 30 kDa component intensified with increasing time *post mortem* and that more tender samples (low Warner-Bratzler shear force values and high sensory scores) exhibited a more rapid *post mortem* appearance than in less tender samples. Desmin were investigated further with Western-blotting (Table 4.22), because desmin play an important role in organizing and maintaining the integrity and strength of the contractile myofibrils and the overall cytoskeleton structure of the skeletal muscle cell (Huff-Lonergan *et al*., 1996a).

The results represented in Table 4.21 and Table 4.22 are in line with the MFL results and indicate that the biochemical basis of tenderisation during *post mortem* ageing involves the breakdown of certain myofibrillar proteins including nebulin and desmin. This is in accordance with the study of Koohmaraie and Geesink (2006).

5.7 Extent of muscle contraction

No significant differences ($p > 0.05$) for muscle area, fibre areas or fibre type distribution were found between the three different crossbreds (Table 4.19). However, the average fibre areas correspond to the study of Frylinck and Heinze (2003), indicating that Brahman breed had larger fibre areas, as is the case in the current study and finding that larger areas are a characteristic of tougher meat. The study of Frylinck and Heinze (2003) indicated that the Simmentaler breed had significantly higher percentage white fibres compared to the Brahman and Nguni. The percentage intermediate fibre values were similar between the breeds and the Simmentaler breed had the highest percentage red fibres compared to the other breeds.

The sarcomere lengths in this study were mostly under 1.7 µm, which indicates marginal shortening (caused by either hot or cold temperatures). Thus high energy content in the muscle and a fast decrease in carcass temperature (Table 4.1) could lead to cold shortening, especially in the Nguni-crosses (i.e. causing the short sarcomere lengths in the Nguni-crosses) (Table 4.24). The study of Frylinck and Heinze (2003) indicated that the sarcomeres of the Brahman and Simmentaler breeds did show a tendency to be marginally shorter. According to Wheeler and Koohmaraie (1994) sarcomere length may have an indirect effect on tenderisation during ageing due to its effect on initial tenderness. Denoyelle and Lebihan (2003) reported that meat tenderness depends on the properties of the muscle fibres and the amount and type of connective tissue.

5.8 Connective tissue

The connective tissue status did not have a significant effect on the differences in tenderness between the different crossbreds evaluated (Table 4.25). However, the percentage collagen solubility in the Nguni-crosses tended to be higher than that of the other crossbreds, which could explain the tendency for their more favourable meat tenderness. As in the case of the calpain system the ratios of the different components, soluble and insoluble collagen, play an important role in the ultimate tenderness and not one component alone.

5.9 Correlation between muscle characteristics

Correlation coefficients reported previously for calpastatin activity and tenderness (WBSF) determined 24 hours *post mortem* were $r = 0.27$ (Shackelford *et al*., 1995) and this corresponds with $r = 0.25$ at 7 days *post mortem* (Table 4.26), 0.66 and 0.39 (Whipple *et al*., 1990b and Shackelford *et al*., 1991). However, these correlations indicate that the calpain system plays an important role during the ageing process (tenderisation). The correlation between WBSF and the sarcomere lengths indicates that muscle shortening (cold shortening) influenced tenderness in the current study, which corresponds with the previous data in Table 4.24 (sarcomere lengths ~ 1.6 µm). Although MFL is also an indicator of toughness, it was rather

difficult to determine at day 1. The reason for better correlations at 7 days *post mortem* between WBSF and muscle characteristics is because the differences between the characteristics at that stage are a better indication.

The study of Frylinck and Heinze (2003) also indicated that the percentage intermediate fibres correlated negatively with WBSF values. This phenomenon indicates that more intermediates fibres favour meat tenderness, which corresponds with the characteristics of the Nguni.

However, when all the mechanisms (calpain system, collagen (total- and soluble collagen) and muscle contraction proteins (fibre typing, myofibre areas and sarcomere length) are considered, it can be concluded that the expression of the calpain system was counteracted by cold shortening for example in the Nguni-crosses. This means that the muscle contraction protein mechanism played a major role in the resultant / final tenderness of the various crossbreds evaluated. The muscle contraction protein mechanism also had a more positive effect on the tenderness of the Brahman-crosses resulting in more favourable tenderness measurements (WBSF), but which is still regarded as tough (Table 4.2).

It can be concluded from this study that meat quality, and in particular meat tenderness, is manifested through a complexity of events in the muscle and their interactions with many environmental stimuli in both the live animal and during the *post mortem* period. The knowledge gained from genomic approaches can be an additional aid in defining and optimising management systems for better meat quality.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

The post World War II food production world has been replaced with a modern consumer world where the emphasis in the food chain is top down (customer driven) not bottom up (producer driven) (Catlett, 2006). Thus, a current concern in the production of beef cattle is tenderness of the meat product, which has a major impact on consumer satisfaction. Strydom (1998) suggested that in any country the whole red meat industry's mind (research, producer, processor, food service) should be set on: supplying a product with maximum edible yield (muscle and fat), according to consumer preference and with the best consistent eating qualities per unit price.

A comparison of crossbreds with regard to the histological, physical and genetic characteristics has not before been attempted in South Africa. This study of beef animals included three genotype crosses (Brahman-crosses, Simmentaler-crosses and Nguni-crosses), classified according to the three breed groups (*Bos indicus*, *Bos taurus* and *Sanga*). Weaned, young bulls (7 - 8 months) were transported to the ARC-Irene, where they were kept under feedlot conditions until the time of slaughter. The animals were slaughtered at an age of 10 - 12 months, so as to produce a carcass in the A-age class with a fatness code of two to three (lean-medium fat) according to the current South African Beef Classification System (Government Gazette, No. 5092, 1993).

At slaughter the carcasses were not electrical stimulated (NS) because electrical stimulation (ES) influences the processes of meat tenderness, and the emphasis was on the expression of the inherent tenderness characteristics without external *post mortem* influences. After exsanguination the carcasses were dressed down, halved and chilled at 4 °C within 2 hours of *post mortem*. The *M. longissimus dorsi* (L1-L6) (LL) of each carcass on both sides were used for sampling. Three 20 – 25 cm thick whole-loin cuts were removed from specific positions along the *M. longissimus thoracis et lumborum* (LT and LL) on the right and left sides for shear force evaluations. Each of the three cuts were vacuum packed, and aged for 1 day, 7 and 14 days *post mortem* at ± 4 °C, then frozen, and stored (-25 °C) for later determination of Warner-Bratzler shear force (WBSF). The muscle pH and temperature decline were measured at the second last lumbar vertebrae at 1-hour, 2, 3, 6, 8 and 20 hours *post mortem* to diagnose pale, soft and exudative (PSE) type (PSE is only found in pork, but a similar phenomenon exist in beef where a high drip loss takes place after a quick pH decline and a slow temperature decline) or dark, firm and dry (DFD) phenomena at slaughter. DFD can influence the ageing process of the meat. The pH and temperature were determined with a digital handheld meat pH meter (Unitemp) fitted with a polypropylene spear type gel electrode. Muscle samples for other procedures were removed from *M. longissimus dorsi* (L1-L6) (LL) at specified positions and preserved at either -25 °C or -80 °C after ageing at 1 day, 7 and 14 days *post mortem.*

The results of the present study showed that differences exist in meat quality of the different crossbreds. Breed had a significant effect on the meat quality characteristics especially with regard to tenderness. Differences in tenderness do occur between *Bos indicus*, *Bos taurus* and *Sanga* breeds due to

differences in the properties of the proteolytic enzyme system (Whipple *et al.*, 1990a; Shackelford *et al.,* 1991). However, there will always be a place for *Bos indicus* cattle because of their adaptability to tropical climates and environments as well as their resistance to tropical parasites (Strydom, 1998).

It is generally accepted that proteolysis of myofibrillar proteins is, at least partly, responsible for *post mortem* tenderisation. However the exact mechanisms are still largely unknown and a number of questions remain to be solved. A large number of muscle proteins have been previously identified and are degraded during the ageing process, but the relative importance of these proteins with regard to the maintenance of the structural integrity of meat remains to be determined.

The activities of the calpain system seem to support some of the similarities and differences in meat tenderness of the crossbreds evaluated. According to Koohmaraie and Geesink (2006) the calpain proteolytic system plays a central role in *post mortem* proteolysis and tenderisation. Calpains are calcium-activated proteases with an optimum activity at a neutral pH. In skeletal muscle, the calpain system consists of at least three proteases, μ-calpain, m-calpain and skeletal muscle-specific calpain, p94 or calpain 3 and an inhibitor of μ- and m-calpain, calpastatin.

It is important to keep in mind that sarcomere length and myofibrillar fragment length are not the only determining factors for tenderness. Other factors, such as collagen content and solubility and the water-holding capacity should also be considered in explaining differences. It is also possible that proteases other than calpains, such as the cathepsins (that forms part of the tenderness model study but are not presented in this study) released from the lysosomes (Ouali *et al.,* 2006), and the multicatalytic proteinase complex (Rivett, 1989) could affect tenderness of the different breeds.

According to Ouali *et al.* (2006) attentions of meat scientists were mainly focused on the two best-known enzymatic systems, i.e. calpains and the cathepsins. Ouali *et al.* (2006) concluded that apoptosis on cells engaged in programmed cell death brings possible answers to many questions regarding the conversion of muscle to meat. It is also suggested that even if the participation of caspases does not fully explain muscle tenderisation, it is probably an essential element facilitating the action of other intracellular proteolytic systems such as cathepsins and there also may be other proteolytic enzymes not considered so far as potential effectors of meat tenderness. In this study not all the enzyme systems and their role on meat tenderness were evaluated, but considering research studies (Koohmaraie and Geesink, 2006; Ouali *et al.,* 2006) it can be concluded that there is more than sufficient evidence to suggest that μ-calpain in combination with calpastatin is the only proteolytic system that influence tenderness (Koohmaraie and Geesink, 2006).

However, when all the mechanisms (calpain system, collagen (total- and soluble collagen) and muscle contraction characteristics (fibre typing, myofibre areas and sarcomere length) are considered, it can be concluded that the expression of the calpain system in this study was overshadowed by cold shortening, especially in the Nguni-crosses (SL = 1.6 μm). This means that the muscle contraction protein mechanism played a major role in the tenderness outcome of the various crossbreds evaluated. The muscle contraction protein mechanism may not have been affected by cold shortening in the Brahman-crosses (SL = 1.7 μm),

thus the reported more favourable tenderness measurements (WBSF, but is still regarded as tough). With this in mind the influence and role on tenderness outcome, considering a genetic approach was evaluated.

According to Bradley *et al*. (1998) the measurement and manipulation of genetic characteristics in cattle has a long history; for example general characteristics such as horn morphology and coat colouring have long been noted. There has been significant interest in genetic selection to decrease problems with variations in meat tenderness. However, the problem of variability in meat tenderness has not diminished, in part because of an inability to accurately select for increased tenderness. Previous studies (Barendse, 2002; Page *et al*., 2002; Page *et al*., 2004; White *et al*., 2005) have independently evaluated markers at the *CAST* and *CAPN1* genes. These studies have shown an association of individual markers at *CAST* and *CAPN1* with meat tenderness in beef cattle, as in the present study. Considering all the marker data used in this study (interaction between the genes), it can be concluded that overall the Nguni-crosses had the highest potential to inherit tender meat, the Brahman-crosses inherited the alleles for tougher meat and the Simmentaler-crosses were intermediate.

One of the aims of this study was to investigate the relationship between breed and muscle characteristics, considering a physiological and genetic approach. It is recommended that purebred animals rather than crossbreds should be used to determine these factors, because the animals were classified according to phenotype and this could have resulted in a genetic "error" made in the study. From this study it can be concluded that even if the animal has the potential to produce tender meat, it is not for sure a tender end product i.e. Nguni-crosses. This is due to the various mechanisms and environmental factors that are involved, thus enhancing or suppressing mechanisms can play a role and give another outcome compared to the genetic make up. This means that attention should also be placed on other methods and factors playing a role to improve tenderness (for example electrical stimulation, *pre-slaughter* handling and management) and one should not over emphasize the genetic potential of an animal and only consider it as an aid in selecting for the desired trait. The reason is that the estimated contribution of gene technology on meat tenderness was found to be 10 - 12 % and the results of this study suggests that researches should take into account the many other environmental and non-environmental conditions that play a role in meat tenderness. It is therefore necessary to integrate the available knowledge in our future projects on meat tenderisation, including the identification of predictive markers. The introduction of meat quality (meat tenderness) in the genetic selection programmes in South Africa i.e. Best Linear Unbiased Prediction (BLUP) system should be considered.

It can be concluded that meat quality and the composition, nutritional value, wholesomeness and consumer acceptability of beef are largely determined by events and conditions encountered by the embryo, the live animal and the *post mortem* musculature. The control of these qualities, and their future enhancement, are thus dependant on a better understanding of the commodity at all stages of its existence - from the initial conception, growth and development of the organism to the time of slaughter and to the ultimate processing, distribution, preparation, cooking and consumption. Though not tested in this study, the use of various *post mortem* tenderisation technologies in South Africa may be more cost-effective than

attempts to improve tenderness in beef cattle genetically. Results from this study indicates that modern technologies available for predicting the potential tenderness of beef animals is not adequate to be of commercial value for the producer. The scientific information obtained from this study can be used to explain inconsistencies in meat tenderness experienced in the beef industry and help develop technologies to manipulate and standardise meat tenderness and the tenderising processes.

Future endeavours should focus on a more co-ordinated strategy between the various research disciplines in the beef industry (genetics, feeding, animal welfare, economics, processing and meat quality) to ensure that the different sectors have the knowledge to supply an end product, which is not only accepted but also preferred, by consumers' worldwide. Addressing one trend (characteristic / trait) is no longer sufficient; consumers want it all. The influence of cell death (necrosis and apoptosis) on meat quality could be researched in the future. Further research that should be considered in South Africa is the role of genetic contribution in meat quality with emphasis on proteomics. The proteome is expressed from the genome, influenced by environmental and processing conditions, and can be seen as the molecular link between the genome and the functional quality characteristics of meat (Hollung *et al.,* 2007). Proteomics is a promising key in meat science to unleash the molecular mechanisms behind different genetic backgrounds or processing techniques of meat. Understanding the variations and different components of the proteome with regard to a certain meat quality trait will lead to knowledge that can be used to optimise the tenderisation process. South Africa should focus on the understanding and identification of markers (i.e. genetic and protein markers) for meat quality (i.e. tenderness), thus an integrated functional genomic approach should be considered.

Addendum A

The establishment of cattle breeds which are now indigenous to Africa is believed by historians to be very closely associated with man, his development, migration and specific behaviour from 6 000 years BC (Payne, 1964; Oliver, 1983; Strydom, 1998; Scheepers, 1999). Due to this movement and behaviour, the cattle breeds can be classified into three groups: *Bos indicus, Bos taurus* and the *Sanga*. It is accepted that cattle arrived on the continent through three main routes from Asia (Figure 1).

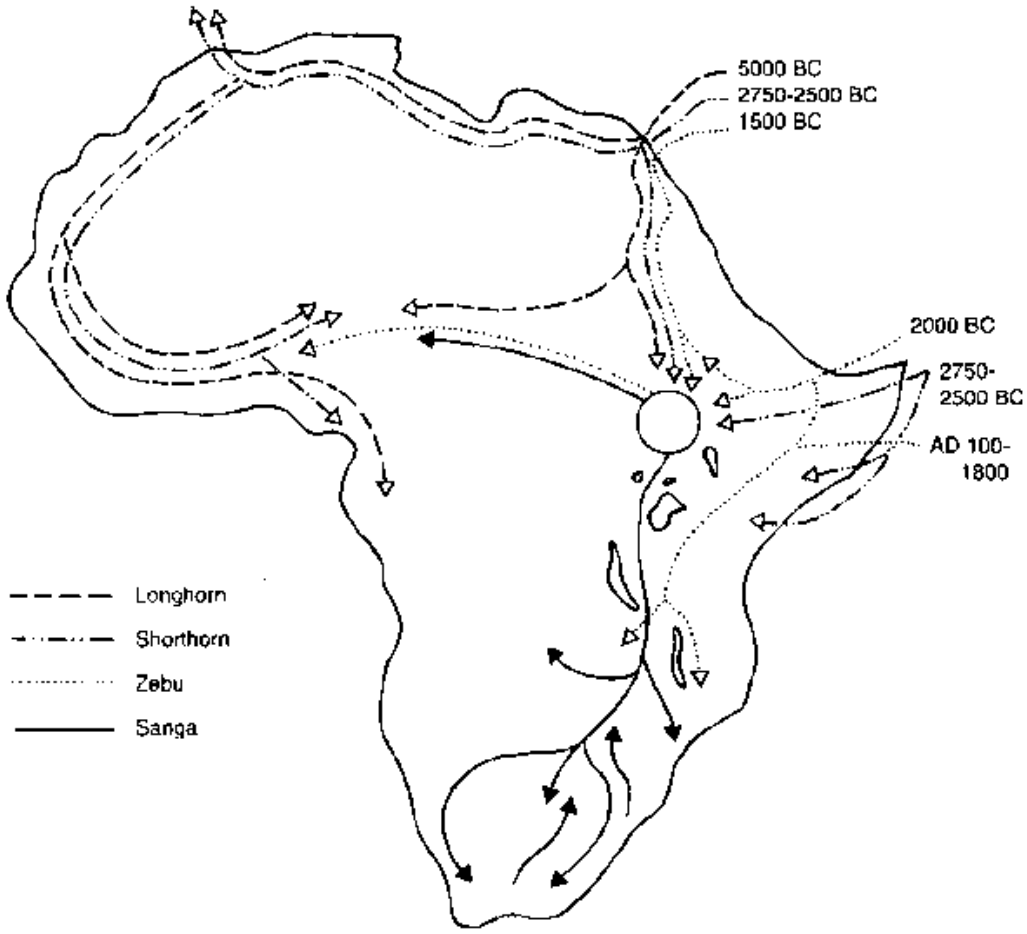

Figure 1: Origin and migration routes of domesticated cattle in Africa (Oliver, 1983)

Longhorn cattle are believed to be the first cattle to be domesticated from the wild *Bos primigenius primigenius* (wild aurochs) in Western Asia (the origin of domestication) about 6000 BC. They appeared in Africa around 5000 BC. Shorthorn cattle originated in the same area as the longhorn cattle and were introduced in to Africa approximately 2500 BC. Both longhorn and shorthorn cattle were of taurine type (hump less) and are often classified as *Bos taurus longifrons* and *Bos taurus brachyceros* (Oliver, 1983; Payne and Hodges, 1997; Strydom, 1998). The third group of cattle introduce into Africa were the Zebu

cattle about 1500 BC and later introductions around 670 BC. Two major types of Zebu cattle (*Bos indicus*) are known; neck humped (cervico-thoracic) and shoulder humped (thoracic types). According to Payne and Hodges (1997) the African *Sanga* first evolved in Northeast and East Africa over a period of time (2000 BC - 400 AD) (Figure 1), as their three ancestral cattle types (longhorn, shorthorn and zebu) entered the region.

Although it is accepted that cattle were domesticated around 2100 BC in the Middle East and migrated south with the tribes that kept them (reaching the area presently known as South Africa around 700 AD) (Gertenbach and Kars, 1999). The first migration southwards was probably that of Sudanic- or Cushitic-speaking tribes (probably the later Koisan people of southern Africa that originated from inbreeding of Bushmen and Sudanic / Cushitic people) and their cattle were long horned *Sanga* types. When Europeans came to the southern tip of Africa, indigenous cattle (*Sanga*) were utilised as draught animals as they could survive the harsh climatic conditions, in contrast to *Bos taurus* breeds. The Nguni is a *Sanga* type named after the Nguni people, which lived on the East coast of South Africa. According to Gertenbach and Kars (1999) the *Sanga* have a sub-metacentric Y-chromosome in contrast to the acrocentric Y-chromosome of the Zebu (*Bos indicus*) types. The *Sanga* (Nguni) cattle breeds are also well known for their high fertility and tick resistance (Maule, 1973; Scholtz, 1988; Schoeman, 1989). Due to the development of new agricultural practices over the years, *Bos taurus* (i.e. Simmental) breeds were imported for their perceived higher income together with better growth performance and carcass quality. The first Simmentalers were imported into South Africa in 1905 and the breed took a minor position until early sixties when it's superior performance in interbreed trials and growth test centres became known (Scholtz *et al*., 1999). Thus, when commercial feedlots started in South Africa and became common practice to finish cattle for the market, supplementing grass feeding, later maturing *Bos taurus*, such as Simmentaler, became even more popular. In addition, the Brahman (*Bos indicus*) was introduced 1954 into South Africa. The breed was now used as a dam as well as a sire line to overcome the climatic challenges to which the *Bos taurus* breeds could not adapt, and also for its pronounced heterosis with non-indicus breeds (Strydom, 1998).

Addendum B

Table 1: Major characteristics and potential roles of desmin, titin and nebulin in muscle (Robson *et al*., 1991).

Protein	Major characteristics	Potential roles in muscle	Importance in *post mortem* muscle
Desmin	1. Desmin is one of the major types / isomers of proteins comprising 10 nm diameter intermediate filaments (IFs) that, in turn, are part of the cytoskeleton of virtually all animal cells. 2. Insoluble, myofibrillar / cytoskeletal protein (Mr of subunit = 53,000) present in skeletal, cardiac and most smooth muscle cells of vertebrates. 3. The purified protein has the ability, via several structural intermediates, to self-assemble into synthetic, 10nm diameter, very long (> 1-2 µm) filaments. 4. Comprises a set of IFs that encircle the Z-line periphery and radiate out perpendicular to the myofibril axis to ensnare and connect adjacent myofibrils. 5. Link myofibrils to sub cellular organelles, such as nuclei, mitochondria and to the cell membrane skeleton.	1. In developing muscle cells, desmin IFs may help align and tie together adjacent myofibrils, but this remains to be proven. 2. In the developed muscle cell, desmin IFs may help appear to play an important cytoskeletal role in connecting the myofibrils and, in turn, tie or anchor the myofibrils to sub cellular organelles and the cell membrane, i.e. desmin IFs may play a significant role in maintaining overall integrity and organization of the skeletal muscle cell	1. Unknown
Titin	1. Insoluble, giant myofibrillar protein (Mr = 2.8 x 10^6). 2. Present in skeletal and cardiac muscle cells of vertebrates and invertebrates. 3. Comprises about 8% to 10% of total myofibrillar protein in vertebrate skeletal muscle. 4. Titin is a very long (≥ 1µm) molecule, with a globular head and a very long thin tail. 5. A titin molecule spans one half the width of a sarcomere, i.e. from the M-line to the Z-line and, thus forms a third filament within the myofibril.	1. In developing muscle, titin may play a role as part of morphogenetic scaffolding during sarcomeric organisation. 2. In the mature myofibril, titin forms a third filament system that provides sarcomeric alignment (e.g. keeps myosin filaments in register, possibly regulates the length of the thick filaments). 3. Helps maintain overall structural integrity of the sarcomeres, myofibrils and muscle cells.	1. Titin is the third most abundant myofibrillar protein and plays a significant cytoskeletal role in determining the degree of integrity (strength) of myofibrils, muscle cells and muscle tissue. 2. Titin is the only myofibrillar protein that is present from Z-line to Z-line. 3. Is degraded *post mortem* by endogenous proteinases, presumably including the calpains.

Table 1: Major characteristics and potential roles of desmin, titin and nebulin in muscle (Robson *et al*., 1991).

Titin	6. The part of titin located within the sarcomeric A-band, where it appears bound to the outside of the thick filament shaft, is relatively inelastic. 7. The part of titin located within the sarcomeric I-band is "elastic"		4. *Post mortem* alterations in titin and titin extractability appear to be associated with increased beef and pork muscle water-holding capacity.
Nebulin	1. Insoluble, very high molecular weight myofibrillar protein (Mr = 6 to 9 x 10^5). 2. Present in skeletal muscle cells of vertebrates where it accounts for about 3-4% of total myofibrillar protein. 3. Nebulin is apparently a very elongated (~ 1µm) molecule. 4. Nebulin's N-terminus is near the distal (free) end of the thin filament and its C-terminus is located at the Z-line 5. Nebulin's repeating domains can bind to F-actin and perhaps via its C-terminal domain, to α-actinin (Z-line).	1. In developing muscle cells, nebulin may play an important role in organisation of the thin filaments during myofibrillogenesis. 2. In the mature sarcomere, nebulin may contribute to act as a template for assembly (i.e. regulate thin filament length) and / or scaffold for stability of the thin filaments. Nebulin might even comprise part of the thin filament structure. 3. Nebulin may help link / anchor the thin filament firmly to the Z-line structure.	1. Nebulin is rapidly degraded *post mortem*, even faster than titin. This degradation may trigger subsequent *post mortem* alterations in the myofibril. 2. The degradation of nebulin that occurs *post mortem* is presumably due to endogenous proteinases, including the calpains, but this must be carefully documented. 3. Because nebulin is a long, fibrous, structural protein of the sarcomere, and may anchor thin filaments to Z-lines, nebulin's demise *post mortem* may decrease overall cytoskeletal integrity of the myofibrils, muscle cells and muscle tissues. These exact relationships, however, remain nebulous.

Addendum C

Figure 1: Vacuum packed samples.

Figure 2: Mighty Small Transphor Tank Transfer Unit (TE 22, Amersham Pharmacia Biotech).

Figure 3: Nanodrop (ND-1000 Spectrophotometer).

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