

The conditioning of springbok (*Antidorcas marsupialis*) meat: changes in texture and the mechanisms involved

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DECLARATION

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SUMMARY

The purpose of this study was to describe the nature of springbok (*Antidorcas marsupialis*) muscle and the changes that take place in the *longissimus thoracis et lumborum* (LTL) and *biceps femoris* (BF) muscles *post-mortem* (PM); thereby providing recommendations for the handling of the meat.

Springbok muscle contained 64 - 78% type IIX fibres, suggesting that it is considerably more glycolytic than bovine muscle. In males the BF contained more type I and fewer type IIA fibres than the LTL and it appeared that female springbok contained a greater proportion of type IIX fibres than males. The cross-sectional areas (CSA's) of the fibres were low but within the range reported for domestic species. There was an increase in the CSA with the glycolytic capacity of the fibres in males (I < IIA < IIX) but no difference between fibre-types in females.

Springbok muscle cooled rapidly and acidified slowly relative to recommended set points for domestic species, with this being most evident in the female LTL. Differences in cathepsin and calpain activity between the genders and muscles were evident, with the higher calpain activity in the BF and male springbok likely a reflection of the fibre-type composition of these samples. The cathepsin BL activity increased PM, possibly due to the degradation of the lysosomal membranes. Calpain and calpastatin activity declined PM, with correlations ($r = -0.64$; $p < 0.01$) between the pH decline rate and the change in calpastatin activity indicating that more rapid acidification results in a greater decrease in calpastatin activity.

No further improvement in the Warner Bratzler shear force (WBSF) of springbok LTL or BF took place from five to 21 days of ageing. The cathepsin activity increased during the ageing period, with the high activity in the absence of a decline in WBSF suggesting that the cathepsins did not contribute to tenderization. The calpain and calpastatin activity declined to negligible levels by five days PM, suggesting that they were activated *in situ* and were involved in tenderization. Higher WBSF values were found for the BF throughout the ageing period.

Springbok LTL increased in sensorial tenderness and sustained juiciness and decreased in residue from three to eight days PM; however ageing to 28 days increased a number of undesirable aroma and flavour attributes and decreased beef-like aroma. This was most likely due to oxidative and proteolytic changes. The WBSF was low for all ageing periods, with no significant change being found. Gender did not have a large influence on the sensory quality of the meat.

The results of this study indicate that springbok meat tenderizes rapidly PM, with ageing periods of five to eight days being recommended to avoid detrimental flavour changes. The chilling rate appears to have a greater effect on the meat than any differences in the fibre-type composition, with the temperature and pH declines PM indicating a risk of cold-shortening.

However the WBSF values found question the necessity of specialized handling techniques being used.

OPSOMMING

Die doel van hierdie studie was om die aard van springbokvleis en die veranderinge wat plaasvind na dood in die *Longissimus thoracis et lumborum* (LTL) en *biceps femoris* (BF) spiere te omskryf. Sodoende word voorstelle vir die korrekte hantering van springbok vleis voorsien.

Die springbokspiere bevat 64 - 77% tipe IIX vesels wat aandui dat dit aansienlik meer glikolities van aard is in vergelyking met die van bees. In die manlike diere het die BF meer tipe I en minder tipe IIA vesels gehad as die LTL. Daarmee saam het die vroulike springbokke 'n hoër hoeveelheid tipe IIX vesels gehad as die manlike. Die opeervlakte van die springbok vesels was klein, maar steeds binne die omvang van wat gemeld is vir gedomestiseerde diere. Tesame met 'n toename in die glikolities kapasiteit was daar 'n toename in die vesel oppervlakte van die manlike diere ($I < IIA < IIX < IIX$), maar geen verskil is egter gevind vir die vroulike diere.

Die springbok spiere het snel verkoel en redelik stadig versuur, relatief tot die van gedomestiseerde spesies. Dit was die mees voor die hand liggend in die vroulike LTL spier. Verskille in die katepsien en kalpaïen aktiwiteit tussen die geslagte was duidelik en die hoër kalpaïen aktiwiteit in die BF van die manlike diere is waarskynlik as gevolg van die samestelling van die veselipes. Die katepsien BL aktiwiteit het toegeneem na-dood wat moontlik te wyte is aan die afbraak van die lisosomale membrane. Kalpaïen en kalpastatien aktiwiteit het verlaag na-dood en korrelasies ($r = -0.64$; $p < 0.01$) tussen die tempo van die pH daling en die verandering in die kalpastatien aktiwiteit het aangedui dat 'n snel versuring lei tot 'n groter afname in kalpastatien aktiwiteit.

Daar was geen verbetering in die instrumentele sagtheid van die springbok LTL of BF vanaf vyf dae veroudering tot en met 21 dae. Die katepsien aktiwiteit het toegeneem tydens die verouderings tydperk. As gevolg van die hoë aktiwiteit tesame met die afwesigheid van 'n afname in instrumentele sagtheid wil dit voorkom of die katepsiene geen bydrae gelewer het tot versagting. Beide die kalpaïen en kalpastatien aktiwiteit het weglaatbaar afgeneem teen vyf dae van veroudering wat aandui dat hierdie ensieme moontlik geaktiveer is *in-situ* en daarom betrokke was by versagting. Die BF spier het hoër instrumentele taaiheid waardes getoon reg deur die verouderings tydperk.

Die springbok LTL het 'n toename in sensoriese sagtheid en verlangde sappigheid tesame met 'n afname in residu getoon vanaf drie tot ag dae veroudering. Die veroudering tot en met 28 dae het egter verskeie ongewenste aroma en geur eienskappe na vore gebring. Die paneel het ook 'n afname in die bees aroma opgetel. Die voorkoms van hierdie ongewenste sensoriese eienskappe is heel waarskynlik as gevolg van oksidatiewe en proteolitiese veranderinge tydens veroudering. Die instrumentele sagtheid was redelik laag reg oor die verouderingstydperk en geen

beduidende verskille is gevind. Geslag het geen verskil gehad op die sensoriese kwaliteit van die vleis nie.

In geheel toon die resultate van hierdie studie dat springbokvleis snel verouder. Die aanbevole verouderingstydperk is tussen vyf en agt dae om sodoende nadelige aroma en geur veranderinge te vermy. Dit wil blyk of die verkoelingstempo 'n groter invloed op die vleis het as enige verskil in die samestelling van die vesel tipes. Die temperatuur en pH dalings na-dood dui wel op die risiko van kouekrimping maar die resultate rondom die instrumentele sagtheid bevestig wel die noodsaaklikheid van gespesialiseerde hanteringstegnieke.

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NOTES

This thesis is presented in the format prescribed by the Department of Animal Sciences at Stellenbosch University. It is structured to form several research chapters (papers prepared for publication) and is prefaced by an introduction chapter with the study objectives, followed by a literature review chapter and culminating with a chapter containing the general discussion and recommendations.

Language, style and referencing format used are in accordance with the requirements of *Meat Science*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has therefore been unavoidable.

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CHAPTER 1

1.1 General Introduction

With human populations expected to reach 9 billion within the next few decades, there is pressure on agricultural scientists to find new ways of meeting food requirements (Tscharntke *et al.*, 2012; Cawthorn & Hoffman, 2014). This challenge is further complicated by the predicted climate changes as a result of global warming as well as the increasing competition between agriculture and industry as well as residential areas for land (McMichael, Woodruff & Hales, 2006; Godfray *et al.*, 2010).

In most cases efforts to overcome the challenge of increasing food production have led to the intensification of farming, precision farming and the genetic improvement of traditional meat species (Gregory, Ingram, & Brklacich, 2005; Cawthorn & Hoffman, 2014). The inefficiencies of meat production (i.e. 7 kcal of grain are required to produce 1 kcal of meat on average) have also led to calls for a vegetarian future based on the large-scale use of plant-proteins (Godfray *et al.*, 2010; Tscharntke *et al.*, 2012). However, each of these strategies has several short-comings that limit their potential to address food security issues.

Intensive farming methods usually require the use of higher quality feed-materials, which increases the competition between people and animals for products such as maize and soya. So-called 'factory-farming' is also under fire from welfare groups, with a growing awareness among consumers of the realities of mass meat production (Cawthorn & Hoffman, 2014). The potential future gains through conventional breeding practices are also limited by the concomitant decline in genetic diversity within domesticated species (Godfray *et al.*, 2010). Biotechnology and genetic modification may provide a means to overcome this limitation; however, despite wide-spread utilisation in the field-crop industry it continues to battle public opinion in Europe and has yet to make significant inroads into the livestock industry (Azadi & Ho, 2010; Godfray *et al.*, 2010).

The proposal of replacing animal protein with plant protein sources is also not a feasible option as in many areas of the world crop production is not a viable option. In arid to semi-arid countries such as South Africa large areas of land do not have the soil, water-supply or infrastructure to make crop-production possible (Cawthorn & Hoffman, 2014). The value of ruminant species to utilise vegetation in these areas as a food source for extensive meat production should not be ignored (Godfray *et al.*, 2010).

Consumers are also becoming increasingly aware of the importance of conservation and biodiversity, putting pressure on farmers to increase production while reducing the environmental impact of their activities (Godfray *et al.*, 2010). This pressure has resulted in a move away from large-scale, monoculture-type agriculture and is one of the drivers for the

current organic-farming revolution. The use of novel animal species indigenous to a particular area may reduce the impact of meat production on the resident ecosystem and aid in ensuring the sustainable use of both plant and animal resources as well as contributing to biodiversity (Dlamini, Fraser & Grové, 2012)

In South Africa, areas unsuitable for crop production or other forms of agriculture have traditionally been used for the extensive farming of cattle and sheep. However, these species evolved and were selected for very different conditions, and often do not make the best use of the available vegetation or produce optimally. This inefficiency has resulted in profit margins dwindling in the face of increased production costs. Along with increased problems of stock theft and predation in recent years this has seen many farmers make the transition from cattle and sheep farming to game ranching (Sherry, 2009).

Meat production is not currently the focus of the South African game ranching industry, as indicated by the very small proportion of the total annual income (1% in 2007) derived from formal meat sales (Hoffman, 2007). However, if the industry is developed properly, game meat has the potential to supplement the production of red meat by conventional livestock while also allowing the more effective utilisation of marginal grazing lands and reducing environmental degradation (Dlamini & Fraser, 2010). In addition, it can benefit the ecotourism industry, as many foreign visitors consider the consumption of game meat to be part of the so-called "Safari" experience (Hoffman & Wiklund, 2006). The export of game meat can also potentially aid in reducing the existing deficit between imports and exports of red meat and bring capital into the country (Thomas, 2012).

However, in order for the game meat industry to be developed and a stable and sustainable market created the quality of the products supplied needs to be high and consistent (Hutchison, Mulley, Wiklund & Flesch, 2010). One of the most important and, unfortunately, most variable attributes of meat quality is tenderness (Bailey, 1972; King, Wheeler, Shackelford & Koohmaraie, 2009).

The structural integrity of meat and thus the force required to shear it depends on the connective tissue content and the nature of the muscle fibres (Bailey, 1972). The connective tissue content is primarily determined by *ante-mortem* factors, such as the age of the animal, over which game meat producers have limited control (Purslow, 2005), whereas the resilience of the myofibrillar component can be greatly influenced by *peri-* and *post-mortem* factors in addition to the nature of the animal or muscle at its death (Hertzman, Olsson, & Tornberg, 1993). *Peri-mortem* factors include the degree of stress experienced by the animal shortly prior to its death, as well as the changes that occur in the muscle during the rigor period. *Post-mortem* factors on the other hand include processing or handling such as freezing or ageing.

The conversion of muscle to meat is influenced by both the metabolic and contractile nature of the muscle and the handling of the animal prior to and the carcass shortly after slaughter (Hertzman *et al.*, 1993; Klont, Brocks, & Eikelenboom, 1998; Lefaucheur, 2010). The pronounced changes in the chemical and physical conditions in the muscle that occur during the rigor period in turn affect the nature of the myofibril at the end of the rigor period and the further changes that take place during ageing (Warriss, 2000; Hwang & Thompson, 2001). It is therefore necessary to fully understand the nature of meat in order to determine the carcass and meat handling methods that will ensure optimal tenderness and overall sensory quality.

The purpose of this study was therefore to develop a greater understanding of the changes that take place during both the *peri-* and *post-mortem* periods in springbok meat. This knowledge will allow more precise recommendations to be made to the industry, which at this point tends to simply follow procedures determined for similar domesticated species or else procedures that are logistically ideal.

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CHAPTER 2

Literature Review

2.1 Introduction

Internationally, meat that is legally derived from species of animal not generally considered to be domesticated, or not considered to be conventional livestock species, is known as venison. However, in many countries this definition has narrowed over the years to refer only to the meat from various species of deer or *Cervid*. For the purpose of this review, the latter definition will be used, while meat from African ungulates will be referred to as *game* meat.

Game meat is produced as a primary product on game ranches, or as a secondary product in the case of trophy hunting and culling in wildlife reserves. Game ranching is defined by Pollock (1969) as the scientific management of many species of wild animals in their natural habitat on large tracts of land without any effort to domesticate them. This differs from game *farming*, which is described as involving the ‘domestication’ of previously wild species, and the farming thereof in a more intensive manner involving only one or two species (Pollock, 1969). An example of this is the relatively intensive farming of *Cervid* species for meat and antler velvet in North America and New Zealand (Hoffman & Wiklund, 2006).

This difference in production systems lends a number of additional positive attributes to game meat that venison has, over the years, lost. Game ranching by its nature is both “organic” and “free-range”, and this should be made full use of in marketing in order to exploit these lucrative markets (Hoffman, 2007). Other important attributes of game meat are its low fat and cholesterol content as well as high protein content (Hoffman, 2007). The characteristics of game meat and springbok meat in particular will be explored more extensively in section 4.

2.2 The South African game ranching industry

The game ranching industry in South Africa has developed extensively in the last 25 - 30 years, with an increasing number of farmers moving from cattle or sheep farming to game ranching. This change has been driven by a number of factors, both environmental and economic.

A large proportion of South Africa is simply not climatically suitable for conventional farming practices, with low rainfall and poor grazing leading to cattle and sheep farming enterprises having low returns on capital investment (Thomas, 2012). Game species are

better adapted to these areas and use the available vegetation more effectively. It has also been suggested that properly managed game ranches may aid in the recovery of degraded grazing land, as is found in many of the more arid regions of South Africa (Dlamini & Fraser, 2010).

The multiple sources of income available to game ranchers, such as ecotourism, hunting and meat production, also help increase returns (The National Agricultural Marketing Council, 2006; Thomas, 2012). In the Kalahari for example, the capital return on game ranches is around 8.3%, while sheep farming in the same area will only give around 7%. In the poor quality low-veld, returns on cattle farms are estimated to only be around 0.9%, while for game ranching they are around 3% (Thomas, 2012). The increasing problem with stock theft, in the sheep industry in particular, has also pushed farmers away from conventional livestock farming and towards game ranching (Sherry, 2009).

At present the game ranching industry is the sixth largest in the agricultural sector, with around R7.7 billion being generated in 2008 and an estimated R9 billion in 2012/2013 (Thomas, 2012; Dry, 2013). It is estimated that this revenue has grown by *circa* 20.3% per annum over the past 15 years. In 2006 there were reported to be approximately 9000 game ranches in South Africa (The National Agricultural Marketing Council, 2006), with this apparently increasing to over 10000 by 2012 (Thomas, 2012). Around 6330 of these are exempted game ranches, with exemption indicating that suitable fencing for the containment of game species is present and the landowner is effectively granted ownership of any present wildlife (The National Agricultural Marketing Council, 2006). The hunting, capture and sale of wild species on exempted farms is not limited by a hunting season (The National Agricultural Marketing Council, 2006).

Game farms utilise an estimated 20.5 million hectares (mha) of South Africa's 84 mha of grazing land, or 16.8% of the total land area of the country (The National Agricultural Marketing Council, 2006). This shows a significant increase from approximately 14.79 mha in 2005 and 7.04 mha in 1993 (The National Agricultural Marketing Council, 2006).

As previously mentioned, the game ranching industry has several sources of income which can be broadly classified as: trophy or safari hunting, biltong hunting, cropping for the game meat market and live capture and sales (Hoffman, 2007). Although the order of importance varies according to the source of the estimate and the figures on which it is based, the unanimous consensus is that meat sales contribute the least to the income of the game industry as a whole (Hoffman, 2007). In 2000 the estimated annual turnover of game ranching in Limpopo alone was R221 million, with only R7 million of this being generated by meat production (Hoffman, 2007). According to the National Agricultural Marketing Council

the national turnover in 2007 was around R 4.7 billion, with meat production hardly even contributing at 1% (Table 2.1) (Hoffman, 2007).

Table 2.1

The estimated contributions of the different game industry subsectors to the total annual revenue generated (The National Agricultural Marketing Council, 2006)

	Million rand	Percentage of total
Recreational hunting industry	3100	66
Translocation	750	16
Trophy hunting industry	510	11
Taxidermists	200	4
Live animal sales	94	2
Meat production	42	1
Total	4696	100

While these figures may suggest that investment into meat production may not be the best use of funds, several factors need to be taken into account when considering the future of the game ranching industry. Ecotourism and trophy and leisure hunting all represent non-essential activities, and are thus vulnerable to changes in the economic environment. This was seen during the recent global recession, with the numbers of foreign hunters declining between 2008 and 2012 (Thomas, 2012). There is also a limit on the income from trophy hunting as only a relatively small proportion of the total population qualify as trophy animals.

With regards to live sales, the joint contribution of the sale and transport of live game is currently relatively high, especially for rarer species or specimens such as the different colour-variants (Dry, 2013). However, this is a market capable of being saturated, and a decline in the price obtained for the more common species has been observed in recent years (Hoffman, Muller, Schutte & Crafford, 2004).

The production of game meat can provide a more reliable source of income as well as aiding in the population control of species such as springbok (*Antidorcas marsupialis*) and blesbok (*Damaliscus pygargus phillipsi*). In addition, with South Africa currently importing around 5.59% of its beef and 10.99% of its mutton it seems wasteful to not make use of game ranching to produce meat for both domestic and international consumption (DAFF Directorate Statistics and Economic Analysis, 2013). Local sales could potentially supplement the red meat market and reduce the amount of other red meats imported, while the export of game meat would help balance the country's import-export margins (Thomas, 2012).

According to estimates made in 2006, South Africa exports approximately 450 tons of game meat with a value of R15 million per annum, with the majority being destined for the European Union (The National Agricultural Marketing Council, 2006; Thomas, 2012). Current estimates place game meat exports at 2000 tons per annum, with a value to R200 million (Dry, 2013). However, considerable fluctuation in the value of the export market has been experienced, with it dropping from 553 tons in 1987 to only 50 tons in 1995, before rising back to 180 tons in 1998 (Hoffman, 2007). Both domestic and foreign factors were responsible for these fluctuations. In some cases drought or disease (particularly Foot-and-Mouth Disease) resulted in problems with supply and export legislation, while in others marketing problems and competition with other venison sources were responsible (Hoffman, 2007).

Despite the lower per kilogram value of domestically consumed game meat (R20 per kg versus R33 per kg) the local market was estimated in 2006 to consume around R27 million worth of game meat per annum (approximately 1350 tons of meat) (The National Agricultural Marketing Council, 2006). It must however be noted that relative to the amount of red and white meat imported into South Africa annually (approximately R4 billion worth) the value of local game meat consumption and export is relatively low (Thomas, 2012).

Of the 160'000 carcasses exported in 2005, more than 80% were springbok, with significant contributions by blesbok and kudu (*Tragelaphus strepsiceros*) and smaller numbers of zebra (*Equus burchelli*), blue wildebeest (*Connochaetes taurinus*), impala (*Aepyceros melampus*) and gemsbok (*Oryx gazella*) (Hoffman & Wiklund, 2006). Van Rensburg (1997) similarly estimated that 529 of the 553 tons of game meat exported in 1987 was springbok meat (Hoffman, 2007). While more recent figures appear to indicate that the contribution of springbok has declined, it still represents the majority of the game species harvested (Table 2.2).

Springbok are also one of the most popular species for biltong hunting (Hoffman, 2007) and are often farmed in combination with sheep in the Karoo (Milton, Dean & Marincowitz, 1992). It is therefore evident that this species is an important contributor to the game industry.

Table 2.2

The number and percentage of each game species harvested in 2013 by Camdeboo Meat Processors (private communication with P. Neethling)

Species	Number	Percentage
Springbok (<i>Antidorcas marsupialis</i>)	12606	66.52
Kudu (<i>Tragelaphus strepsiceros</i>)	332	1.75
Blesbok (<i>Damaliscus pygargus phillipsi</i>)	1444	7.62
Zebra (<i>Equus burchelli</i>)	1367	7.21
Gemsbok (<i>Oryx gazella</i>)	401	2.12
Blue wildebeest (<i>Connochaetes taurinus</i>)	126	0.66
Black wildebeest (<i>Connochaetes gnou</i>)	1797	9.48
Red Hartebeest (<i>Alcelaphus buselaphus caama</i>)	51	0.27
Impala (<i>Aepyceros melampus</i>)	10	0.05
Water buck (<i>Kobus ellipsiprymnus</i>)	3	0.02
Eland (<i>Taurotragus oryx</i>)	23	0.12
Wild ostrich (<i>Struthio camelus</i>)	792	4.18
Total	18952	100

2.3 The springbok

Springbok are one of the most distinctive game species in South Africa and are internationally associated with the country. One of the smaller wild ungulates, they are strikingly coloured and both males and females have heavily ridged lyrate horns (Smithers, 1983). On average adults stand at a shoulder height of around 75 cm, with males reaching a mature mass of around 41 kg and females around 37 kg (Smithers, 1983).

There are three main subspecies currently recognised, namely *Antidorcas marsupialis marsupialis* (Southern or Karoo Springbok), *A. m. hofmeyri* (Kalahari springbok) and *A. m. angolensis* (Angolan springbok) (Van Aswegen, Labuschagne & Grobler, 2012). The Karoo springbok has the most southern distribution pattern of the three, the Kalahari springbok is found in southern Namibia and Botswana and northern South Africa and the Angolan springbok is found in Angola (Smithers, 1983; Van Aswegen *et al.*, 2012). Significant size variations have been found between the Karoo and Kalahari springbok subspecies, with Karoo springbok having an average shoulder height of 73 cm and a mature body mass of 30.6 kg, while Kalahari springbok are considerably larger, with males having an average height of 77 - 87 cm and mature body mass of 41 kg (Van Aswegen *et al.*, 2012). This difference in size may be linked to differences in the lengths of the alleles at the *BMP4* tandem repeat region (Van Aswegen *et al.*, 2012).

Springbok are the only species of gazelle found south of the Zambezi (Dorst & Dandelot, 1972) and naturally occur in open, dry grassland and other semi-arid areas with open terrain (Smithers, 1983). They are mixed feeders, with grasses (such as *Aristida* and *Schmidtia*) and forbs being preferred but various shrubs, trees, roots and bulbs also being utilised (Dorst & Dandelot, 1972; Milton *et al.*, 1992; Stapelberg, Van Rooyen, Bothma, Van der Linde & Groeneveld, 2008). Springbok are selective and preferential feeders and will tend to change consumption in response to changes in the availability and nutrient content (Stapelberg *et al.*, 2008). They have also been observed to utilise natural licks (hard, exposed clay or soil), possibly to supplement their dietary intake of nutrients (Stapelberg *et al.*, 2008).

Springbok are highly agile and athletic antelope and at full gallop can reach 88 km/hr (Smithers, 1983). One of their most distinctive behaviours is a characteristic movement called 'pronking' or 'stotting'. These are stiff-legged leaps into the air with the back arched and the crest of hair along the dorsal line fanned out. A height of 3.5 m may be attained when pronking and the movement is usually repeated five to six times in a row (Dorst & Dandelot, 1972; Lawrence, Barker, Fairall & Maclay, 1989).

Although in the past springbok were observed to form huge herds, migrating by the hundreds of thousands in search of the best pasture (Dorst & Dandelot, 1972; Smithers, 1983), this is no longer the case. Reduction of numbers by hunting and the rinderpest (caused by Morbillivirus of family *Paramyxoviridae*), as well as the division of land into discrete farms has led to springbok seldom being found in herds greater than 100 animals (Smithers, 1983; Lawrence *et al.*, 1989). They are highly gregarious however (Dorst & Dandelot, 1972), and will form larger herds if possible. They also often associate loosely with other species of herbivore such as wildebeest, blesbok and ostriches (Lawrence *et al.*, 1989).

Springbok remain in groups for most of the year (Skinner & Louw, 1996). However, the size and composition of these groups is not fixed. Herds are usually smaller during dry times when food is scarce, while very large mixed herds can aggregate in areas of new growth after rains (Smithers, 1983). Apart from these mixed herds four types of social groups or arrangements can be distinguished: harem herds, territorial males, bachelor herds and nursery herds (Skinner & Louw, 1996).

Harem herds form during the breeding season, and generally consist of a single adult male and females with offspring (Dorst & Dandelot, 1972; Skinner & Louw, 1996). Older, stronger males can also become territorial during these times, defending specified areas from all other males and attempting to retain female herds within their territory by herding (Smithers, 1983). Territorial areas often include vital resources such as water-points and by

occupying high-value areas dominant males can increase their chances of mating (Skinner & Louw, 1996). Defending a territory does come at a cost however, with territorial males being more vulnerable to exposure and starvation as well as predation (Skinner & Louw, 1996). They also expend a large amount of energy defending their territory from other males (Skinner & Louw, 1996). The period of occupation of a territory differs from region to region and according to prevailing environmental conditions, but can be anything between 6 and 26 months (Skinner & Louw, 1996). Territorial males are seldom under 3 to 4 years of age, as prior to this they are unable to acquire and defend a territory (Skinner & Louw, 1996).

Bachelor herds of between two and 50 individuals are also found, consisting of predominantly adult and yearling male springbok but also occasionally yearling females (Smithers, 1983; Skinner & Louw, 1996). Nursery herds consist of females and young and can number between 11 and 150 individuals, often depending on the type of environment or the total population size. Nursery and bachelor herds usually form with the disruption of the mixed herds at the onset of the lambing season (Smithers, 1983).

Springbok are iteroparous (produce more than one offspring in their life-time) and generally monotonous, with twins being rare (Mentis, 1972). Around 80% of young ewes have been observed to conceive for the first time at around seven months of age, which often coincides with the autumn rutting season (Mentis, 1972; Smithers, 1983). Males mature significantly later than females, with reproduction first becoming possible at around 12 - 13 months of age (Mentis, 1972; Smithers, 1983).

While springbok do not adhere to a strict annual breeding season, with males being available to mate does in oestrus throughout the year, peaks in activity are generally found (Skinner & Louw, 1996). These vary according to the prevailing climate of the area, with springbok in Kimberly and the North-West Province often having an autumn to late autumn (May) rutting season, and springbok in the winter-rainfall area of the Western Cape having high levels of breeding activity in March, and lambing in July (Skinner & Louw, 1996). Springbok in Angola have breeding peaks around December and January, while those in Etosha breed mostly between June and August (Mentis, 1972).

While some studies report that the timing as well of the length of rut is random and unrelated to any specific factor (Hoffman, Kroucamp & Manley, 2007a), it is thought that good rainfall and thus the supply of high quality pasture may contribute (Smithers, 1983; Skinner & Louw, 1996). Rams are the initiators of rutting seasons, with oestrus in does being stimulated through the ram affect and a corresponding synchronised spike in lambing occurring (Skinner & Louw, 1996).

This breeding behaviour is thought to be an adaptation to the arid areas which they inhabit, allowing springbok to multiply rapidly and opportunistically in times of favourable

climatic conditions. The synchronisation of lambing is thought to have the additional advantage of decreasing the negative effect of predation on total lamb survival (Skinner & Louw, 1996). In exceptionally good years two ruts may occur, allowing does to have an annual lambing rate of 200%; however in years of drought there may not be a rutting season at all (Skinner & Louw, 1996).

Springbok have a gestation period of 167 - 171 days (Mentis, 1972) and lambs are on average 3.82 kg at birth (Smithers, 1983). Initially the lambs remain hidden while the does graze and are easily caught by hand, but from 2 to 3 days of age onwards they become more active and will run away if startled (Smithers, 1983). By three to four weeks of age they are running with the rest of the herd and remain with their dam throughout the day (Smithers, 1983).

Does produce only a small quantity of milk daily, with a value of 170 ml per milking being reported by Skinner and Louw (1996). However, the concentration of both protein and butterfat is more than twice that of cow's milk and the lactose content is similar to that of goat and sheep milk and slightly lower than that of cow's milk (Table 2.3) (Osthoff, Hugo & De Wit, 2007). Lambs are completely dependent on milk for the first two weeks of life, where after they begin to utilise some plant material (Osthoff *et al.*, 2007). Grazing behaviour increases from six weeks onwards but lambs are only weaned fully at around 120 days of age (Skinner & Louw, 1996; Osthoff *et al.*, 2007).

Table 2.3

Nutrient composition of springbok milk (%) relative to ovine, caprine and bovine milk (Osthoff *et al.*, 2007).

Nutrient	Springbok	Sheep	Goat	Cow
Water	74.8	83.2	87.2	87.1
Fat	14.5	7.0	5.2	3.9
Protein	7.4	5.3	3.6	3.3
Lactose	4.2	4.3	4.2	4.8

Springbok are reported to have potential annual population growth rates of 35 - 45% (Stapelberg *et al.*, 2008). It is therefore recommended that cropping rates of around 30% are normally used, with this being increased to 40% in periods of favourable environmental conditions when ewes have two lambing seasons in a single year (Skinner & Louw, 1996). It must however be noted that these recommendations are primarily based on anecdotal evidence and further, definitive research is still required.

2.4 Springbok meat production and quality

Springbok grow most rapidly up to the age of 28 weeks, at which point rams and ewes have reached 88% and 92% of their mature mass respectively (Skinner & Louw, 1996; Hoffman, 2007). The dressing percentage of springbok carcasses has been found to be relatively similar to that of domesticated livestock, with Hoffman (2007) reporting dressing percentages of 56.1% and 58.8% in males at 3 - 6 months and over two years, respectively. The corresponding values for female springbok were 53.3% and 55.0%. These values are in agreement with Skinner & Louw (1996), who reported a dressing percentage of 56% in 12 week olds. Van Zyl, Von La Chevallerie and Skinner (1969) reported a similar value for springbok rams (59%), but lower dressing percentage for springbok ewes (51%).

At 12 weeks of age the carcass consists of 83% lean, 13% bone and 4% fat (Skinner & Louw, 1996). While a marginal increase in the fat content with age does occur, it still very seldom exceeds 4%, which is low relative to domesticated species (the carcass lipid content of beef is reported as being 24.1%) (Skinner & Louw, 1996; USDA, 2014a). One of the most distinctive characteristics of springbok carcasses are their disproportionately large *Longissimus thoracis et lumborum* (LTL) muscles, with the cross-sectional area of the muscle in 12 week old male springbok being 16.6 cm², in comparison to only 12.6 cm² in well-fed sheep (Skinner & Louw, 1996). This is thought to be due to their high speed gallop and distinctive “pronking” or “stotting” movement, and is of economic importance considering the high value of this muscle or carcass cut. Apart from this exception springbok carcasses have been found to have a similar composition of cuts as 20 week old sheep. This is in terms of the percentage of loin plus rump and shoulder plus chine in the carcass (Skinner & Louw, 1996).

The proximate composition of the lean meat gives an indication of the nutritional value of the meat. Springbok lean has been found to have a proximate composition of 73.4 - 74.4% moisture, 18.8 - 21.2% protein, 1.3 - 3.5% intramuscular (IM) fat and 1.1 - 1.4% ash (Hoffman, Kroucamp & Manley, 2007b). This indicates a similar protein content to beef (20.94 - 21.79%) but a slightly lower IM fat content (4.87 - 6.33%) (Sales & Hayes, 1996; USDA, 2014b). This low fat content is one of the main selling points for game meat, as is its favourable polyunsaturated to saturated fatty acid ratio (PUFA:SFA) (Hoffman, Kroucamp & Manley, 2007c).

The World Health Organization recommends a PUFA:SFA ratio of at least 0.4, and springbok meat has been found to have values ranging from 0.96 to 1.18, with an average of 1.06 (Hoffman, 2007). As a point of reference, the PUFA:SFA ratio for grass-fed beef has been reported to be 0.07 - 0.75 (Daley, Abbott, Doyle, Nader & Larson, 2010). The high PUFA content of springbok meat is most likely as a result of its pasture rather than grain-

based diet as well as the low total IM fat content of the meat. Raising animals on pasture rather than grain has been reported to increase the proportion of polyunsaturated fatty acids (Fisher *et al.*, 2000; Wiklund, Manley, Littlejohn & Stevenson-Barry, 2003), and neutral storage lipids tend to have a diluting effect on the structural phospholipids present in meat, which are predominantly unsaturated (Lawrie & Ledward, 2006).

In addition to the high total PUFA content (36.3 - 41.4%), springbok meat has also been reported to have an omega-6:omega-3 ratio of below 4:1, which is favourable as ratios below 5:1 are suggested to reduce blood pressure and inflammation (Hoffman *et al.*, 2007c). The predominant omega-6 polyunsaturated fatty acid was linoleic acid, with α -linolenic acid contributing the most to the omega-3 fatty acids (Hoffman *et al.*, 2007c).

Springbok meat is reported to have cholesterol levels of 54.45 - 59.34 mg/100g, compared to 41 mg/100g for grass-fed beef top loin and 66 mg/100g for grass-fed lamb loin (Hoffman *et al.*, 2007c; USDA, 2014b; USDA, 2014c). There is however considerable variation in reported cholesterol levels and grain-fed animals tend to have higher levels although actual data demonstrating this is limited (Daley *et al.*, 2010).

The two most prevalent amino acids in springbok meat are glutamic and aspartic acid, with leucine and lysine being the essential amino acids with the highest concentrations (Hoffman *et al.*, 2007b). This is similar to the amino acid composition found for beef and ostrich, with the exception that in these two meats lysine has the highest concentration of the essential amino acids, whereas in springbok meat the leucine concentration is higher (Sales & Hayes, 1996). Grass-fed beef also seems to have higher levels of glutamic acid relative to aspartic acid than springbok meat (USDA, 2014b).

Potassium and phosphorous had the highest concentrations of the minerals tested for in springbok meat, with calcium being the next most prevalent mineral (Hoffman *et al.*, 2007b). In comparison to beef springbok loin was found to have a much lower potassium (beef: 350 mg/100g; springbok: 126.7 mg/100g), phosphorous (beef: 180 mg/100g; springbok: 144.5 mg/100g), sodium (beef: 61 mg/100g; springbok: 14.09 mg/100g) and zinc (beef: 4.3 mg/100g; springbok: 1.3 mg/100g) (Sales & Hayes, 1996; Hoffman *et al.*, 2007b). In contrast, springbok contained considerably more calcium (beef: 7 mg/100g; springbok: 68.16 mg/100g) and slightly more iron (beef: 2.1 mg/100g; springbok: 2.8 mg/100g) (Sales & Hayes, 1996; Hoffman *et al.*, 2007b). A number of factors can influence the mineral content of meat however, one of which is the animal's diet (Hoffman *et al.*, 2007b). It is therefore difficult to say whether these differences represent a species effect or are simply due to different rearing methods being used.

As can be seen in Table 2.4 springbok meat shear force values were found to be lower than those for meat from other game species such as impala, kudu and mountain reedbuck,

and considerably lower than values reported for beef. The shear force was also found to be inversely correlated with tenderness and sustained juiciness as rated by a sensory panel, with correlations of -0.70 and -0.43 respectively being reported (Hoffman, Kroucamp & Manley, 2007d).

Table 2.4

Reported shear force values for springbok meat relative to beef and impala, kudu and mountain reedbuck meat.

Species	Shear force (N)	Source
Springbok	15.8 - 17.9	Hoffman <i>et al.</i> (2007a)
	12.9 - 20.7	Kroucamp (2004)
	22.38	Van Rensburg (1997)
Beef	47.3 - 65.0	Crouse & Koohmaraie (1990)
	31.0 - 54.2	Shackelford, Wheeler & Koohmaraie (1997)
Lamb	21.6 - 73.9	Hopkins <i>et al.</i> (2011)
Impala	31.9	Hoffman, Mostert, Kidd & Laubscher (2009)
Kudu	32.1	Hoffman <i>et al.</i> (2009)
Mountain reedbuck	23.0	Hoffman, Van Schalkwyk & Muller (2008)

Conditioning for three to seven or three to ten days has been suggested as resulting in optimum tenderness in springbok meat (Skinner & Louw 1996; Van Rensburg, 1997). In addition to being tender springbok meat has also been found to generally have a finer texture than other game meats, with a fibre-diameter of 45.5 μm being reported by Skinner & Louw (1996).

Colour measurements on springbok meat were found to be relatively typical of game meat. L^* values were below 40, a^* values were high and b^* values were low (L^* : 30.71 - 34.93, a^* : 12.05 - 18.36, b^* : 7.80 - 9.01) (Hoffman *et al.*, 2007a). In this study gender was found to have a significant ($P < 0.05$) effect, with females having higher a^* and chroma values (a^* females: 14.95 vs males: 13.29; chroma females: 17.35 vs males: 15.63) (Hoffman *et al.*, 2007a).

2.5 Harvesting and processing

Methods used for the harvesting and processing of springbok meat can be divided into two types according to whether the meat is destined for export or the local market. While exported meat is strictly regulated according to rules imposed by the EU (Commission Regulations no. 178/2002, 1441/2007, 2073/2005, 2075/2005, 854/2004, 852/2004 and

853/2004; Commission Decision 2008/752/EC; Council Directives 98/83/EC, 2002/99/EC and 2003/99/EC) there is still relatively little control over meat produced for local consumption.

Game ranches wishing to supply animals to export abattoirs are required to register with the Provincial Veterinary Authority, and certain minimum requirements must be met for this application to be approved. These minimum requirements relate to factors such as the occurrence of Foot-and-Mouth and other diseases, animal welfare, the use of growth enhancers and therapeutic remedies, and stocking and cropping on the ranch (DAFF National Directorate Veterinary Quarantine and Public Health, 2010a). If these minimum requirements are adhered to the owner of the game ranch is supplied with a Registration Certificate for export. Ranches are inspected annually to determine whether they still comply with the regulations (DAFF National Directorate Veterinary Quarantine and Public Health, 2010a).

Harvesting for export is done according to Veterinary Procedural Notices VPN/09/2010-01, VPN/08/2010-01 and VPN/10/2007-01 among others, which are developed and updated annually by DAFF and are designed to align with EU export requirements (DAFF, 2007; DAFF, 2010b; DAFF, 2010c). The harvesting of game animals for export usually involves high intensity cropping, with each team cropping at least 20 animals at a time (Hoffman & Wiklund, 2006).

Springbok are shot at night by professional marksmen on vehicles, with high-powered spotlights being used to locate and blind the animal. Using this method a single marksman can crop 30 - 80 springbok per night with minimal stress to the remainder of the herd. Light-calibre rifles and head and neck shots are used to minimise stress and the loss of usable meat from the carcass (Hoffman & Wiklund, 2006). Body shots can lead to large losses of saleable meat, while shots to the neck generally only result in the loss of 3% of the carcass, with almost no loss with head shots (Skinner & Louw, 1996).

Once the springbok has been shot the jugular vein and carotid artery are severed and the carcass is hung from the side of the vehicle to minimise contamination and aid exsanguination (Hoffman & Wiklund, 2006). Within two hours of death the carcass is delivered to the game depot, where evisceration takes place. It is then weighed and, along with the pluck, undergoes primary inspection before being placed in a refrigerated truck held at 5°C (Van der Merwe, Jooste & Hoffman, 2011; Van der Merwe, Hoffman, Jooste & Calitz, 2013). The carcasses are required to be placed in a refrigeration unit within 4 hours *post-mortem* at ambient temperatures above 12°C and within 12 hours *post-mortem* at ambient temperatures below 12°C (Van Schalkwyk & Hoffman, 2010; Van der Merwe *et al.*, 2013).

Depending on the harvesting team the refrigerator truck is usually full within three days, with the carcasses then being transported to a game meat processing facility (Private communication with Piet Neethling from Camdeboo and Charl de Villiers from Mosstrich). The internal muscle temperature is required to remain between 4°C and 7°C throughout transport (Van Schalkwyk & Hoffman, 2010). In order to deactivate the Foot-and-Mouth virus game meat carcasses destined for export are required by law to be held at an ambient temperature above 2°C, with an internal muscle temperature of 4°C to 7°C and pH of below 6 for 24 hours prior to deboning (Van Schalkwyk & Hoffman, 2010). Commercial game meat producers in South Africa usually carry out this maturation period on the arrival of the carcasses at the processing facility (Private communication with Piet Neethling from Camdeboo and Charl de Villiers from Mosstrich). The carcasses also need to be skinned and undergo a second inspection prior to further processing (Van der Merwe *et al.*, 2013). The combined time periods required for harvesting, transport, maturation and deboning normally result in carcasses being deboned between three and seven days *post-mortem* (Private communication with Piet Neethling from Camdeboo and Charl de Villiers from Mosstrich).

The deboned meat is further processed into a variety of products, most of which are exported. Cuts produced include strip loin, tenderloin, fillets, primal leg cuts, deboned shoulders, goulash and trimmings, while a few value-added products are also produced, such as steaks, sausages, hamburger patties, cubes, kebabs, deboned legs that have been larded and barded and shanks (Private communication with Piet Neethling from Camdeboo and Charl de Villiers from Mosstrich). Only boneless cuts may be exported to the EU due to the possible presence of a strain of tuberculosis that is transmitted in the bone (Hoffman, 2007). The remainder of the carcass, which mainly consists of off-cuts, is sold locally. Most of the meat is sold frozen, with meat from Mosstrich abattoir having a frozen shelf-life of three years. Camdeboo Meat Processors also sell meat frozen to fresh, with a shelf-life of 7 days. At present neither of the two main export abattoirs sells whole carcasses (Private communication with Piet Neethling from Camdeboo and Charl de Villiers from Mosstrich).

Unlike the export market, there are currently few regulations controlling the production of game meat for the local market. While there is a section in the Meat Safety Act (Act 40 of 2000) regarding game and crocodile meat, the enforcement of these regulations can be challenging and there are serious logistical problems with the supply of independent meat inspectors to inspect all the game meat produced in the country (Van der Merwe *et al.*, 2011; Van der Merwe *et al.*, 2013).

Variation in the cropping method used can potentially affect meat quality by increasing the *ante-mortem* stress level. Studies on kudu and impala have found that animals are

stressed more by day than night harvesting (Kritzinger, Hoffman & Ferreira, 2003; Hoffman & Laubscher, 2009). This can result in the meat having a higher ultimate pH, which shortens the shelf-life and increases the risk of spoilage. Alternative methods involving shooting from helicopters or using helicopters to herd the buck into bomas, where they are subsequently shot, are also occasionally utilised (De Bruyn, 1993; Hoffman & Laubscher, 2010). However the high cost per animal of these methods has reduced their popularity

While levels of hygiene are not necessarily poorer on non-registered ranches producing meat for the local market, the maintenance of the cold chain is often lacking. Game meat produced for export is required to be placed in a refrigerator unit within four hours *post-mortem*; while in systems that do not have to comply with VPN, this time period is often closer to 12 to 13 hours (Van der Merwe *et al.*, 2013). However, in a study by Van der Merwe *et al.* (2013) assessing microbial levels on game meat it was found that the meat is not necessarily of poor quality or unsafe for human consumption. The highly extensive nature of game farming likely contributes to the relatively clean status of game meat, even when harvesting and dressing procedures do not comply to Good Hygiene Practices or Good Manufacturing Practices (Van der Merwe *et al.*, 2011).

Overall it can be said that while game meat produced for the local market is not necessarily of lower quality than exported meat, the risk of poor quality products is higher because of the current lack of regulation, enforcement and traceability.

2.6 Factors effecting meat tenderness and texture

Tenderness is one of the most important eating qualities for the consumer as well as one of the most variable attributes of meat (Bailey, 1972; Kerry & Ledward, 2009). It is defined according to sensory evaluation as a combination of three sensations: firstly the ease with which the teeth initially penetrate the meat, secondly the ease of fragmentation during chewing and thirdly the abundance of residue remaining after chewing (Lawrie & Ledward, 2006). The perception of the texture of meat is inseparable from that of tenderness and yet there is seldom a simple or direct relationship between the two (Lawrie & Ledward, 2006). Texture is defined in older texts as the visually assessed grain of the meat, as determined by the size of the fibre-bundles as well as the quantity of connective tissue surrounding the fibres and fibre-bundles (Purslow, 2005; Lawrie & Ledward, 2006).

While instrumental methods such as the Warner Bratzler shear force have been developed in an effort to assess tenderness without the time and cost of a sensory panel, these do not take into account secondary factors such as sustained juiciness that can influence the perceived tenderness. This has led to some studies not finding the expected or desired correlations between sensory ratings for tenderness and instrumental measures of

texture (De Huidobro, Miguel, Blázquez & Onega, 2005). Merely measuring the cross-sectional area of the muscle fibres or the size of the fibre-bundles also does not satisfactorily describe the texture of a bite of meat.

A wide variety of animal and production factors play a role in determining meat texture and tenderness. These factors can be divided into three main groups: those that are related to the structural and biochemical composition of the meat, as determined by the nature of the animal or how it was reared (*ante-mortem* factors), those that are influential around the time of death (*peri-mortem* factors) and those that play a role in the changes in the meat during ageing (*post-mortem* factors) (De Huidobro *et al.*, 2005; Lefaucheur, 2010).

2.6.1 *Ante-mortem* factors

2.6.1.1 *Fibre-type and diameter*

Mammalian muscle tissue can be organised into primary, secondary, tertiary and quaternary levels of organization. The primary structure, the contractile elements or myofibrils, are contained in muscle cells or fibres (secondary), which are further grouped into fibre bundles or fascicles (tertiary), and then finally entire muscles (Bailey, 1972). While the basic biochemical structure and function of different muscle fibres is relatively similar, they do differ in several important ways. These include the preferred energy source, dominant pathway for the release of energy from these substrates and rate of contraction; thus leading to the classification of muscle fibres into type I and type IIA, IIX and IIB (Lawrie & Ledward, 2006; Curry, Hohl, Noakes & Kohn, 2012).

Muscle fibres can be classified on several different levels, or according to different criteria, namely macroscopic appearance, metabolic type, contraction rate and histochemically (Klont, Brocks & Eikelenboom, 1998; Warriss, 2000; Lefaucheur, 2010). Macroscopically muscles are often described as being either red or white, thus leading to the similar classification of the fibres themselves (Warriss, 2000). The physical appearance of a muscle fibre is primarily determined by the myoglobin and mitochondrial (and therefore cytochrome) content, both of which are red in colour. The myoglobin and cytochrome content of a fibre is directly related to its metabolism, with high levels generally indicating a dependence on oxidative rather than glycolytic metabolism (Warriss, 2000). This allows the redefinition of red and white fibres more accurately as being either aerobic or anaerobic respectively.

Red (aerobic) fibres are typically more prevalent in muscles required for slower, more continuous activity (such as postural muscles); thus making oxidative rather than glycolytic energy production favourable (Klont *et al.*, 1998; Warriss, 2000; Lefaucheur, 2010). High concentrations of myoglobin make the sufficient supply of oxygen possible, while large

numbers of mitochondria and high activity levels of enzymes such as cytochrome oxidase, succinic dehydrogenase (SDH) and citrate synthase (CS) are indicative of high levels of aerobic metabolism (Klont *et al.*, 1998; Warriss, 2000; Curry *et al.*, 2012). The activity of SDH has been used as a reference enzyme for the oxidative capacity of fibres, allowing their classification as red, white or intermediate (Klont *et al.*, 1998; Lefaucheur, 2010).

The capability of red fibres to produce energy through glycolysis alone is relatively limited; with glycolytic enzymes such as lactate dehydrogenase (LDH), phosphofructokinase (PFK) and creatine kinase (CK) having low activity levels (Curry *et al.*, 2012). In order to cater for the increased demand for oxygen, as well as significant heat production, red muscle fibres are narrower and more closely associated with a larger number of blood capillaries than white fibres (Klont *et al.*, 1998; Lawrie & Ledward, 2006; Lefaucheur, 2010). The energy sources of red and white fibres also differ, with the glycogen present in red fibres resembling amylopectin and that in white fibres amylose. Red muscle fibres are also capable of utilising lipids as a primary energy source during prolonged activity; with fat droplets commonly being present (Klont *et al.*, 1998; Lawrie & Ledward, 2006). High activity levels of 3-hydroxyacyl-CoA dehydrogenase, an enzyme involved in the β -oxidation of fatty acids, is further indicative of the importance of fatty acids for energy production in these fibres (Curry *et al.*, 2012).

White (or anaerobic) fibres are generally adapted to intense, short term contraction (Lefaucheur, 2010) and are therefore predominantly glycolytic, have a large cross-sectional area and contain lower concentrations of myoglobin and mitochondria (Lawrie & Ledward, 2006). White fibres contain high concentrations of glycogen to fuel anaerobic metabolism and have high levels of both ATPase and phosphorylase activity. Phosphocreatine also provides an important source of energy for contraction (Lefaucheur, 2010). The activity of enzymes involved in oxidative metabolism is however low (Warriss, 2000).

Fibres classified as intermediate are also found, typically having high levels of ATPase activity (similar to white fibres), but low phosphorylase activity levels (as is found in red fibres) (Lawrie & Ledward, 2006).

The second basis of fibre typing is according to contraction rate, with fibres being classified as either fast- or slow-twitch. Slow-twitch fibres are also known as type I fibres and are relatively analogous with red fibres, whereas fast-twitch fibres are specified as type II fibres and share many traits with so-called white fibres (Warriss, 2000). Slow-twitch fibres are typically associated with muscles used for the maintenance of posture while fast-twitch fibres are used for less sustained but rapid movement (Warriss, 2000). While slow-twitch fibres are relatively fatigue resistant, they generally cannot produce as much force as fast-twitch fibres, and have low maximum force generation values (Curry *et al.*, 2012). Type I

fibres are also highly sensitive to calcium and thus have low excitation thresholds (Lefaucheur, 2010).

The classification of fibres according to both contraction rate and metabolic type originally led to the subdivision of type II into type IIA and B. Type IIA fibres are aerobic but also fast-twitch, having all the typical characteristics of a red fibre but also containing high levels of glycogen (Warriss, 2000). The glycogen levels in type IIA fibres are also maintained and returned to normal relatively rapidly following depletion, making the fibres relatively stress resistant. While classified as oxidative, type IIA fibres are however not as efficient at oxidative energy production as type I fibres (Lawrie & Ledward, 2006).

Type IIB fibres on the other hand are almost purely glycolytic, containing relatively high levels of glycogen but lower levels than type IIA (Lefaucheur, 2010). Glycogen levels in type IIB muscles are also slow to return to normal, making the fibres extremely susceptible to stress (Warriss, 2000). The few mitochondria present in this type of fibre tend to be clustered around the I-band rather than distributed throughout the fibre (Lawrie & Ledward, 2006).

The final and most specific definition of the different types of fibres relates to the type of myosin heavy chain (MHC) isoform present in the myofibril (Lawrie & Ledward, 2006). Four different types of MHC have been identified in adult mammalian skeletal muscle tissue (MHC I, IIA, IIX and IIB), leading to the specification of a further fibre type, namely type IIX. This has led to some confusion as in older literature the fibres referred to as type IIB (according to contraction rate and metabolic type) are most likely now specified as type IIX (according to MHC isoform). This is seen in the description of the fibre type composition of bovine LTL found in Lawrie & Ledward (2006), as 46 - 59% of the fibres are reported to be type IIB, while more recent research has indicated that this type seldom occurs in the skeletal muscle of large mammals (Greenwood, Harden & Hopkins, 2007; Lefaucheur, 2010; Curry *et al.*, 2012). When found to be present they are usually restricted to muscles with specialized functions, such as those controlling the eye (Curry *et al.*, 2012). The situation is confused further by some literature specifying fibres as type I, IIA, IIBR (SDH+) and IIBW (SDH-), based on the combination of ATPase staining with variable pH pre-incubation and SDH staining (Lefaucheur, 2010). In this thesis the fibre types will henceforth be referred to as type I, IIA, IIX and IIB, with biological characteristics as indicated in Figure 2.1. In some cases type IIX and type I/IIA fibres are also mentioned; these are intermediate between types IIA and IIX, and types I and IIA, respectively (Greenwood *et al.*, 2007).

There is a progressive increase in the rate of contraction as well as the myosin ATPase activity from type I to type IIA, IIX and IIB (Lawrie & Ledward, 2006). Type IIX fibres are abundant in the skeletal muscles of large mammals, including those of springbok (Curry *et al.*, 2012). They are classified as being fast-twitch glycolytic in terms of contraction rate

and metabolic type; however their oxidative capacity is generally higher than that of type IIB fibres and can vary greatly (Greenwood *et al.*, 2007; Lefaucheur, 2010; Curry *et al.*, 2012). As is typical of most glycolytic fibres they contain relatively few mitochondria and show high activity levels for enzymes forming part of the glycolytic pathway (Curry *et al.*, 2012). As previously mentioned, it appears that type IIX is similar to the type previously known as type IIB, while type IIB (immunohistochemically determined) is also classified as a fast-twitch glycolytic fibre but is mostly restricted to small mammals such as rodents (Curry *et al.*, 2012). Fibre-types I and IIA are analogous to the fibres specified as slow oxidative and fast oxidative-glycolytic respectively, as classified according to enzyme-activity based histochemistry (Greenwood *et al.*, 2007).

As a result of the biochemical differences between the fibre-types the proportional composition of a muscle has a distinct effect on the behaviour of that muscle *post-mortem*, with this including both the changes during rigor as well as those during more extensive ageing. The decline in pH and temperature during the *peri-mortem* period will be discussed more extensively in section 6.2.1 but considering the role played by fibre-type it will be considered to some extent in this section as well.

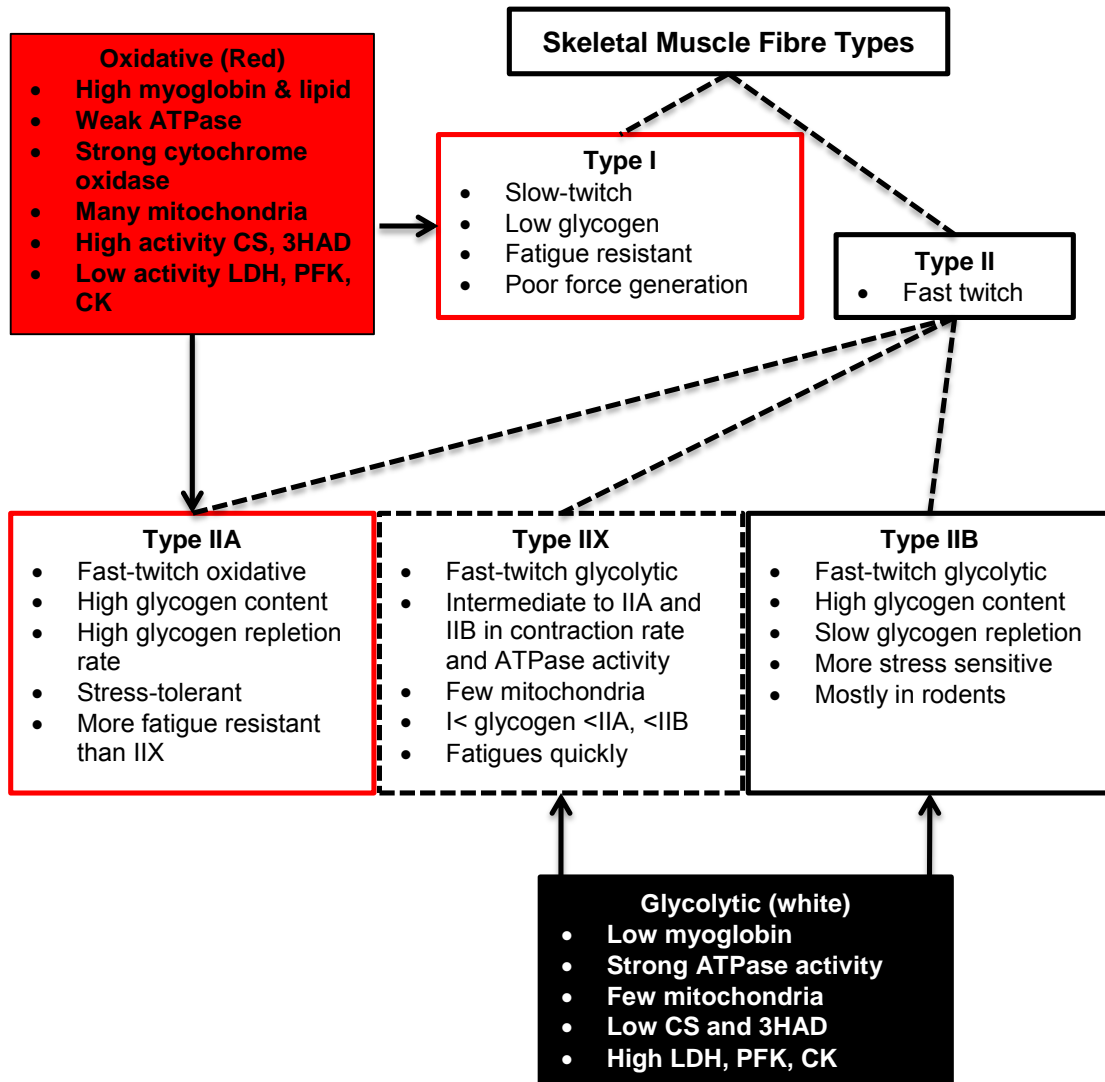


Figure 2.1 The contractile, metabolic and physical characteristics of skeletal muscle fibre types as classified according to MHC isoform type (Klont *et al.*, 1998; Warriss, 2000; Lefaucheur, 2010; Curry *et al.*, 2012).

(CS - citrate synthetase; 3HAD - 3-hydroxyacyl CoA dehydrogenase; LDH - lactate dehydrogenase; PFK - phosphofructokinase; CK - creatine kinase).

The rate and extent of the change in the pH of a muscle *post-mortem* influences numerous quality-related factors, including meat colour, water-holding capacity and tenderness (Kim, Warner & Rosenvold, 2014); (Lefaucheur, 2010). This change is as a result of the build-up of lactic acid and usually involves a decline from around pH 7.1 (*in vivo*) to 5.4 - 5.5 (pH_u) (Lawrie & Ledward, 2006). Lactic acid is produced from glycogen by the glycolytic process as the muscle cells attempt to maintain ATP levels and thus homeostasis (Lefaucheur, 2010). In the absence of a depletion of the glycogen content the pH will

continue to decline until it inhibits the activity of the enzymes involved (Lawrie & Ledward, 2006).

Glycolysis is stimulated by the increased concentration of inorganic phosphorous produced from ATP by non-contractile myosin ATPase activity attempting to maintain body temperature and the structural integrity of the muscle (Lawrie & Ledward, 2006). While ATP can for a short period be produced from the reaction between ADP and creatine phosphate, and some oxidative metabolism can continue while myoglobin reserves still supply oxygen, these systems are quickly depleted, leaving glycolysis and thus lactic acid production as the only alternative (Lawrie & Ledward, 2006).

Muscle cells containing greater quantities of myoglobin (type I), should therefore maintain a higher pH for longer than the more glycolytic muscle fibre types. This effect is seen most distinctly in whale muscles, with pH levels remaining high for an extended period *post-mortem* due to the high myoglobin content of the muscle (Lawrie & Ledward, 2006). Muscles with a high proportion of slow oxidative fibres ("red" muscles) have also been found to have higher ultimate pH levels than muscles containing more glycolytic fibres (Klont *et al.*, 1998; Lefaucheur, 2010). The higher activity levels of the glycolytic enzymes, as well as the presence of greater quantities of glycogen, may also contribute to the more rapid decline in pH and more rapid onset of rigor in so-called 'white' muscles (Klont *et al.*, 1998). The buffering capacity of the muscle fibres can also have a significant effect on the rate of pH decline (Lefaucheur, 2010).

Type IIA and IIB fibres have also been found to shorten to a lesser extent than oxidative fibres if unrestrained during rigor (Klont *et al.*, 1998). This is thought to be due to the more extensive and efficient sarcoplasmic reticulum network found in fast-twitch fibres, which potentially gives them a greater ability to control the concentration of calcium within the sarcoplasm and thus the degree of contraction (Lefaucheur, 2010). Slow twitch type I fibres also have lower sarcoplasmic reticulum ATPase activity, which reduces their ability to maintain sarcoplasm calcium concentrations (Lefaucheur, 2010).

In addition to the effect of variation in fibre type the environmental conditions present during the slaughter of the animal and the processing of the carcass also have a large effect on the pH and temperature changes in the meat. The different fibre types have also been found to respond differently to various stressors and conditions, with type I fibres being more susceptible to cold stress and type IIA and IIB/X fibres being more affected by lairage and agonistic-related stressors (Klont *et al.*, 1998; Lawrie & Ledward, 2006). Type IIB fibres are also generally more susceptible to stress and glycogen depletion as a result of their slow glycogen repletion rate (Lawrie & Ledward, 2006). This indicates that different muscles may

respond best to different handling protocols, something which should be taken into account when setting up handling procedures for meat.

The relationship between the decline in pH and the decline in temperature has a dual effect on the final tenderness of the meat. Not only does it greatly determine the degree of shortening that takes place during the onset of rigor (Bailey, 1972; further discussed in section 6.2.2) but it may also affect the rate of enzymatic tenderization of the meat. The activation of the calpains, which are one of the most important proteolytic enzymes involved in changes during ageing, is extremely pH and temperature dependant (Ertbjerg, Henckel, Karlsson, Larsen & Møller, 1999; Huff Lonergan, Zhang & Lonergan, 2010). Calpain I is also thought to be most active during the first 24 - 48 hours *post-mortem* (Dransfield, 1994; Lawrie & Ledward, 2006). This will be discussed further in section 6.3.2.

Apart from influencing the conversion of muscle to meat, fibre type may also have a more direct effect on ageing. It has been found that type I fibres have a 2 - 5 times greater rate of protein turnover than type II fibres (Lawrie & Ledward, 2006). A higher rate of protein turnover generally indicates greater concentrations of proteolytic enzymes, and this has indeed been found, with slow-twitch red muscles having the highest concentrations of calpains (Klont *et al.*, 1998). However, a correlation between the slow MHC isoform present in type I fibres and levels of calpastatin activity has also been found and the calpain:calpastatin ratio has been reported to be higher in fast twitch glycolytic muscle in pigs, sheep and cattle (Lawrie & Ledward, 2006; Lefaucheur, 2010). This indicates that the rate of calpain-driven tenderization will not be as high as one would expect based on the calpain content alone (Lawrie & Ledward, 2006). This is supported by the finding that slow-twitch red muscles have the slowest rates of tenderization (Klont *et al.*, 1998). There is considerable controversy on the effect of fibre type on meat quality and tenderness however, with some studies reporting positive correlations between the type I fibre content and tenderness while others suggest that increasing the proportion of type II fibres would be beneficial (Lefaucheur, 2010).

In addition to influencing biochemical factors, fibre-type can also have an effect on the physical nature of the meat as a result of variation in fibre diameter between the fibre types (Klont *et al.*, 1998). A negative correlation between oxidative capacity and fibre diameter has been reported, with type I fibres having the smallest diameter and type IIB the largest (Klont *et al.*, 1998). Fibre diameter can have a significant, if not always predictable, effect on the tenderness of meat (Lefaucheur, 2010).

Assuming a constant endomysial thickness it would seem logical that a finer texture (i.e. smaller muscle fibres) would result in tougher meat due to the higher ratio of connective tissue to fibre area (Lawrie & Ledward, 2006). However, many studies suggest that

increasing the cross-sectional area of muscle fibres would have a detrimental effect on meat sensory quality and that fine-grained meats are preferred (Purslow, 2005; Lefaucheur, 2010). However, despite correlations between increased muscle fibre cross-sectional area and reduced textural quality being found, numerous other factors have often been involved, casting doubt on whether the correlation is in fact causative as well (Lefaucheur, 2010).

Apart from the influence of fibre type, fibre diameter is also extremely dependant on the function of the muscle (Lawrie & Ledward, 2006). For functional reasons muscles that are required to make small, precise adjustments are finer in texture, while those that are more required for brute force than finesse consist of fibres with greater cross-sectional areas (Lawrie & Ledward, 2006).

A number of factors such as age, gender and species influence the proportion of fibres of each type in a specific muscle. In addition considerable variation has been found between muscles in the same animals as well as at different positions within the same muscle (Lefaucheur, 2010).

Significant differences in fibre type composition have been reported between different species (Table 2.5). Higher levels of type I fibres in bovine rather than porcine *longissimus* muscles have been ascribed to the slower-moving and more fatigue-resistant nature of cattle (Lawrie & Ledward, 2006; Lefaucheur, 2010). The effect of breeding has also been indicated, with wild pig muscles containing greater numbers of type IIA fibres, which have a greater capacity for oxidative energy production than the other type II variants, than domestic pig muscles (Lawrie & Ledward, 2006). This may also be as a result of the rather more sedentary life of modern domestic pigs, while wild pigs are still required to search and travel for their food. Alternatively the selection for increased growth rate and greater lean meat production may have resulted in a shift in fibre type from oxidative towards glycolytic, as has been found in other studies (Lefaucheur, 2010). This selection has also been suggested to be responsible for the larger cross-sectional areas found in domesticated species (Lefaucheur, 2010).

Table 2.5

The fibre type composition (%) of the *Longissimus* muscle from several species

	I	IIA	IIAX	IIIX/IIIB	Source
Springbok	3 - 9	15 - 29	-	64 - 80	Curry <i>et al.</i> (2012)
Fallow deer	5 - 9	33 - 51	-	41 - 61	Curry <i>et al.</i> (2012)
Cattle	29.5 - 33.1	14.9 - 29.0	-	39.9 - 52.6	Vestergaard, Oksbjerg & Henckel (2000); Hwang & Joo (2010)
Sheep	5.7 - 6.5	16.7 - 27.0	8.4 - 10.1	56.1 - 65.4	Greenwood <i>et al.</i> (2007)
Pig	6.0 - 11.0	6.5 - 12.3	-	78.4 - 87.5	Choe <i>et al.</i> (2008)

Studies on fallow deer (*Cervus dama*) and springbok (*Antidorcas marsupialis*) have indicated a strong positive correlation between the maximum sprinting speed of a species and the abundance of type IIX fibres present in the muscle (Curry *et al.*, 2012). Both the *Longissimus thoracis et lumborum* and *Vastus lateralis* muscles of springbok had higher proportions of type IIX and lower proportions of type IIA and type I relative to the same muscles in fallow deer observed by Curry *et al.* (2012). However, springbok muscles also had higher activity levels of enzymes involved in oxidative energy production. These differences may reflect the relatively high stamina and high sprinting speed of springbok (Curry *et al.*, 2012).

Breed or genotype differences have also been found, with this being mainly attributed to differences in the intensity of selection and the selection parameters used during the development of the breed (Lefaucheur, 2010). Breeds selected for high growth rates and reduced fat deposition have been reported to have lower proportions of type I fibres and greater quantities of type IIB fibres in both pigs and sheep (Lefaucheur, 2010). These results have been most consistent when comparing extremes of selection however (Lefaucheur, 2010).

The fibre type composition of muscles has also found to be influenced by the gender of the animal, with larger quantities of type IIB/X fibres being found in steers or oxen, while intact bulls tend to have larger proportions of type IIA fibres (Lawrie & Ledward, 2006).

The determination of differences in fibre-type composition between animals is complicated by the large amount of between muscle and within muscle variation (Lawrie & Ledward, 2006). In a study looking at variation within bovine LTL muscle it was found that the proportion of red fibres was 11% higher at the 6th thoracic and 5th lumbar vertebra than it was at the 11th thoracic vertebra (37% and 26%, respectively), with similar amounts of variation in the other fibre types (Lawrie & Ledward, 2006). Similar within muscle variation was found in cattle *Semitendinosus* muscle (Klont *et al.*, 1998). It has also been found in pigs that limb muscles situated closer to the bone tend to contain more slow oxidative fibres (type I), while muscles found closer to the skin contain more glycolytic fibres (Klont *et al.*, 1998).

Environmental pressures on the animal can also influence the final fibre-type composition at slaughter. Long-term endurance type exercise has been reported to result in a gradual shift from glycolytic to oxidative fibre types; however this change was only considerable in miniature pigs and requires further study (Lefaucheur, 2010). The exposure to high or low temperatures has been found to result in increased proportions of slow twitch fibres in white muscles, while hot temperatures reduced overall metabolic rate (oxidative and glycolytic) in white porcine muscles (Lefaucheur, 2010).

As is indicated in this section, the fibre-type composition of a muscle can have an influence on the quality of the resulting meat. It is also clear that a number of factors can affect the composition of a muscle and that extrapolating results across species, muscle and method of rearing is not ideal. However, despite this only a single study on the fibre-type composition of springbok meat has been performed at this stage, and this study did not consider the effect that muscle fibre-type and size could have on springbok meat quality. More research into this facet of springbok meat quality is thus required.

2.6.1.2 Connective tissue content and type

The connective tissue content of a muscle is one of the primary factors influencing the final tenderness or background toughness of a cut of meat (Bailey, 1972; Purslow, 2005). It is present in three discrete layers in muscle tissue, namely the epimysium, perimysium and endomysium (Bailey, 1972). The epimysium is the sheath of connective tissue surrounding the muscle as a whole, and is most clearly seen in cuts containing more than one muscle as the division between two adjacent muscles (Bailey, 1972). The perimysium extends off the epimysium into the muscle itself and separates the muscle fibres to form primary and secondary fascicles or fibre bundles (Purslow, 2005). The endomysium is the finest layer of connective tissue, surrounding each individual muscle fibre (Bailey, 1972).

These three layers are made up of two main types of connective tissue, namely collagen and elastin, embedded in an amorphous proteoglycan-based ground substance (Bailey, 1972; Purslow, 2005). Collagen is the more prevalent protein - typically making up 1% - 15% of mammalian muscle tissue on a dry basis - and has fibres that are straight and non-branching, inelastic and yellow in colour (Bailey, 1972; Warriss, 2000; Purslow, 2005). Elastin on the other hand is true to its name, consisting of branched, elastic fibres. Elastin only contributes around 0.6% - 3.7% of dry muscle tissue (Bailey, 1972; Purslow, 2005).

The collagen fibre itself is the quaternary organisation of the protein, with primary, secondary and tertiary structures also being defined (Lawrie & Ledward, 2006). At the primary level it consists of a specific sequence of amino acids (about 1000 in total), as determined by the genetic code controlling its synthesis. This amino acid chain typically consists of a repeating sequence of glycine, proline, hydroxyproline and one other amino acid (Lawrie & Ledward, 2006). The identity of this last amino acid is the distinguishing element in the classification of the currently recognized 12 different types of collagen. A unique trait of collagen is its high content (approximately 12.8% in mammals) of hydroxyproline relative to that found in other proteins (Warriss, 2000; Lawrie & Ledward, 2006)

The tertiary structure of collagen refers to the organization of three primary alpha chains, each of which has a secondary left-handed helical structure (polyproline helix), into a tropocollagen molecule (Bailey, 1972). Tropocollagen forms a right-handed triple alpha helix and is also referred to as the collagen protofibril (Lawrie & Ledward, 2006). These protofibrils or tropocollagen molecules further assemble to form first fibrils and then complete collagen fibres. In the muscle the collagen fibres not only form a supportive structure or framework for the muscle fibres but also connect them to the bones they are required to move (Lawrie & Ledward, 2006).

The orientation of the collagen fibres differs between the different connective tissue layers. The endomysium consists of fine fibres in a randomly orientated felt-like layer which can easily shift to accommodate the change in muscle fibre length that takes place during contraction (Purslow, 2005). In contrast, the fibres of the perimysium are neatly arranged, lying parallel to one another in two separate layers, each of which is at an angle to the axis of the muscle fibre (approximately 54° at rest) (Purslow, 2005). By changing the angle of the two layers to one another and to the muscle fibres the perimysium can also adjust to elongation and contraction of the muscle (Purslow, 2005). The organization of the epimysium differ between muscles, with some showing a similar cross-hatched arrangement to the perimysium while in others the fibres are aligned parallel to the muscle axis (Purslow, 2005).

It appears that of the three layers the perimysium contributes the greatest to the toughness of meat (Purslow, 2005). This is based on the relative ease of splitting the endomysium from the perimysium, while the disruption of the perimysial layers themselves requires more force (Purslow, 2005). This disruption is necessary for the complete breakdown of the muscle structure, as is found during mastication, indicating that the strength of the perimysium will affect perceived tenderness (Purslow, 2005). There is also more variation between muscles in the collagen content, the proportional contributions of the different types of collagen, the size of the collagen fibres and the types of crosslinks present in the perimysium than the endomysium (Purslow, 2005). While the epimysium may make a considerable contribution to the shear force of multi-muscle low value cuts such as shin, in higher value steaks it is usually removed and therefore does not play a role.

Of the 12 currently defined types of collagen, only four are thought to contribute significantly to the structure of muscle tissue, with these being type I, III, IV and V (Lawrie & Ledward, 2006). The distribution of these types between the three layers of connective tissue is found in Table 2.6.

Table 2.6

The collagen-type composition of the three connective tissue layers in skeletal muscle tissue (Lawrie & Ledward, 2006)

	Collagen types present	Predominant type
Endomysium/Basement membrane	I, III, IV, V	IV
Perimysium	I, III, V	III
Epimysium	I, III	I

Apart from the types of collagen as determined by the identity of the variable amino acid in the primary structure, collagen is also specified as being either heat stable or heat labile (Lawrie & Ledward, 2006). This is based on the degree to which the collagen gelatinizes with prolonged heating at 60 - 63°C (Warriss, 2000). The importance of the nature rather than quantity of collagen to meat quality can be demonstrated by the difference in tenderness between beef and pork. Pork is typically considered to be more tender than beef, suggesting a lower collagen content. The opposite is however true, with the hydroxyproline content of pork muscles generally being higher than that of their beef counterparts (Lawrie & Ledward, 2006).

The heat stability of collagen is primarily determined by the nature of the cross linkages present. Collagen molecules form bonds both inter- and intramolecularly, with three types of bonds being found. These are disulphide bonds, divalent bonds and more complex or mature cross-linkages (Lawrie & Ledward, 2006). Divalent bonds are formed through the action of the enzyme lysyl oxidase which catalyses the oxidative deamination of lysine and hydroxylysine residues to form aldehyde groups (Bailey, 1972). In some cases two lysine aldehydes will react in a condensation reaction to form aldols, however these require a very specific relative positioning of the two molecules and therefore only form intramolecularly. Intermolecular divalent bonds can form either between lysine aldehydes and hydroxylysine or between hydroxylysine aldehydes and hydroxylysine (Lawrie & Ledward, 2006). In the former reaction the aldehyde group of lysine reacts with the ϵ -amino group of the hydroxylysine to form an aldamine link (Schiff base), whereas in the latter case an oxo-imino link is formed, which is in turn reduced to dihydroxylysinorleucine (Bailey, 1972; Lawrie & Ledward, 2006). While dihydroxylysinorleucine is acid stable, aldamine bonds are heat labile and sensitive to pH changes, and thus do not contribute to the toughness of cooked meat (Bailey, 1972).

It is not the absolute number of intermolecular cross-links present that determines the connective-tissue-dependent toughness of cooked meat, but rather the degree to which these have formed mature, non-reducible cross-links that bind the collagen fibre together

into a strong, resistant network (Lawrie & Ledward, 2006). Disulphide and divalent bonds are less important in the development of toughness than the complex bonds, which form intermolecularly between three or more different alpha-chains (Lawrie & Ledward, 2006). While more research into the exact biochemical nature of these mature linkages is still required, there is evidence that hydroxyaldohistidine and pyridinoline may be involved, with mature cross links proposed to consist of hydroxylysylpyridinoline and histidinohydroxylysinonorleucine. The glycosylation of lysine residues may also be involved (Lawrie & Ledward, 2006).

The second important connective tissue protein, elastin, is the primary component of the elastic fibres present in meat. Elastin is an amorphous protein consisting of 40% glycine, 40% hydrophobic amino acids and small amounts of proline and hydroxyproline. Elastin also forms cross-linkages, primarily between large numbers of lysine residues (Lawrie & Ledward, 2006). However, unlike the highly organized structure of collagen, elastin protein chains coil randomly (Bailey, 1972).

Elastic fibres are in the minority in meat connective tissue, and are mostly present in the capillaries (Bailey, 1972). However, their behaviour during heating makes them important to consider nonetheless (Lawrie & Ledward, 2006). Unlike collagen fibres, which gelatinize and become soluble during cooking, elastic fibres shrink and remain tough. Both coarse and fine elastic fibres are found, with coarse fibres having an average diameter of 5 - 10 μm , while fine fibres are 1 - 2 μm in diameter. The coarse fibres are predominantly present in the epi- and perimysium, and are arranged parallel to the muscle fibre itself (Lawrie & Ledward, 2006). Fine fibres are aligned to the network of collagen fibres, and are therefore at an angle to the axis of the muscle. Some evidence of variation in muscle content of coarse elastin fibres has been found (Lawrie & Ledward, 2006).

The final components of connective tissue are the proteoglycans and glycoproteins that make up the ground substance (Lawrie & Ledward, 2006). Proteoglycans form an extended structure capable of holding large quantities of water and are thought to be responsible for controlling the size and alignment of the connective tissue matrix (Lawrie & Ledward, 2006). Glycoproteins on the other hand help hold the whole muscle together, linking the cells to the basement membrane and the collagen of the various connective tissue layers (Lawrie & Ledward, 2006). Specific proteoglycans identified in intramuscular connective tissue include decorin, heparan sulphate-containing proteoglycans (basement membrane) as well as chondroitin sulphate and dermatan sulphate (perimysium) (Purslow, 2005).

The contribution of the connective tissue to the toughness of meat depends upon whether the shear force is measure on the raw or cooked meat as well as the temperature to

which the meat is cooked (Purslow, 2005). The strength of the perimysium has been found to increase up to a temperature of 50°C in beef, leading to the conclusion that the connective tissue has the greatest contribution to shear force on heating to temperatures between 20 and 50°C (Purslow, 2005). Considering that the majority of cooked meat is heated to at least an internal temperature of 60°C the role of connective tissue may thus not be as great as one would expect (Purslow, 2005). The effect of cooking on collagen also negates any changes that take place in the connective tissue during ageing, resulting in conditioning not having any practical influence on the connective tissue-based shear force of cooked meat (Purslow, 2005).

The amount and solubility of the collagen present in meat is dependent on a variety of factors such as species, breed, animal age and muscle (Purslow, 2005).

The most commonly discussed factor is the age or rather the physiological maturity of the animal (Purslow, 2005). As an animal matures, so do its tissues, and for collagen this involves an increase in the average collagen fibril diameter and a change in the nature of the cross-linking bonds that hold the alpha chains together (Purslow, 2005; Lawrie & Ledward, 2006). While there is not necessarily an increase in the number of intermolecular bonds with age, the proportion of these bonds that are non-reducible and heat- and acid-stable tends to increase (Bailey, 1972; Purslow, 2005). This results in an increasing amount of the collagen not gelatinizing and becoming soluble during cooking, causing meat from older animals to be tougher on consumption (Bailey, 1972; Lawrie & Ledward, 2006).

The strengthening of the connective tissue with age is related to the rate of growth and turnover of the collagen in the muscle. Animals with a rapid growth rate and thus a high turnover tend to have meat with greater quantities of soluble collagen and fewer mature cross-links (Lawrie & Ledward, 2006). Correspondingly, the decrease in the rate of turnover with maturity is related to the increased stability of the collagen and the increased toughness of the meat. A half-life of 45 days has been reported for collagen (Purslow, 2005). It must however be noted that while the heat-stability of collagen and meat toughness increases with age the relative contribution of connective tissue to muscle decreases (Lawrie & Ledward, 2006).

The effect of growth rate is also seen in the variation in collagen characteristics found between animals reared in different systems or with different levels of nutrition. It has been found that under nutrition results in meat with a higher intramuscular collagen content as well as reduced levels of soluble collagen, while animals slaughtered after a period of rapid growth have a lower contribution by connective tissue to toughness (Purslow, 2005; Lawrie & Ledward, 2006). This may be particularly applicable to game species, where the likelihood

of low nutritional conditions existing are higher, and may indicate that different handling methods could be required when using meat from animals harvested after a drought period.

Various deficiencies, most significantly in vitamin C or copper, can also interfere with the enzyme activity responsible for the post-translational modification of collagen. This results in reduced stability in the collagen network in the muscle and other tissues and unfortunately a number of health problems (Lawrie & Ledward, 2006). The presence of lathyrogenic agents in the diet - such as beta-amino proprionitrile or those found in sweet peas and chickpeas - also inhibits the activity of lysyl oxidase. This causes skeletal deformities and other problems due to faults in the synthesis of collagen and elastin (Warriss, 2000).

The collagen content, type and solubility of different muscles in a carcass also differ, as can be experienced in the tenderness of the *Psoas major* muscle (1% collagen) or the toughness of the *Sternomandibularis* (10% collagen) (Bailey, 1972; Lawrie & Ledward, 2006). One study clearly correlated this variation with the difference in the Warner Bratzler shear force values obtained for a number of muscles (Lawrie & Ledward, 2006). This variation in connective tissue structure and content is a reflection of the function of the muscle (Purslow, 2005). The elastin content also varies significantly, with the *Semitendinosus* muscle containing the greatest quantity (40% of total connective tissue) (Lawrie & Ledward, 2006).

In addition to the quantitative effect of collagen amount and type on the toughness of meat as measured instrumentally as the shear force, the nature of the connective tissue structure in the meat can also influence the texture of the meat. The grain of meat has been related to the size of the fibre bundles or fascicles as well as the thickness of the perimysium delineating these bundles (Purslow, 2005). There is considerable confusion in literature as to whether a coarse or fine grain is preferable, with some articles associating a coarse grain with less desirable meat but other studies correlating a coarser grain to greater tenderness (Purslow, 2005). In the majority of studies it appears that there is a negative correlation between size of the muscle fibre bundles and tenderness (Purslow, 2005).

There is considerable variation in the grain of meat between muscles, genders, breeds and species. Muscle differences have been related to the function of the muscle as well as the rate of post-natal growth, with muscles required for power rather than precision and those with the highest post-natal growth rate having the coarsest texture (Purslow, 2005; Lawrie & Ledward, 2006). The size of muscle fibre bundles is also reported to increase with the age of the animal and be the highest in rams and lowest in ewes (Purslow, 2005). It has also been suggested that physically larger animals have larger fibre bundles (Purslow, 2005).

There is currently a considerable lack of knowledge on the collagen content of springbok muscle, making the comparison of springbok meat to the meat from other species difficult in this regard. Further research on the collagen content of springbok meat as well as the nature of the collagen present is therefore necessary.

2.6.1.3 Fat content

Carcasses are normally described as consisting of three tissue types, namely lean (meat), fat and bone. The carcass fat content is highly variable, in terms of both the total content of the carcass and the distribution within the carcass. Four primary fat depots are distinguished, with these being the subcutaneous, peri-nephric, omental and inter- and intramuscular fat depots (Warriss, 2000).

In terms of their influence on meat quality the intra-/intermuscular and subcutaneous depots are the most important, as they are most often included in the saleable cuts and have both direct and indirect effects on meat tenderness.

An increase in the intramuscular fat content, otherwise known as marbling, has long been associated with improved eating quality, with this including tenderness, juiciness and flavour (Kempster, 1981; Faucitano, Rivest, Daigle, Lévesque & Gariépy, 2004; Fortin, Robertson & Tong, 2005). The increased tenderness found in highly marbled meat may be as a result of the diluting effect of the fat on the other components of the meat, particularly the connective tissue (Fiems *et al.*, 2000). Intramuscular fat is also predominantly found within the perimysium; consequently an increase in the fat content may separate and reduce the integrity of the perimysial connective tissue (Fortin *et al.*, 2005). The presence of lipids in the meat during chewing also increases the juiciness of the meat through its stimulatory effect on saliva production (Fiems *et al.*, 2000; Fortin *et al.*, 2005).

The positive effect of the intramuscular fat content on meat quality has led to the recommendation that a minimum of 1 - 4% is required in pork for acceptable eating quality to be maintained (Van Laack, Stevens & Stalder, 2001; Fortin *et al.*, 2005). Considerable variation in this recommendation does however exist (Fortin *et al.*, 2005), and a number of studies have failed to find significant correlations between the intramuscular fat content and eating quality (Van Laack *et al.*, 2001; Fortin *et al.*, 2005). Current views of the health-related consequences of a high fat diet have also resulted in consumers rejecting meat products with a high quantity of marbling (Fortin *et al.*, 2005).

The subcutaneous fat layer on a carcass acts as thermal insulation, and can thus have an indirect effect on meat quality by changing the rate of cooling of a carcass. Carcasses with little subcutaneous fat can cool very quickly, increasing the risk of cold shortening (section 6.2.1) (Fiems *et al.*, 2000; Savell & Baird, 2005). Minimum fat depths to prevent cold

shortening in lamb and beef have been reported as 2.5 mm and 6.2 mm, respectively (Savell & Baird, 2005).

On the other hand, an excessively thick layer of fat retards the loss of heat from the carcass. This could result in low pH values being obtained while the temperature of the carcass is still high, increasing the denaturation of proteins and consequently weep loss (Savell & Baird, 2005). The persistence of high temperatures in the deep tissues of a carcass also increases the risk of microbial spoilage and bone taint (Warriss, 2000; Lawrie & Ledward, 2006). From an economic perspective the presence of excessive amounts of subcutaneous fat also indicates a less than optimal efficiency of production due to the high energy cost of fat deposition (Fiems *et al.*, 2000). Such products are also less appealing to consumers (Fiems *et al.*, 2000). It therefore seems likely that an ideal subcutaneous fat depth exists that will allow sufficient cooling to prevent excessive denaturation while still providing enough insulation to prevent cold shortening and promote enzymatic tenderization (Savell & Baird, 2005).

Species differ in the total amount of carcass fat and the proportional importance of the various depots (Kempster, 1981; Warriss, 2000). Mutton or lamb carcasses have the highest carcass dissectible fat content of the common domesticated species, with 30.2% being reported, relative to 21.1% for pigs and 15.6% for cattle (Warriss, 2000). The distribution of this fat also differs, with a greater proportion of the total carcass fat in pigs being found subcutaneously (68%), with lamb having around 43% and cattle 24% in this particular depot (Warriss, 2000). Game species are well known to have relatively low carcass fat contents, with Van Zyl & Ferreira (2004) reporting whole carcass fat contents of 3.5 - 8% for springbok, 6.1% for blesbok and 7.0 - 7.5% for impala. The intramuscular fat content of meat from game species is also low, with values of 1.32 - 3.46% being reported (Hoffman *et al.*, 2007b). This indicates that the carcasses of game species may be particularly vulnerable to cold-shortening, as well as that juiciness and tenderness may be compromised to some extent in game meat as a result of the low intramuscular fat content.

In addition to the differences observed between species, breed and gender differences have also been found (Kempster, 1981). These both appear to be primarily a reflection of whether genders or breeds are classified as early or late maturing, in other words at what age the animal attains physiological maturity. Fat is the last of the body tissues to develop, and the subcutaneous and intramuscular depots are the second to last and last locations at which deposition takes place (Fiems *et al.*, 2000; Lawrie & Ledward, 2006). Early maturing animals will therefore tend to start depositing fat at a younger age, and will accumulate fat in these locations sooner than late maturing animals. The higher fat contents found for breeds

such as Dorper sheep or Aberdeen Angus cattle are thus as a result of their early-maturing nature (Warriss, 2000).

Specific effects of intensive artificial selection for certain traits are also found in the differences between breeds. In cattle this is seen in the difference in the distribution of fat deposition between the beef and dairy breeds. Dairy cattle generally deposit more fat internally, while the subcutaneous depot is favoured in beef cattle (Kempster, 1981; Warriss, 2000). Similar results have been found for sheep, with improved meat breeds generally having a higher proportion of intra-/intermuscular fat than “mountain” breeds (Kempster, 1981). This may be as a result of the specific selection for meat quality and increased growth rate, as a high intramuscular fat content generally improves the organoleptic quality of meat (Faucitano *et al.*, 2004). Alternatively, the greater quantities of internal fat found in dairy and “mountain” breeds may reflect their higher metabolic requirements, as internal fat is thought to be more metabolically active than subcutaneous fat (Kempster, 1981). There has also been a striking effect of breeding in pigs in recent years, with the selection for lean growth in response to consumer demands resulting in a remarkably rapid transformation in the carcass composition of popular pig breeds (Warriss, 2000; Faucitano *et al.*, 2004).

In terms of gender differences females are generally early maturing and intact males late maturing, with castrated males falling intermediate to these two (Warriss, 2000). This is supported by the findings of higher fat contents in female springbok, blesbok and impala carcasses (Van Zyl & Ferreira, 2004). An exception to this is found in pigs, where barrows are earlier maturing than gilts (Faucitano *et al.*, 2004). The effect of gender is however most prominent after the onset of sexual development, at which point the control of growth shifts away from the pituitary growth hormone and the steroid hormones from the testes and ovaries start to have a greater effect (Lawrie & Ledward, 2006).

The nature of fat as a late maturing tissue also explains the finding that the carcass fat content tends to increase with age (Warriss, 2000; Lawrie & Ledward, 2006). The relative importance of the various depots also changes, gradually moving from kidney fat dominated to intramuscular fat-dominated deposition (Lawrie & Ledward, 2006).

Another factor that must be taken into account when comparing different animals is the manner in which they were reared. Intensively farmed animals generally experience a higher plane of nutrition, consuming nutrient and calorie-dense foods such as grains. In contrast, extensively farmed animals obtain the majority of their nutrients from grasses and other vegetation and typically have a lower plane of nutrition. This results in intensively farmed animals growing more rapidly and having a swifter transition from muscle-dominated to fat-dominated growth (Lawrie & Ledward, 2006). Intensively farmed animals will therefore typically have greater quantities of fat in all depots (Lawrie & Ledward, 2006). When

compared at the same total fat content a greater proportion of fat was found in the subcutaneous depot in grain fed than extensively reared cattle (Kempster, 1981).

Considerable within and between muscle variation in the intramuscular fat content has also been found (Faucitano *et al.*, 2004).

2.6.2 *Peri-mortem* factors

Once an animal reaches the correct mass, maturity or degree of fatness it is typically sent to an abattoir for slaughter. Alternatively, in the case of game species, it is harvested at night using a high-powered rifle once it has been deemed sufficiently mature. At this point factors such as the fat content, collagen content and fibre type and diameter are fixed and the maintenance of meat quality becomes the responsibility of the abattoir or harvesting team. Factors related to the handling of the carcass and meat during the *peri-* and *post-mortem* periods influence whether the potential quality of the meat as determined by the intrinsic nature of the animal is reached (Lawrie & Ledward, 2006).

2.6.2.1 *pH and temperature decline post-mortem*

The declines in pH and temperature *post-mortem* are two of the most important factors determining the final tenderness and eating quality of meat (Kim *et al.*, 2014); with this being reflected by the inclusion of a 6 hour pH measurement in the Meat Standards Australia grading systems for beef and mutton (Hopkins *et al.*, 2011). The nature of the changes in pH and temperature are in turn influenced by a number of genetic and environmental factors such as the fibre type composition of the muscle, the morphology of the carcass and the *ante-* and *peri-mortem* conditions. In order to understand how and why the biochemical changes *post-mortem* affect meat tenderness, as well as how they themselves are influenced, one needs to understand the physiological basis of these changes.

Living muscle normally has an *in vivo* pH of 7.1 - 7.2, with the homeostatic efforts of the regulatory organs acting to maintain this. However, when the jugular vein and carotid arteries are severed at death the loss of blood flow results in tissues no longer being supplied with oxygen and nutrients or having their metabolic waste products removed (Warriss, 2000). Despite this, the individual cells continue to attempt to maintain their normal metabolism and structural integrity (Lawrie & Ledward, 2006). Unfortunately this requires a constant supply of ATP (adenosine triphosphate), with its degradation to ADP (adenosine diphosphate) and inorganic phosphate (P_i) providing the energy needed for homeostasis (Lawrie & Ledward, 2006). In the living animal an increase in the concentration of P_i stimulates ATP production, with this taking place through three possible mechanisms:

aerobic respiration, anaerobic glycolysis and ATP regeneration from ADP and creatine phosphate (Huff Lonergan *et al.*, 2010).

In *post-mortem* muscle the cell's ability to utilise aerobic respiration is severely limited due to the lack of oxygen supply via the blood (Lawrie & Ledward, 2006). While the provision of oxygen by myoglobin can allow a certain degree of respiration this is generally very short-lived. In the absence of active respiration the reserves of creatine phosphate are also quickly depleted, resulting in anaerobic glycolysis remaining as the only possible source of ATP synthesis by the cell (Warriss, 2000; Huff Lonergan *et al.*, 2010).

The breakdown of glycogen for energy production via glycolysis, though inefficient, is a normal process in living cells and is not in itself detrimental to homeostasis. However, in the absence of blood flow the waste products of glycolysis - most particularly lactic acid - cannot be removed from the muscle as they would normally be *in vivo* (Warriss, 2000). The subsequent build-up of lactic acid in the muscle is the primary cause of the decline in pH from 7.1 - 7.2 to 5.4 - 5.8 observed in muscle *post-mortem* (Warriss, 2000; Huff Lonergan *et al.*, 2010). This decline ultimately inhibits the very enzymes responsible for it, resulting in the complete cessation of ATP synthesis (Warriss, 2000). The gradual decline in ATP results in the permanent binding of the two main myofibrillar proteins (myosin and actin) and thus the development of rigor mortis (Kim *et al.*, 2014).

As one can imagine the complete alteration in the temperature (38°C - 39°C to 0°C - 5°C) and pH conditions in the muscle that takes place during the first 24 - 48 hours *post-mortem* has a far-reaching effect on the nature of the resulting meat (Kim *et al.*, 2014). In terms of tenderness these effects can be categorised as those that influence the structural changes in the muscle during rigor (sarcomere shortening) and those that affect the proteolytic changes that take place during conditioning (Kim *et al.*, 2014).

The temperature at which a muscle enters *rigor* has an important influence on the degree of contraction that takes place (Bekhit, Farouk, Cassidy & Gilbert, 2007). A certain minimum degree of sarcomere shortening is normal, with this being in the range of a 10% decrease in sarcomere length (Huff Lonergan *et al.*, 2010). However, specific combinations of pH and temperature can exacerbate this and thus increase the toughness of the meat, with muscle contraction being found to increase at temperatures below 14°C and above 20°C (Huff Lonergan *et al.*, 2010).

Cold shortening occurs when the temperature of the muscle is below 10 - 15°C while the pH is still above 6.0 - 6.2 (indicating the possibility of ATP still being available) and can result in a decrease in sarcomere length of up to 50% (Bailey, 1972; Thompson, 2002; Huff Lonergan *et al.*, 2010). It is thought to be as a result of low temperatures increasing the leakage of ions from the sarcoplasmic reticulum (SR) into the sarcoplasm, accelerating the

increase in the calcium ion concentration of the sarcoplasm (Bailey, 1972; Kim *et al.*, 2014). Although a gradual release of calcium ions is normal during rigor, by accelerating this process the necessary calcium ion concentration for muscle contraction occurs while ATP is still available to power the contraction process (Thompson, 2002; Huff Lonergan *et al.*, 2010). This results in the shorter sarcomeres found in cold-shortened muscle and an increase in toughness (Bailey, 1972; Hopkins *et al.*, 2011). Cold shortening is most commonly a problem in small, lean carcasses such as those from game species, as the lack of insulation and bulk results in the internal temperature declining very rapidly (De Bruyn, 1993). It can also occur in cases where hot-deboning has been done or when carcasses are blast-frozen pre-rigor (Bailey, 1972).

Heat shortening (or heat-induced toughening) on the other hand occurs when muscles or carcasses reach a pH of 6 while still at a temperature of above 35° (Thompson, 2002; Kim *et al.*, 2014). This is most likely to occur in the deep muscle tissues of large or very fat carcasses (Huff Lonergan *et al.*, 2010). The observed shortening of the sarcomeres at high temperatures is not as extreme as those found for cold-shortening, with shrinkage of around 30% being reported (Huff Lonergan *et al.*, 2010; Kim *et al.*, 2014). However, the reduced tenderness found in muscles subjected to these conditions has been ascribed to a combined effect of both decreased sarcomere length and the depletion of the proteolytic enzymes (Thompson, 2002; Kim *et al.*, 2014). This results in not only reduced initial tenderness but also limits the extent to which this can be corrected by ageing (Kim *et al.*, 2014).

The relative contributions of sarcomere shortening and enzyme depletion to toughening appears to depend on the rate of glycolysis in the muscle, with the combined effect of high temperatures and rapid glycolysis reducing the importance of the sarcomere length (Kim *et al.*, 2014).

Apart from the direct effect of temperature on shortening it also interacts with the pH, with a high temperature during rigor often resulting in a more rapid decline in the pH (Bekhit *et al.*, 2007). Considerable controversy on the effect of this on meat quality exists.

To some extent a rapid decline and low ultimate pH (< 5.7) has been reported as increasing tenderness and overall quality (Silva, Patarata & Martins, 1999; Thompson, 2002; Hopkins *et al.*, 2011). This has been attributed to increased rates of proteolysis (Bekhit *et al.*, 2007; Hopkins *et al.*, 2011), with two explanations having been proposed. Some researchers suggest that the reduced pH favours the activation and release of the lysosomal enzymes, or cathepsins, which have acidic pH optima (section 6.3.2) (Silva *et al.*, 1999). Other studies have maintained that the calpains are still responsible, and that the rapid onset of rigor results in the increased release of calcium ions and thus the activation of the calpains (Bekhit *et al.*, 2007).

However, the early combination of high temperatures with a low pH reduces the stability of proteolytic enzymes (Bekhit *et al.*, 2007), and in most cases the improvement in tenderization has only been evident within a relatively short period of ageing. After longer periods of ageing the difference between samples with a normal pH decline and those with a more rapid decline decreased and became insignificant. This indicates that while the rate of tenderization may be increased the overall extent remained unchanged, or, in some cases may have in fact been reduced due to the early exhaustion of the enzymes (Kim *et al.*, 2014).

This is reflected in the reduced tenderness and overall quality of meat in which the high temperature/low pH conditions have been too extreme ($\text{pH}_{45} < 6.0$; temperature $> 25 - 35^\circ\text{C}$) (Kim *et al.*, 2014). This is the result of increased protein denaturation (Kim *et al.*, 2014). Accelerated glycolysis is most commonly found in pigs, where it is known as PSE for the Pale, Soft and Exudative meat it produces (Kim *et al.*, 2014). PSE can occur as a result of acute *peri-mortem* stress; in addition it has been linked to a specific gene in pigs, known as the halothane gene (Kim *et al.*, 2014). While particularly prevalent in pork, PSE conditions are by no means limited to this species. Various studies have reported similar phenomenon in beef, sheep, venison and poultry (Kim *et al.*, 2014). In some cases this may be as a result of incorrectly applied electrical stimulation (Kim *et al.*, 2014).

The ultimate pH obtained can also affect long term ageing. To some extent a high ultimate pH (> 6.2) will tend to increase tenderization by favouring the activity of the calpains, which have pH optima above 6 (Silva *et al.*, 1999). This has been reported in studies looking at different ultimate pH levels, with an increase in pH_u from 6.0 to 7.0 being reported to increase the tenderness of beef (Bouton, Carroll, Fisher, Harris & Shorthose, 1973). Meat with an ultimate pH of 5.8 - 6.2 has been found to be the least tender (Bouton *et al.*, 1973; Silva *et al.*, 1999). This may be due to these pH levels falling outside the optimum ranges for both the calpains and the cathepsins (Silva *et al.*, 1999).

Meat with a high ultimate pH is known as DFD meat, for its dark, firm and dry appearance (Warriss, 2000). DFD meat forms as a result of the depletion of the glycogen content to below the minimum required to reduce the pH to 5.5, with this being $57 \mu\text{mol/g}$ in cattle (Silva *et al.*, 1999; Thompson, 2002). In domesticated species this can be as a result of the stress of transporting the animals to the abattoir, mixing different groups in lairage, extended lairage times or adverse climatic conditions (Silva *et al.*, 1999; Thompson, 2002). Harvesting methods that result in high stress levels, such as shooting from vehicles during the day, can cause DFD in game meat (De Bruyn, 1993). The occurrence of DFD is more common in male than female animals, with young bulls being particularly liable to develop this condition (Dransfield, 1994).

The statement that *ante-mortem* stress can result in both PSE and DFD, two completely opposite conditions, may seem contradictory at first. However, the types of stress causing these two conditions differ. As previously mentioned, DFD is as a result of the depletion of glycogen. In order for this to occur the animal must be stressed or physically active for a considerable period prior to death. In other words DFD results from chronic stress prior to slaughter (Warriss, 2000). In contrast, PSE occurs as a result of a short period of acute stress directly prior to slaughter (Warriss, 2000). Rather than depleting glycogen this is thought to stimulate glycolysis in the *post-mortem* muscle (Warriss, 2000).

The susceptibility of animals to PSE and DFD can be influenced by the fibre-type composition of their muscles. The effect of fibre-type is related to both the metabolic nature of the fibre and the role of the muscle or fibre in the living animal (Kim *et al.*, 2014). Type I muscle fibres (oxidative) contain low levels of glycogen. This makes them more susceptible to glycogen depletion, which consequently leads to higher ultimate pH levels and increased risk of DFD (Klont *et al.*, 1998). In addition, type I muscle fibres are particularly susceptible to cold stress, with this most likely reflecting the role they play in thermoregulation in the living animal (Lawrie & Ledward, 2006). Oxidative fibres have also been reported to be more susceptible to cold-shortening than glycolytic fibres, possibly reflecting a difference in the efficiency of the sarcoplasmic reticulum network between the fibre types (Huff Lonergan *et al.*, 2010; Kim *et al.*, 2014).

Muscles moreover differ in susceptibility due to their physical location in the carcass. Deeper-lying muscle will tend to take longer to cool due to the insulating effect of the overlying tissue; this will increase the risk of a low pH and high temperature existing simultaneously and thus the occurrence of PSE (Kim *et al.*, 2014). In addition to reduced heat loss, muscle temperatures can also be increased *post-mortem* through metabolic heat production, further increasing the risk of PSE (Kim *et al.*, 2014).

Another factor that can influence the pH decline *post-mortem* is the ingestion, or lack thereof, of feed prior to slaughter (Silva *et al.*, 1999). It is common during the commercial slaughter of livestock for animals to be deprived of food for some time prior to slaughter, either incidentally during transport and lairage or deliberately to clear the gut (Warriss, 2000). Unfortunately this may increase the risk of DFD meat in stressed animals due to the depletion of muscle glycogen during fasting (Lawrie & Ledward, 2006). While this management practice is obviously not practicable in the game industry at present, the amount and nature of the vegetation available to the animals prior to harvest may be a factor worth considering. It has been found that grass-fed cattle are more susceptible to glycogen depletion than cattle reared on grain (Thompson, 2002).

In an effort to prevent cold shortening as well as promote the tenderization process the technique of electrical stimulation has been developed (Lawrie & Ledward, 2006). This involves passing a current through the carcass shortly after death, thereby stimulating the activity of the contractile actomyosin ATPase in the muscle by triggering the release of calcium ions into the sarcoplasm, much like the effect of a natural nerve impulse (Lawrie & Ledward, 2006). This increases ATP usage and therefore glycolysis, thereby increasing the rate of the pH decline *post-mortem* (Thompson, 2002; Savell & Baird, 2005). By reducing the pH to below 6 within a short space of time *post-mortem* it becomes possible to more rapidly chill carcasses without the risk of cold shortening (Lawrie & Ledward, 2006).

2.6.2.2 Sarcomere length

Sarcomere length is generally considered to be one of the myriad of factors influencing meat tenderness and is an indication of the degree of overlap of the actin and myosin filaments (Bailey, 1972; Kerry & Ledward, 2009). This in turn is a reflection of the degree of contraction that takes place during the onset of rigor. As was discussed in the previous section, variation in the rate of temperature change of the muscle during the rigor process has an influence on this contraction (Bailey, 1972); this is not however the only factor that needs to be considered.

In order to fulfil their function in the body, most skeletal muscles are attached via tendons to the bones they are responsible for moving (Bailey, 1972; Kerry & Ledward, 2009). In the carcass these tendons and associated bones serve as physical restraints on the contraction of the muscle during rigor (Dransfield, 1994; Kerry & Ledward, 2009). The position of the carcass during hanging determines how each muscle is restricted, depending primarily on the conflicting forces of the suspending hook and gravity. In the most common method of hanging, i.e. from the Achilles tendon (Ahnström, Hunt & Lundström, 2012), the weight of the carcass tends to stretch muscles such as the *Psoas major* and *Latissimus dorsi* more than those in the caudal rump (such as the *Semitendinosus* and *Biceps femoris* muscles) and the dorsal muscles such as the *Longissimus thoracis et lumborum* (LTL) (Bailey, 1972). Greater contraction occurs in the unstretched muscles, resulting in shorter sarcomere lengths (Bailey, 1972).

An alternative hanging method known as “tender-stretch” involves hanging carcasses by the obturator foramen in order to decrease contraction in valuable muscles such as the LTL (Kerry & Ledward, 2009; Ahnström *et al.*, 2012). The suspension of carcasses by the pelvis rather than Achilles has been found to improve the tenderness of some muscles by 15 - 40% (Ahnström *et al.*, 2012). However, this change in the pattern of contraction can also increase shortening in some muscles. Pelvic suspension via the aitch bone or ischium has

been reported to increase sarcomere lengths in the LTL and the rump muscles, but correspondingly increased contraction in the cranial rear leg muscles (Bailey, 1972). An alternative method known as “tendercut” has also been promoted, with this involving cutting the bone and connective tissue in the spine at the 12th thoracic vertebra as well as in the pelvic girdle (often the ischium) (Kerry & Ledward, 2009; Ahnström *et al.*, 2012). By removing the support of these structural tissues the full weight of the carcass is suspended on the muscles themselves, increasing sarcomere length and tenderness (Kerry & Ledward, 2009).

In the event of the complete removal of the restraining effects of the skeleton, tendons and other muscles prior to rigor, such as through hot-deboning, extreme contraction occurs (Dransfield, 1994). This can lead to unpleasantly tough meat. The more rapid cooling of the muscle on removal from the carcass also increases the risk of cold-shortening, further decreasing sarcomere length. Methods of packaging that physically restrict the shortening of the muscle have been developed however, with this including the use of cling-wrap, the Pi-Vac Elastopak system and SmartStretch™ technology (Taylor, Toohey, Van de Ven and Hopkins, 2012).

While the use of alternative hanging methods has been promoted as a method of increasing the tenderness of high-value cuts, the associated increase in chiller space and labour requirements has put some producers off (Kerry & Ledward, 2009; Ahnström *et al.*, 2012). In the specific case of Southern African game meat harvesting the greater the number of carcasses that can be fitted in a refrigerator truck, the more profit can be made, as the cost of transport per animal declines. Tender-stretch methods are therefore not commonly used in the industry and are not focussed on in this thesis.

2.6.3 *Post-mortem* factors

A number of basic *post-mortem* factors are important for the ultimate quality of the meat, including the period of chilled storage and whether or not the meat is frozen. Although other processes such as physical or chemical tenderization, mincing, smoking and salting will of course also alter the nature of the meat, these can be considered to be value-adding or preserving methods, rather than handling methods for ‘fresh’ meat.

2.6.3.1 *Freezing*

With the demand for a constant, reliable supply of ‘fresh’ products, freezing has become a nearly unavoidable process in the meat supply chain (Leygonie, Britz & Hoffman, 2012). Whether cuts are blast frozen shortly *post-mortem*, as is done with many chicken products, or sold unfrozen only to be placed in the house-wife’s chest freezer, the process

has an effect on the quality of the end product (Muela, Sañudo, Campo, Medel & Beltrán, 2012).

The most significant consequence of freezing is the formation of ice crystals within the cells as the intracellular fluid freezes. This results in two important changes in the environment of the cell. The removal of water through freezing causes an increase in the concentration of the solutes present in the sarcoplasm, including proteins, enzymes and ionic salts (Leygonie *et al.*, 2012). This has been suggested to promote protein denaturation and thus increase moisture loss (Leygonie *et al.*, 2012). Protein denaturation has also been associated with accelerated protein oxidation, potentially resulting in detrimental changes in meat quality (Leygonie *et al.*, 2012). Rapid freezing has been reported as causing less denaturation than slow freezing (Leygonie *et al.*, 2012).

The second, more physically destructive consequence of freezing is the rupturing of the cells and various cellular organelles by the ice crystals as well as the disruption of the connective tissue (Shanks, Wulf & Maddock, 2002). The extent of this damage is determined by the number, distribution, size and morphology of the ice crystals, which in turn is determined by the rate and temperature of freezing (Muela *et al.*, 2012). Rapid freezing at low temperatures increases the proportion of the ice crystals that form intracellularly rather than extracellularly, as well as reducing the average size of the crystals (Leygonie *et al.*, 2012; Muela *et al.*, 2012). Smaller crystals cause less physical disruption to the muscle.

The structural damage to the muscle has two main affects, one favourable and the other not (Shanks *et al.*, 2002). As can be expected freezing tends to increase the tenderness of meat (Muela *et al.*, 2012), with meat that has been frozen and thawed prior to cooking having lower shear force values than unfrozen meat (Shanks *et al.*, 2002). In fact similar shear force values have been found in unfrozen meat aged for 21 days and meat aged for seven days and then frozen (Lagerstedt, Enfält, Johansson & Lundström, 2008). However, the rupturing of the cell membranes and the disruption of the physical structure of the muscle also reduces the barriers to moisture loss, decreasing the water-holding capacity (Muela *et al.*, 2012). Increased purge of fluid is thus found when frozen meat is thawed, with this not only increasing mass losses but also being visually unattractive for the consumer.

Lower thaw loss is generally found in meat that has been frozen rapidly, with longer freezing times (> 19.5 minutes) tending to increase purge losses (Leygonie *et al.*, 2012; Muela *et al.*, 2012). Freezing has also been found to increase cooking losses (Crouse & Koohmaraie, 1990; Shanks *et al.*, 2002). The nature of the thawing process also affects moisture losses, with studies reporting that reducing the time taken for the meat to warm from -5°C to -1°C to below 50 minutes reduced thaw losses (Leygonie *et al.*, 2012). Thawing

over a long period at refrigerator temperatures has been found to result in the highest drip losses (Leygonie *et al.*, 2012).

In addition to the rate of freezing, the duration and temperature (average and variation), and the exposure of the meat to light and air during frozen storage can also have an effect on quality (Muela *et al.*, 2012). The duration of storage does not have as much of an effect on the structural integrity of the meat as much as the method of freezing; however it has been suggested that prolonged frozen storage increases the size of the ice crystals and the amount of thaw loss (Muela *et al.*, 2012). Fluctuations in the temperature of storage also tend to increase the detrimental changes in the meat (Muela *et al.*, 2012).

Interactions between changes during ageing and freezing have also been reported (Shanks *et al.*, 2002). Shanks *et al.* (2002) found that the effect of freezing on shear force was greater in muscles that had not undergone prior ageing. Similar results are reported for cooking loss, with freezing increasing the cooking loss more in meat that had aged for a shorter period prior to freezing (Shanks *et al.*, 2002). Freezing of meat prior to ageing also tends to increase the rate of tenderization after thawing (Crouse & Koohmaraie, 1990; Shanks *et al.*, 2002). This has been attributed to the acceleration of proteolysis, as freezing and frozen storage has been reported to reduce calpastatin levels without affecting calpain activity (Crouse & Koohmaraie, 1990).

2.6.3.2 Conditioning/ageing

Possibly one of the oldest meat-handling techniques is that of ageing or conditioning (Dransfield, 1994). This can be roughly defined as the holding of meat, either on the carcass or as individual cuts, at just above its freezing temperature for a certain period of time prior to eating (Lawrie & Ledward, 2006). The main purpose of conditioning is the tenderization of the meat; with the improvement of flavour being a secondary goal (Bailey, 1972; Lawrie & Ledward, 2006).

Considering the physical structure of meat, the decline in shear force generally observed during conditioning could be as a result of the degradation of either the connective tissue or the myofibrils and related cytoskeletal proteins. While the notable contribution of collagen to the structural integrity of meat originally led to the suggestion that tenderization was a result of collagen degradation this hypothesis has ultimately been dismissed. This was based on the finding that the amount of soluble hydroxyproline present in meat did not increase significantly during ageing, indicating that collagen denaturation and proteolysis did not take place to any great extent (Hopkins & Huff-Lonergan, 2004; Lawrie & Ledward, 2006). While some decline in the structural integrity of the connective tissue during extended

ageing has been found the effect of this on the shear force during normal ageing periods is most likely negligible (Ouali, 1992).

It is therefore apparent that the observed increase in tenderness is a result of a weakening of the myofibrillar and cytoskeletal components of the meat (Kemp, Sensky, Bardsley, Buttery & Parr, 2010). However, this weakening is not as a result of the degradation or disassociation of the actomyosin formed during rigor (Hopkins & Huff-Lonergan, 2004). The degradation has been found to rather involve the cytoskeletal proteins responsible for maintaining the organization of the myofibrils and the linkage of the myofibrils to the sarcolemma, resulting in the disintegration of the Z-lines and fragmentation of the myofibrils (Hopkins & Huff-Lonergan, 2004). Troponin T, troponin I, titin, desmin, dystrophin, nebulin, vinculin and meta-vinculin are a few of the proteins found to be degraded during ageing; (Hopkins & Huff-Lonergan, 2004; Nowak, 2011). This degradation has been attributed to a number of factors, with the activity of endogenous proteolytic enzymes being considered the most important (Kemp *et al.*, 2010; Nowak, 2011).

A proteolytic enzyme or enzyme system is considered to potentially contribute to tenderization if it is naturally present in skeletal muscle cells, the proteins it degrades mimic those found to change during ageing and it has access to the myofibrils (Kemp *et al.*, 2010; Nowak, 2011). Several proteolytic enzyme systems are thought to fulfil these criteria, namely the calpains, cathepsins, caspases and proteasome (Nowak, 2011).

Calpains are also known as calcium-dependent proteinases (CDP's), calcium-activated neutral proteinases (CANP's) and calcium-activated sarcoplasmic factors (CASP's). The calpain system consists of a number of iso-enzymes as well as an inhibitor, calpastatin. Fourteen calpain enzymes have been identified thus far; however two forms, known as calpain I or μ -calpain, and calpain II or m-calpain, are generally considered most important in the tenderization of meat (Lawrie & Ledward, 2006; Kemp *et al.*, 2010). Both calpain I and II are heterodimers, consisting of a 28 kDa subunit and an 80 kDa subunit. While the 28 kDa subunit is identical in both isoforms the 80 kDa subunit is coded for by different genes (Huff Lonergan *et al.*, 2010). The function of the 28 kDa subunit is uncertain but it may be involved in the regulation of the enzyme by binding calcium ions. The 80 kDa subunit consists of four domains, two of which are thought to be involved in calcium binding, one catalytic domain, and an N-terminal domain with a unique amino acid sequence (Huff Lonergan *et al.*, 2010). A third calpain isoform, designated calpain 3 or P94, is also expressed in skeletal muscle and has been suggested to contribute to tenderization (Kemp *et al.*, 2010). However a number of studies, including one utilising mice in which the gene for calpain 3 was disabled, have found no link between this enzyme and tenderness (Kemp *et al.*, 2010).

A number of factors are involved in determining the proteolytic activity of calpains in meat, with the calcium ion concentration, inhibitory activity of calpastatin and pH playing the biggest role.

Calpain I requires a calcium ion concentration of 5 - 65 μM and calpain II a concentration of 300 - 1000 μM for half-maximal activity (Lawrie & Ledward, 2006; Huff Lonergan *et al.*, 2010). The calcium ion concentration of the sarcoplasm increases *post-mortem* as a result of the failure of the ATP-dependant calcium pumps in the sarcoplasmic reticulum (Calkins & Seideman, 1988; Lawrie & Ledward, 2006). Calpain I is activated earlier *post-mortem* than calpain II, with the activation of calpain I thought to begin at a pH of approximately 6.3 (normally *circa* 6 hours *post-mortem* in beef) and calpain II at 16 hours *post-mortem* (Dransfield, 1994). However, these concentrations also result in the autolysis of the enzymes. In calpain I this involves the degradation of the 80 kDa subunit to 76 kDa, while in calpain II a 78 kDa product is formed (Huff Lonergan *et al.*, 2010). The 28 kDa subunit is degraded to 18 kDa in both calpains. The brief autolysis of the enzymes results in a decrease in the concentration of calcium required for half-maximal activity, as well as increasing the binding of calpain I to cell organelles such as the myofibrils (Huff Lonergan *et al.*, 2010). However, extended autolysis results in complete inactivation, with calpain I activity declining *post-mortem* (Dransfield, 1994). The finding that the same conditions that allow calpain activity also cause autolysis and inactivation has led to the conclusion that a decrease in measurable activity is an indication of activation in the meat (Huff Lonergan *et al.*, 2010).

Both the absolute pH and the rate of change in the pH have an effect on the activity of the calpains. Calpains have pH optima of above 6, as indicated by the higher calpain I activity found in meat with a high ultimate pH (Ertbjerg *et al.*, 1999). However, the rate of pH decline during early rigor also has an effect on calpain activity and the persistence of activity during ageing (Huff Lonergan *et al.*, 2010). To some extent a more rapid decline in pH, as is found in electrically stimulated meat, has been found to increase the rate of tenderization, with the degradation of calpain substrates occurring earlier *post-mortem* (Calkins & Seideman, 1988; Huff Lonergan *et al.*, 2010). This has been attributed to the earlier activation of calpain I, although the rate of autolysis also increases (Huff Lonergan *et al.*, 2010). However, an excessively rapid decline in the pH has the opposite effect, decreasing the activation and autolysis of calpain I and reducing the degradation of proteins such as desmin and talin.

The specific inhibitor of the calpain system, calpastatin, is encoded by a single gene, but variations in transcription and splicing result in several isoforms being generated (Kemp *et al.*, 2010). This may influence the location of calpastatin within the cell, as well as enabling

the inhibition of multiple calpain isoforms (Kemp *et al.*, 2010). Calpastatin consists of five domains, I to IV are inhibitor domains exhibiting homologous amino acid sequences, while the fifth, domain L, is the alkaline N-terminal (Huff Lonergan *et al.*, 2010). Each inhibitor domain contains three regions - A, B and C - with regions A and C binding the calpain molecule and region B blocking the catalytic site (Kemp *et al.*, 2010). The existence of multiple inhibitory domains indicates that each calpastatin molecule is capable of simultaneously inhibiting multiple calpains (Huff Lonergan *et al.*, 2010; Kemp *et al.*, 2010). This inhibition is reversible and calcium-dependant, with a lower calcium ion concentration required for the half-maximal binding of calpastatin than for the activation of the calpains in either the autolysed or unautolysed forms (Huff Lonergan *et al.*, 2010). The inhibitory activity of calpastatin is also found to decrease *post-mortem* as a result of cleavage by calpains and possibly other proteolytic enzymes such as the caspases (Huff Lonergan *et al.*, 2010; Kemp *et al.*, 2010).

There is strong evidence for the role of calpains in protein degradation during conditioning. Calcium-sequestering agents such as ethylene diamine tetra-acetic acid (EDTA) have been found to inhibit tenderization, while the injection of calcium chloride increases it (Wheeler, Koohmaraie & Shackelford, 1997; Hopkins & Huff-Lonergan, 2004; Huff Lonergan *et al.*, 2010). The pattern of protein degradation found during tenderization also corresponds to the proteins degraded by the calpains, with the cytoskeletal proteins and intermediate filaments such as titin, nebulin and desmin proteolysed but actin and myosin not (Lawrie & Ledward, 2006; Huff Lonergan *et al.*, 2010; Kemp *et al.*, 2010). The different rates of tenderization found in beef, lamb and pork have also been linked to the differences in the calpastatin to calpain ratio between these meats (Kemp *et al.*, 2010). Variation in the activity of calpastatin has also explained the reduced tenderization found in callipyge sheep and *Bos indicus* cattle as well as cattle and sheep fed β -agonists (Johnson, Calkins, Huffman, Johnson & Hargrove, 1990; Dransfield, 1994; Huff Lonergan *et al.*, 2010; Kemp *et al.*, 2010). In addition, calpastatin activity and expression is increased by the increased plasma adrenalin levels found in stressed pigs (Kemp *et al.*, 2010). It therefore seems likely that calpastatin activity levels reflect not only intrinsic differences in meat quality but also serve as a mechanism for the effect of environmental factors. Calpastatin activity at 24 hours *post-mortem* has been reported to be responsible for 40% of the variation in tenderness after conditioning in meat from ruminant species (Kemp *et al.*, 2010).

Of the calpains, calpain I is thought to be the greatest contributor to tenderization (Kemp *et al.*, 2010). This is based on the relatively high calcium concentration required for the half-maximum activity of calpain II, as these conditions are unlikely to occur naturally in meat (Kemp *et al.*, 2010). Calpain II activity has also been found to be retained for a

considerable period during ageing, indicating a lack of conditions favouring its activation and autolysis (Kemp *et al.*, 2010). This has been further supported by studies with calpain I knockout mice, where considerable retardation of tenderization was found (Kemp *et al.*, 2010). Dransfield (1994) suggests that only around 30% of calpain II enzymes are activated during ageing under normal conditions.

While there is strong evidence that the calpains are the first and most influential contributors to tenderization, they also undergo autolysis once activated, resulting in a decline in the activity of calpain I in particular as the time *post-mortem* increases (Lawrie & Ledward, 2006). The ultimate pH found in meat is also not favourable for the activity of the calpains (Lawrie & Ledward, 2006). It therefore appears that a secondary force must be responsible for the continued tenderization seen from 24 - 48 hours *post-mortem* onwards. This is supported by the finding that while tenderization was inhibited in calpain I knockout mice it was not prevented altogether, indicating the possibility of the contribution of other proteolytic enzymes (Kemp *et al.*, 2010). The finding of a significant correlation ($p < 0.10$) between tenderness and calpain I activity at one day *post-mortem* but not three days *post-mortem* also agrees with supposition that calpain I is most active early *post-mortem* (Calkins & Seideman, 1988).

A second group of enzymes that has been extensively studied are the lysosomal enzymes or cathepsins. Seven cathepsin isoforms categorized into three families are found; namely cathepsins B, L, H and X (cysteine peptidases), cathepsin G (serine peptidase) and cathepsins D and E (aspartic peptidases) (Lawrie & Ledward, 2006; Nowak, 2011). Of these, cathepsins B, L, H and D have been studied in terms of meat tenderization (Ouali, 1992; Hopkins & Huff-Lonergan, 2004; Lawrie & Ledward, 2006). Cathepsins H and D are thought to play little to no part in tenderization, as cathepsin H does not degrade native myofibrillar proteins and cathepsin D is only active at a pH of 2.5 - 5.0, which is not normal for meat (Hopkins & Huff-Lonergan, 2004; Lawrie & Ledward, 2006). In contrast, cathepsins B and L could contribute to tenderization as they have been found to rapidly degrade troponin T and I as well as C-protein under normal meat pH conditions. They are also active at a wide range of pH levels, with activity being retained in a pH range of 4 - 6.5 and 3.0 - 6.5 for cathepsin B and L respectively (Hopkins & Huff-Lonergan, 2004; Lawrie & Ledward, 2006). The system also contains specific inhibitors, known as cystatins (Herrera-Mendez, Becila, Boudjellal & Ouali, 2006). The term 'cystatin' actually describes a superfamily of cysteine-peptidase inhibitors made up of four families, the stefins, cystatins, kininogens and glycosylated protein inhibitors (Herrera-Mendez *et al.*, 2006). While low molecular weight proteins resembling cystatins have been identified in skeletal muscle and have been correlated with meat

tenderness extensive study into their role in tenderization is still needed (Herrera-Mendez *et al.*, 2006).

The cathepsins are thought to be responsible for the continued decline in shear force after the inactivation of the calpains (Lawrie & Ledward, 2006). This is based on their low pH optima (below 6), as well as the proposed lysing effect of the decline in pH and depletion of ATP on the lysosomal membranes (Calkins & Seideman, 1988; Lawrie & Ledward, 2006; Kemp *et al.*, 2010). Several studies have also found significant correlations between cathepsin activity and tenderness or changes in shear force (Calkins & Seideman, 1988; Johnson *et al.*, 1990). It has also been suggested that the more rapid tenderization found in electrically stimulated meat could be as a result of the low pH and high temperature conditions favouring cathepsin activity (Calkins & Seideman, 1988).

However, there is by no means consensus on whether or not even cathepsins B and L actually do play a part in tenderization. Nowak (2011) considers the likelihood of their contribution to be doubtful. This is based on the discrepancy between the proteins degraded by cathepsins and those found to break down during ageing (Nowak, 2011), with both cathepsin B and cathepsin L found to degrade both myosin and collagen (Hopkins & Huff-Lonergan, 2004). There is also a lack of conclusive evidence that the cathepsins are released from the lysosomes during ageing (Nowak, 2011), as well as the lack of conclusive evidence of a link between variations in tenderness and the level of cathepsin activity (Kemp *et al.*, 2010). In addition to the containment of the cathepsins within the lysosomes the presence of the cystatins in meat may also prevent cathepsin activity *post-mortem* (Hopkins & Huff-Lonergan, 2004).

There is increasing interest in the proteasome as a role-player in tenderization during conditioning (Nowak, 2011). The proteasome consists of a 19 S regulatory subunit and a 700'000 Da 20 S proteolytic subunit which has been designated the multicatalytic proteinase complex (MCP) (Kemp *et al.*, 2010). The MCP consists of four rings stacked on top of one another to form a cylindrical structure (Sentandreu, Coulis and Ouali; 2002; Nowak, 2011). Seven subunits of 20 - 35 Da each make up each of the rings, with two inner rings consisting of β -subunits and two outer rings consisting of α -subunits (Sentandreu *et al.*, 2002; Nowak, 2011). The catalytic activity of the proteasome is associated with the β -subunits (Sentandreu *et al.*, 2002). Both activator and inhibitor proteins for the MCP have been identified; however their mechanisms of action require further research (Sentandreu *et al.*, 2002). The proteolytic activity of the 26 S proteasome is ATP-dependant and requires the tagging of target proteins with ubiquitin (Kemp *et al.*, 2010). However, once it has dissociated into its subunits the 20 S catalytic subunit can function in the absence of both ubiquitin and ATP, thus potentially

allowing activity *post-mortem* (Kemp *et al.*, 2010). The proteasome is also abundant in skeletal muscle (Kemp *et al.*, 2010).

The role of the MCP in tenderization is based on findings that purified and partially purified extracts were capable of hydrolysing myofibrillar proteins, including nebulin, tropomyosin, troponin C, actin and the myosin light chain, as well as decreasing the integrity of the M- and Z-lines (Sentandreu *et al.*, 2002; Kemp *et al.*, 2010). The inhibition of proteasome activity has also been found to prevent the degradation of several proteins typically degraded during ageing. However, the MCP has been found to have alkaline pH optima of 7 - 9, and is present in the highest concentrations in type I muscle fibres (Ouali, 1992; Sentandreu *et al.*, 2002). This suggests that it may make the greatest contribution to tenderization in high-pH, slow-twitch muscles (Sentandreu *et al.*, 2002). Despite the relatively high optimum pH the MCP does however retain substantial activity at a pH of below 6 (Kemp *et al.*, 2010). It has also been found to maintain activity during ageing of up to seven days *post-mortem* (Kemp *et al.*, 2010). While the MCP quite possibly contributes to tenderization, it is unlikely to be responsible for the majority of the changes, as the pattern of degradation by the proteasome alone does not coincide with that found during ageing (Kemp *et al.*, 2010).

The caspase system is another family of proteolytic enzymes that has been implicated in the tenderization process (Kemp *et al.*, 2010). Fourteen caspases have been identified to date, forming a family of cysteine aspartate-specific proteases which can be divided into those participating in apoptosis and those participating in inflammation. Apoptotic caspase activity involves the degradation of specific proteins including myofibrillar and cytoskeletal proteins and can be activated by hypoxic conditions similar to those found in *post-mortem* muscle (Kemp *et al.*, 2010). Details on the mechanisms of action of the caspases can be found in Kemp *et al.* (2010) and Kemp & Parr (2012).

Caspase 3, 7 and 9 activity has been found to decline during ageing, with the majority of the decline taking place early *post-mortem* (Kemp *et al.*, 2010). This decline was negatively correlated with the shear force, indicating increased tenderization in muscles showing the greatest activation of these caspases (Kemp *et al.*, 2010). Caspase-specific substrates were also degraded during ageing and the incubation of myofibrils with purified caspases has been found to result in the degradation of actin, troponin T, myosin light chain, desmin and troponin I (Kemp *et al.*, 2010). The results of studies examining the role of the caspases do however vary (Kemp, Bardsley & Parr, 2006; Kemp & Parr, 2008; Underwood, 2008; Mohrhauser, 2011).

There is evidence of substantial interaction between the caspase and calpain systems. The caspases are known to degrade calpastatin, which would indirectly increase

tenderization by reducing the inhibition of the calpains (Kemp *et al.*, 2010). The calpains have also been implicated in the activation of the caspases (Kemp *et al.*, 2010). This interaction complicates the explanation of the role of caspases in tenderization as both direct and indirect effects are likely.

Apart from the role it plays in the activation of the calpain system, the increase in the ionic strength *post-mortem* has also been suggested to play a direct role in tenderization (Ouali, 1992). The osmotic pressure of meat doubles over the rigor period as a result of the release of ions from proteins as they denature as well as the failure of the ATP-dependent calcium pumps in the sarcoplasmic reticulum (Ouali, 1992; Calkins & Seideman, 1988; Hopkins & Huff-Lonergan, 2004). This rise in osmolality increases the susceptibility of proteins to proteolysis by increasing their solubility (Ouali, 1992; Hopkins & Huff-Lonergan, 2004). In addition, the high ionic strength may directly cause the fragmentation of nebulin, desmin and titin and weaken the Z-disc by liberating phospholipids from this structure, as well as increasing the lengths of the sarcomeres (Ouali, 1992; Geesink, Taylor, Bekhit & Bickerstaffe, 2001; Hopkins & Huff-Lonergan, 2004). Fast-twitch muscle fibres have also been found to have higher osmolalities *post-mortem*, which may contribute to the greater rate of tenderization found in fast-twitch white muscles (Klont *et al.*, 1998; Hopkins & Huff-Lonergan, 2004). However, at this point it has not been conclusively proven that the ionic strength and calcium ion concentration has a tenderizing effect independently of enzyme activity (Hopkins & Huff-Lonergan, 2004).

While tenderness is one of the most important quality traits for consumers, with greater tenderness generally resulting in greater overall acceptability, longer is not necessarily better when it comes to ageing meat.

A number of studies have found that tenderization is a finite process, with a particular basal tenderness being reached at around 14 days *post-mortem* (in beef), where after little further improvement is found (Calkins & Seideman, 1988; Monsón, Sañudo & Sierra, 2005). This minimum shear force is attributed to the connective tissue content, which is not degraded to any great extent during ageing (Calkins & Seideman, 1988; Sentandreu, Coulis & Ouali, 2002; Purslow, 2005; Kerry & Ledward, 2009). Apart from the lack of any further improvement in texture with ageing beyond the point of maximum tenderness, several undesirable changes have been found to occur during the conditioning process. These include increases in weep loss and desiccation as well as changes in flavour and aroma attributes (Johnson, 1991; Campo, Sañudo, Panea, Alberti & Santolaria, 1999; Stetzer, Cadwallader, Singh, Mckeith & Brewer, 2008).

Depending on the method of ageing used, prolonged storage can result in quantitative losses. When ageing vacuum-packaged, deboned cuts moisture losses are contained within

the packaging and, if the meat is to be sold as-aged, will not directly affect the producer in terms of the mass sold. However, when dry-ageing or ageing on the carcass, moisture losses from the surface of the meat will result in desiccation and the formation of a hard, dark layer, increasing trimming losses (Lawrie & Ledward, 2006; Li, Babol, Wallby & Lundström, 2013). The risk of excessive desiccation is particularly high in lean carcasses, such as those of most game meat species (Lawrie & Ledward, 2006).

In addition to weight losses, over-ageing can result in changes in the flavour and aroma profile of meat (Lawrie & Ledward, 2006; Stetzer *et al.*, 2008). During storage a number of chemical changes occur, including protein and lipid oxidation and the denaturation and degradation of proteins (Campo *et al.*, 1999; Lawrie & Ledward, 2006). These changes influence the nature of the pool of precursor compounds available in the meat for the formation of volatile compounds during cooking (Spanier, Flores, McMillin & Bidner, 1997; Brewer, 2006; Stetzer *et al.*, 2008). Short- to medium-term ageing of beef has been found to increase the overall aroma intensity and the intensity of beef-like, brothy, sweet and browned-caramel aromas and flavours, which are favourable (Monsón *et al.*, 2005; Brewer, 2006). However, more extensive ageing periods have resulted in the increase of liver-like, metallic, gamey, off, rancid, cardboard, painty, bitter and sour attributes; (Spanier, Vercellotti & James, 1992; Brewer, 2006; Yancey *et al.*, 2006; Stetzer *et al.*, 2008). While attributes have been associated with specific components in meat, it is likely that similar processes are responsible for the changes found during ageing, with the most likely options being enzymatic degradation of proteins, nucleotides and lipids, protein denaturation and lipid and protein oxidation (Lawrie & Ledward, 2006; Koutsidis *et al.*, 2008).

Besides the role it plays in textural changes, enzymatic proteolysis has also been linked to flavour changes, with significant correlations between calpain activity and the development of rancid, sour and salty flavours being found (Brewer, 2006). Sour flavours have been associated with the levels of free amino acids such as aspartic acid and histidine and the amino acid amide asparagine in the meat, which could explain the role played by proteolysis in the development of this flavour (Brewer, 2006; Koutsidis *et al.*, 2008). It is also possible that increases in metallic and liver-like flavours during ageing could be partly due to the release of iron from myoglobin by denaturation or proteolysis. The occurrence of liver-like flavours has been linked to the myoglobin content of the meat (Yancey *et al.*, 2006). Liver-like attributes have also been linked to the reaction of carbonyl-containing compounds with sulphur-containing compounds such as thiols, sulphides and thiazoles, which may also increase due to protein degradation (Brewer, 2006; Yancey *et al.*, 2006).

Another likely role-player is the oxidation of both proteins and lipids. Rancidity has been long linked to lipid oxidation, and the various volatile compounds associated with

undesirable aromas and flavours could well be products of oxidation (Brewer, 2006). Several carbonyls are produced, which may directly contribute to off flavours as well as increasing liver-like attributes by reacting with sulphur-containing peptides (Brewer, 2006; Stetzer *et al.*, 2008). Many of the aldehydes produced by the oxidation of phospholipids and polyunsaturated fatty acids are also known to produce strong, unpleasant odours (Stetzer *et al.*, 2008), and several products of lipid oxidation, such as heptanol, hexanal and hexanol have also been linked to the occurrence of liver-like attributes (Brewer, 2006). Game meat is typically high in polyunsaturated fatty acids, both as a result of the animals' diet and the low intramuscular fat content of the meat (Lawrie & Ledward, 2006; Hoffman, Kroucamp & Manley, 2007d). This, along with its high myoglobin content, may make game meat more susceptible to oxidative changes (Brewer, 2006; Lawrie & Ledward, 2006; Yancey *et al.*, 2006). A direct relationship between both the overall unsaturated fatty acid content and the presence of specific unsaturated fatty acids, and the development of liver-like flavours has also been indicated (Yancey *et al.*, 2006). The increased concentration of oxidation products such as pentanal in aged meat may also result in the observed decrease in beef-like attributes through a masking effect (Stetzer *et al.*, 2008). It must however be noted that a lack of correlation between levels of lipid oxidation as indicated by TBARS values and sensory scores for liver flavour found in some studies has led to the suggestion that lipid oxidation does not play a role (Spanier *et al.*, 1997; Yancey *et al.*, 2006). This is in direct contrast to other studies which have proposed that lipid oxidation is the main cause for the decline in desirable and increase in undesirable flavours during ageing (Spanier *et al.*, 1992).

In addition to the increase in free amino acids in aged meat, the development of a sour flavour in vacuum-aged meat can also be as a result of the production of lactic acid by lactic acid bacteria. These bacteria are favoured by the anaerobic environment present in vacuum-packed meat, and are capable of converting sugars such as glycogen to lactic acid (Warriss, 2000; Li *et al.*, 2013). This may result in the development of an off-milk smell and flavour in extensively over-aged vacuum-packed meat, as the lactic acid concentration has been reported to increase with ageing (Jeremiah & Gibson, 2003).

The range of changes that take place during conditioning, both positive and negative, make it clear that an ideal balance must be reached in order to optimise meat quality. It is also clear that this ideal balance will differ depending on the species, breed, gender, animal and muscle, as a result of differences in the proteolytic potential of the enzyme systems present, the early *post-mortem* handling of the carcass, the background toughness of the meat and the susceptibility of the meat to oxidative changes. The initial tenderness of the meat prior to ageing will also determine whether conditioning the meat at all is the best

decision economically, as the cost of production increases with the conditioning time (Dransfield, 1994; Warriss, 2000).

Springbok meat is reported to be relatively tender (Skinner & van Zyl, 1971; Hoffman *et al.*, 2007a). It is also high in polyunsaturated fatty acids and iron, making it potentially high risk in terms of oxidation (Hoffman *et al.*, 2007c; Hoffman *et al.*, 2007b). Furthermore, springbok carcasses contain very little subcutaneous fat, increasing losses to desiccation when ageing on the carcass (Lawrie & Ledward, 2006). All these factors, along with the rate of tenderization, need to be taken into account when making decisions on the conditioning of springbok meat. It is clear that simply extrapolating ageing practices from studies done on beef will not be sufficient to optimise the quality of the springbok meat that reaches the consumer.

2.7 Ageing in industry

In the beef industry the ageing of high-value cuts is a deliberate process and is used to optimise product quality as well as provide a selling point. The importance placed on ageing can be seen in the large number of peer-reviewed publications on everything from the effect of breed, muscle and gender to the biochemistry of ageing (Culler, Smith, Cross & others, 1978; Johnson *et al.*, 1990; Boakye & Mittal, 1993a; Boakye & Mittal, 1993b; Dransfield, 1994; Heinze & Bruggemann, 1994; Campo *et al.*, 1999; Jeremiah & Gibson, 2003; Gruber *et al.*, 2006; Farouk *et al.*, 2007; Laville *et al.*, 2009; Cruzen, Paulino, Lonergan & Huff-Lonergan, 2014). Different methods of ageing have also been investigated, with studies looking at the flavour and textural changes, as well microbiological safety, of traditional dry-aged beef, beef aged in special water-permeable bags and beef aged as vacuum-packaged cuts (DeGeer *et al.*, 2009; Li *et al.*, 2013; Li *et al.*, 2014; Smith *et al.*, 2014). There are also a relatively large number of publications on the meat quality and ageing of venison from species such as red and fallow deer (Drew, Crosbie, Forss, Manley & Pearse, 1988; Taylor, Labas, Smulders & Wiklund, 2002; Wiklund, Sampels, Manley, Pickova & Littlejohn, 2006; Brennand, Nummer & Memmott, 2010; Williamson, Ryland, Suh & Aliani, 2014). In addition to this, many pamphlets and guides for hunters on the handling of venison can easily be found online, with these providing information on everything from skinning to deboning and ageing (Benson, 2010; Clemson Cooperative Extension, 2007; Garden-Robinson & Marchello, 2012).

In contrast, little to no information on the handling of indigenous antelope carcasses in South Africa can be found (Van Rensburg, 1997; Van Rensburg & Zondagh, 1993). While a booklet on the harvesting of game meat for export in Namibia has been published, it only covers the harvesting and slaughter process up until the offloading of the carcasses at the

game processing facility, and does not mention meat ageing protocols (Van Schalkwyk & Hoffman, 2010). Local hunters therefor often either do not age their meat at all or follow what they have been told by friends, family or people involved in the hunting industry. Personal communication with commercial game meat processors has indicated that apart from the required 24 hour hanging period necessary for the prevention of Foot and Mouth disease (Council Decision 79/542/EC) no specific or defined ageing period is used, even for the export market. According to both Piet Neethling from Camdeboo and Charl de Villiers from Mosstrich (the two main game meat exporters in South Africa), carcasses can hang with the skin on for between three and seven days before being deboned. This period is not specified and controlled for the sake of meat quality optimisation but rather simply a result of the logistics of the harvesting process.

2.8 Conclusion

The information presented in this review clearly indicates the importance of the game industry to the agricultural, conservation and tourism sectors of South Africa as well as the status of the springbok within this industry. However, it also emphasizes the current lack of focus on game meat and the quality thereof.

The importance of tenderness for consumer acceptance has been emphasized in numerous studies and it can be seen by the wide scope of this review that the management of tenderness is not a simple task. Due to the nature of game farming the control of *ante-mortem* factors is limited, making the optimisation of conditions during the *peri-* and *post-mortem* periods even more vital. A reliable body of work on the handling of game meat during these periods is therefore required in order for appropriate recommendations to be made to the industry.

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CHAPTER 3

The fibre-type composition and cross-sectional area of springbok (*Antidorcas marsupialis*) *Longissimus thoracis et lumborum* and *Biceps femoris* muscle

Abstract

The purpose of this study was to determine the fibre-type composition and fibre cross-sectional area (CSA) of springbok *Longissimus thoracis et lumborum* (LTL) and *Biceps femoris* (BF) muscle. Samples were taken from seven springbok (four male, three female) at approximately eight hours *post-mortem* and snap frozen in liquid nitrogen. Immunohistochemistry was performed on sequential sections using the primary antibodies A4.74, BA-D5 and BF-35 to type fibres as type I, IIA, IIAX or IIX. The CSA of the fibres was determined using the software Image J. The proportions of each fibre-type differed in all the samples, with type IIX fibres accounting for 64% - 78%. Female springbok had more type IIX and fewer of the other fibre-types than males, but statistical significance could not be determined due to the small number of samples per gender. The fibre-type composition differed between muscles in males, with the BF containing more type I fibres and fewer type IIA fibres than the LTL. Overall the CSA values were relatively low, but still in the range reported for domestic livestock species. Female springbok had higher CSA values but no differences in CSA between the fibre-types or muscles were found. In male springbok there was an increase in CSA with glycolytic capacity (I < IIA < IIAX < IIX). Considering that the CSA values found for springbok muscle were not substantially different from that of other livestock species it is unlikely that this parameter will markedly affect the consumer's decision to purchase the meat.

Keywords: immunohistochemistry, game meat

3.1 Introduction

Skeletal muscle is an extremely complex and highly organised tissue (Kohn, Burroughs, Hartman, & Noakes, 2011). It consists of four levels of organisation, namely the myofibril, the muscle fibre, the fascicle and the whole muscle (Bailey, 1972). Of these structures the myofibril represents the functional unit of contraction while the muscle fibre can be considered as the functional metabolic unit (Bailey, 1972). Variation in the contractile and metabolic nature of the muscle fibres allows a wide variation in skeletal muscle function (Klont, Brocks, & Eikelenboom, 1998; Lefaucheur, 2010).

The different fibre-types can be identified and classified according to a number of methods, with these being based on the pH or formaldehyde sensitivity of myosin ATPase, the activity of the metabolic enzymes or the reactivity of the MHC isoforms with specific monoclonal antibodies (Dingboom & Weijs, 2004). The immunohistochemical method generally results in the specification of types I, IIA, IIX (in some literature this is referred to as IID) and IIB fibres as well as various hybrid fibres (Dingboom & Weijs, 2004). Type I fibres are classified as being slow-twitch oxidative, type IIA fibres as fast-twitch oxidative and type IIX fibres as fast-twitch glycolytic (Kohn *et al.*, 2011). Type IIB fibres are relatively rare in the skeletal muscles of large mammals (Dingboom & Weijs, 2004).

Considerable variation in the proportion of each fibre type in a muscle has been reported, with this being influenced by a number of factors including species, gender and muscle function. This variation in fibre-type composition can affect the contraction of the muscle during rigor, the rate of decline in the pH and the rate and degree of tenderization during ageing (Klont *et al.*, 1998). It is thus one of the most important intrinsic factors determining meat quality (Lefaucheur, 2010).

While fibre-typing has been performed relatively extensively on more common species such as cattle, sheep and pigs (Valin, Touraille, Vigneron & Ashmore, 1982; Henckel, Oksbjerg, Erlandsen, Barton-Gade & Bejerholm, 1997; Vestergaard & Henckel, 2000; Sazili *et al.*, 2005; Moreno-Sánchez, Díaz, Carabaño, Rueda & Rivero, 2008), only one study thus far has identified the fibre-type composition of springbok muscle (Curry, Hohl, Noakes & Kohn, 2012).

Springbok are one of the most important species in the game meat industry, and the game industry is one of the fastest growing in the South African agricultural sector (Hoffman, Muller, Schutte, Calitz, & Crafford, 2005; Hoffman & Wiklund, 2006; Thomas, 2012). The current study was thus done in order to contribute to the existing information on springbok fibre-type composition and cross-sectional area as well as explore the effect that this may have on meat quality.

3.2 Materials and methods

3.2.1 Harvesting and slaughter

Seven mature springbok (four male, three female) were harvested according to standard operating procedure (SOP number SU-ACUM13-00034) at Elandsberg nature reserve near Wellington in the Western Cape of South Africa (33°25'08.0"S 19°01'12.8"E) over nine days in January/February of 2014. The springbok were either harvested in the early morning before dawn or at night after dark in order to allow the use of a spotlight to locate and temporarily immobilise them, thereby minimising stress. They were killed with a shot to the head from a 30-06 or .270 calibre rifle. Each springbok was picked up immediately after being shot and the throat was cut to allow exsanguination. The bled carcasses were subsequently transported to the meat processing facility at the Department of Animal Sciences, University of Stellenbosch, where they were skinned and eviscerated within two hours *post-mortem* (PM). Once dressed the warm carcasses were placed in a cool room at 3 - 6°C to undergo rigor. All carcasses were suspended by both Achilles tendons in order to ensure equal contraction of the muscles in both sides of the carcass.

Ethical clearance for this study was issued by the Stellenbosch University Animal Care and Use Committee (Ethical clearance number SU-ACUM13-0034).

3.2.2 Sampling

At approximately eight hours PM portions from the centre of each *Longissimus thoracis et lumborum* (LTL) and *Biceps femoris* (BF) muscle were removed from the carcass. Several small blocks of muscle (approximately 0.5 cm³) were cut from each portion such that the direction of the muscle fibres aligned with the dimensions of the block. The blocks were subsequently snap-frozen in liquid nitrogen and stored at -80°C until sectioning.

3.2.3 Immunohistochemistry

The fibre-type identification was done using immunohistochemical methods as described by Kohn *et al.* (2011).

Serial cross-sections of 10 µm each were cut perpendicular to the direction of the fibres using a cryostat set to -25°C. The sections were fixed in acetone for 2 minutes and allowed to dry. They were subsequently rehydrated in phosphate buffered saline (PBS), pH 7.40, and blocked with 5% donkey serum (Sigma-Aldrich, St Louis, USA) for 40 minutes at room temperature.

After blocking, the sections were incubated with primary antibody cocktails overnight at 4°C in a humidifying chamber. All primary antibody cocktails contained anti-dystrophin

(MANDYS1 CLONE 3B7; Developmental Studies Hybridoma Bank, Iowa, USA) and either A4.74 (specific to myosin heavy chain IIa and IIx), BA-D5 (specific to MHC I) or BF-35 (specific to MHC I and IIa isoforms) primary antibodies (diluted 1:50 in PBS). The primary antibodies were monoclonal antibodies raised in mice (Developmental Studies Hybridoma Bank, Iowa).

The sections were then washed in PBS for 2 minutes, prior to being incubated with Cy3 donkey anti-mouse secondary antibody (diluted 1:500 in PBS) (Jackson ImmunoResearch Laboratories, Pennsylvania, USA) for 1 hour at room temperature. In addition to the secondary antibody the sections were also incubated with diluted Hoechst (Sigma-Aldrich, St Louis, USA) for ten minutes prior to washing to visualise the nuclei. Sections were thoroughly rinsed in PBS and mounted using fluorescent mounting medium MOVIOL with anti-fade.

All slides were visualised and photographed using a fluorescent-capable Nikon Eclipse 80i and a Canon 650D camera.

3.2.4 Image analysis

The fibres in each sample were identified by comparing the intensity of the fluorescent staining for each primary antibody in the sequential sections (Fig. 3.1). They were classified as type I, I/IIA, IIA, IIAX or IIX and the number of each type in the image was counted. Between 500 and 1400 fibres were counted for each sample.

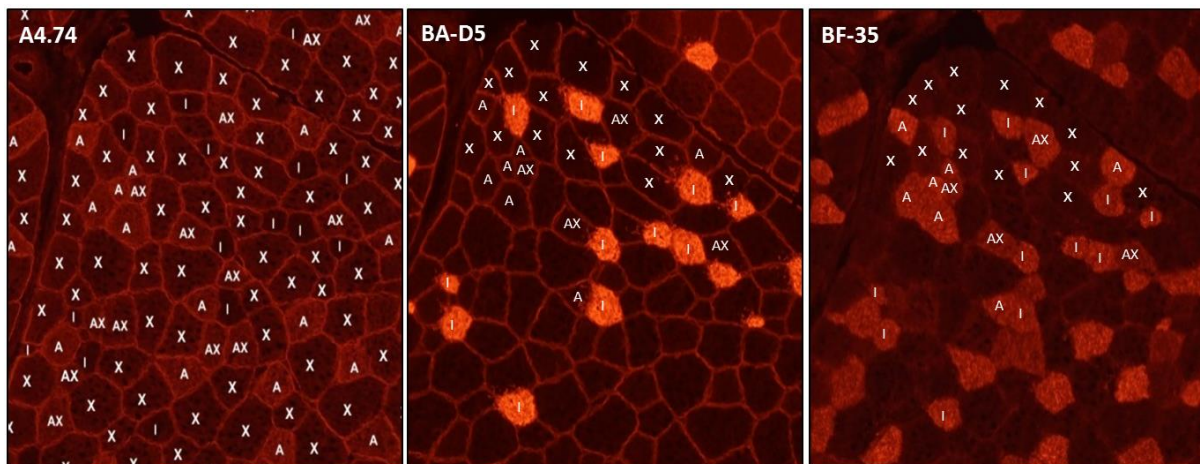


Figure 3.1 Histochemistry of springbok muscle samples as stained using the primary antibodies A4.74, BA-D5 and BF-35. Fibre types: I - type I, A - type IIA, AX - type IIAX, X - type IIX.

Once the fibres had been identified and labelled the average cross-sectional area (CSA) of each type was determined. This was done using the program Image J (version 1.47, <http://rsb.info.nih.gov/ij/>), with each fibre being outlined and the area enclosed by the outline determined by the program. In the event that fewer than 100 fibres of a specific type

were present in the image the CSA was determined for all the fibres, otherwise 100 fibres were measured.

3.2.5 Statistical analysis

The trial had a completely randomised design with the main effects, muscle and fibre-type, as well as their interaction, being tested. Gender was not tested as a main effect due to the limited number of samples per gender. The software program Statistica (version 12, Statsoft inc. 2013) was used for the analysis of the data. Prior to further analysis the data was tested to determine whether the assumptions of normality and homoscedasticity were fulfilled using normal probability plots and Levene's test respectively. The Variance Estimation, Precision and Comparison (VEPAC) function of Statistica was then used to perform repeated measures of analysis of variance (ANOVA's) on the data to determine the significance of each main effect.

The data for male and female springbok was analysed separately in order to determine whether the genders differed in the effect of muscle on the fibre-type composition or CSA. This method of analysis also allowed for the comparison of the genders, although the significance of any differences observed could not be determined.

3.3 Results and discussion

While the fibres were typed as type I, I/IIA, IIA, IIAX or IIX, very few type I/IIA fibres were identified, and they were not present in all the samples. They will thus not be discussed to any great extent. It must however be noted that all of the samples in which type I/IIA fibres were identified were from male springbok, and a maximum percentage of 3.9% was found in a single male BF sample.

3.3.1 Fibre-type composition

The proportions of each fibre type differed ($p < 0.001$) in all the samples, with the vast majority of fibres being type IIX, followed by type IIA, type IIAX and type I (Fig. 3.2, Table 3.1). Similar results were reported by Curry *et al.* (2012). When comparing these results to those reported for blesbok (*Damaliscus pygargus phillipsi*), black wildebeest (*Connochaetes gnou*), blue wildebeest (*Connochaetes taurinus*) and kudu (*Tragelaphus strepsiceros*), it appears that the fibre-type composition of springbok is more similar to that of kudu than the other game species analysed (Kohn, Hoffman, & Myburgh, 2007). Springbok muscle also appears to contain considerably more type IIX fibres than reported for Svalbard reindeer (*Rangifer tarandus platyrhynchus*; Kiessling & Kiessling, 1984), fallow deer (*Dama dama*; Curry *et al.*, 2012) and cattle (Vestergaard, Oksbjerg & Henckel, 2000).

Table 3.1

The fibre-type composition and cross-sectional area (CSA) of muscle fibres in springbok *Longissimus thoracis et lumborum* and *Biceps femoris* muscle (LSMean \pm SEM)

		Female		Male	
		LTL	BF	LTL	BF
Fibre-type composition (%)	Type I	1.8 ^C \pm 0.76	6.6 ^C \pm 0.71	3.1 ^d \pm 0.61	10.4 ^c \pm 1.21
	Type IIA	13.5 ^B \pm 0.57	11.9 ^B \pm 1.57	21.2 ^b \pm 2.53	16.9 ^c \pm 0.56
	Type IIAX	7.0 ^C \pm 1.84	5.8 ^C \pm 1.15	10.9 ^{bcd} \pm 2.26	7.7 ^{cd} \pm 2.09
	Type IIX	77.7 ^A \pm 1.84	75.7 ^A \pm 2.79	64.7 ^a \pm 5.09	63.9 ^a \pm 4.00
Fibre CSA (μm^2)*	Type I	2112 \pm 324.0		1367 ^c \pm 91.0	
	Type IIA	1904 \pm 219.1		1636 ^{bc} \pm 153.3	
	Type IIAX	2101 \pm 213.3		1904 ^b \pm 147.9	
	Type IIX	2777 \pm 401.2		2521 ^a \pm 183.9	

LSMean: least squares mean; SEM: standard error of the mean.

LTL: *Longissimus thoracis et lumborum*; BF: *Biceps femoris*.

A, B, C; a, b, c, d Within each gender, least square means reported with different superscripts differ significantly from each other ($p \leq 0.05$).

*The values for the individual muscles have not been given for the fibre CSA due to no significant difference between the muscles being found.

The high proportion of fast-twitch glycolytic fibres found in this study is in agreement with the considerable sprinting ability of springbok, with top speeds of 88 km/h being reported (Smithers, 1983; Kohn *et al.*, 2011). This, along with the extremely high activity of the glycolytic enzymes in springbok muscle as reported by Curry *et al.* (2012), indicates that the muscle is capable of rapidly providing large amounts of energy through anaerobic mechanisms (Curry *et al.*, 2012).

The high proportion of fast-twitch fibres does not however concur with the manner in which the springbok were reared or the physical nature of springbok meat. A number of studies have found that cattle, sheep, horses and pigs farmed extensively or subjected to endurance-type exercise tend to have higher proportions of oxidative fibres (Serrano, Quiroz-Rothe & Rivero, 2000; Vestergaard, Oksbjerg & Henckel, 2000; Dingboom & Weijs, 2004). A lower level of nutrition - as is found in free-ranging, unsupplemented game - has been found to have a similar effect, reducing the number of glycolytic fibres (Vestergaard, Oksbjerg & Henckel, 2000). In addition, springbok meat is known to be high in iron and have a relatively low L* value, both of which have been associated with a high proportion of type I and IIA fibres (Henckel *et al.*, 1997; Dingboom & Weijs, 2004; Hoffman, Kroucamp & Manley, 2007a; Hoffman, Kroucamp & Manley, 2007b; Choe & Kim, 2008). It thus appears that the low type I content of springbok muscle is in direct conflict with a number of other characteristics of the meat.

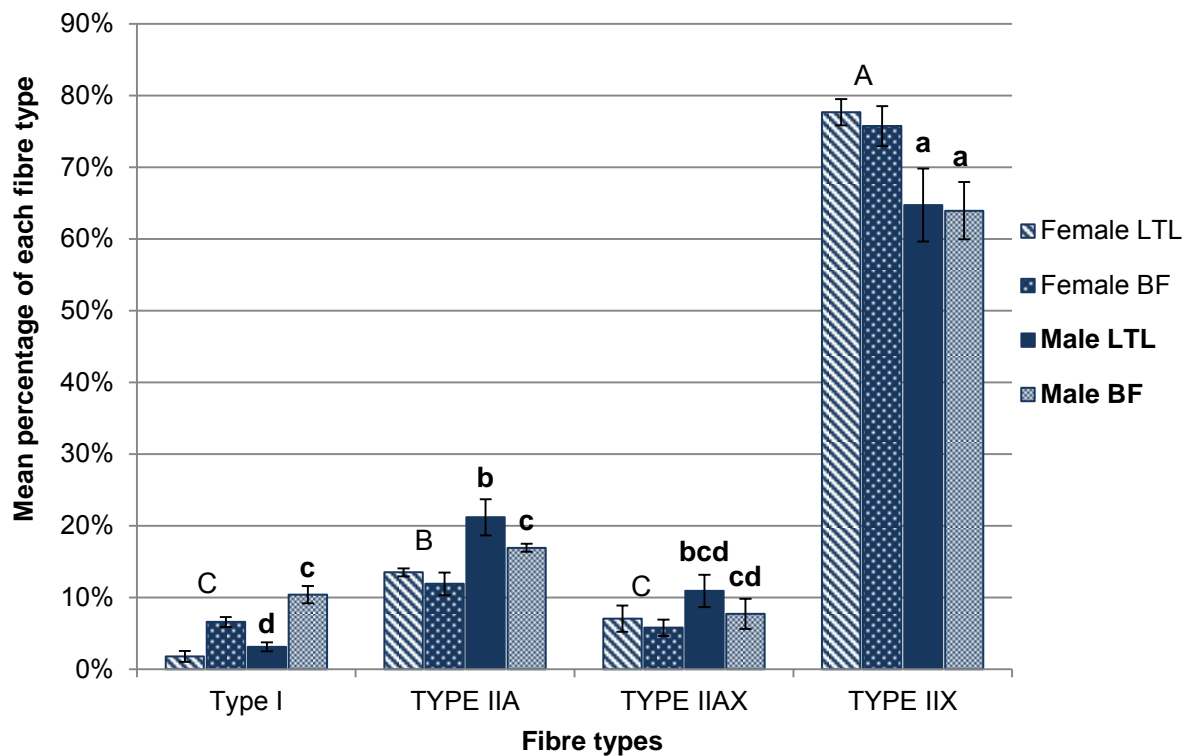


Figure 3.2 The fibre-type composition of male and female springbok *Longissimus thoracis et lumborum* (LTL) and *Biceps femoris* (BF) muscle.

Different letters indicate significant differences ($p \leq 0.05$) between the proportions of each fibre type in each muscle. Significance is not compared across the genders. Error bars indicate the standard error of the mean for each group.

However, it must be noted that while immunohistochemistry allows the grouping of fibres into discrete types, variation in the metabolic and thus biochemical characteristics does exist within each myosin ATPase-based type (Maltin, Balcerzak, Tilley, & Delday, 2003; Dingboom & Weijs, 2004; Choi & Kim, 2009). Vestergaard, Oksbjerg and Henckel (2000) reported that a change in the oxidative capacity of a muscle may take place without a corresponding change in fibre type. This has also been reported in pigs, with endurance exercise increasing the oxidative capacity without resulting in a significant change in the fibre-type composition of the muscle (Choi & Kim, 2009). Moreover, it has been found that in some species, such as the horse, the oxidative capacity of type IIA fibres is in fact greater than that of type I fibres (Dingboom & Weijs, 2004).

The type II fibres in springbok may thus have a higher oxidative capacity than normally found for these fibre-types in domesticated species. This would increase the fatigue-resistance of the fibres and muscle, providing springbok with valuable stamina in conjunction with speed. This is supported by the results of Curry *et al.* (2012), who found that despite the

high proportion of type IIX fibres the activity of the enzymes involved in oxidative metabolism was relatively high in springbok muscle, and comparable to that found in endurance-trained humans. A large amount of variation in the oxidative capacity of the fibres was also found within the type IIX group (Curry *et al.*, 2012). More extensive research on the relationship between the MHC isoforms and the metabolic nature of muscle fibres in different species is thus required.

Although a statistical comparison of the genders could not be made, as insufficient samples were analysed, one can see that the genders did have numerically different fibre-type compositions (Table 3.1, Fig. 3.2). Female springbok had considerably more type IIX fibres and fewer of the other types than male springbok. This is in agreement with the finding in sheep that muscles from ewes contained more type IIX fibres and fewer type IIA fibres than castrates (Greenwood, Harden & Hopkins, 2007). It also supports the finding in previous studies that male animals are more susceptible to DFD than females (Dransfield, 1994), as the low-glycogen content of oxidative fibres increases the risk of glycogen depletion and thus DFD (Dingboom & Weijs, 2004). However, it must be noted that the proportion of oxidative fibres in both genders was very low.

In addition, significant negative correlations between the proportion of type IIA fibres and all sensory eating quality attributes, including overall acceptability, have been found (Henckel *et al.*, 1997). Furthermore, the proportion of type IIB fibres (analogous with type IIX fibres) was positively correlated with both colour and flavour in pork (Henckel *et al.*, 1997). This suggests that, based on the fibre-type composition, female springbok have the potential to have higher quality meat than male springbok.

No significant interaction between fibre-type and muscle was found for female springbok ($p = 0.125$), indicating that the two muscles did not differ in fibre-type composition. In contrast, there was interaction ($p = 0.007$) in male springbok, with the BF containing a higher proportion of type I fibres and a lower proportion of type IIA fibres (Table 3.1). While this could influence the function of the muscle the effect on its PM behaviour may be negligible, as both type I and type IIA fibres are classified as oxidative according to their succino-dehydrogenase activity (Lefaucheur, 2010; Curry *et al.*, 2012).

The fibre type composition of the muscle can also influence the physical changes that take place during the rigor period. Type I fibres have been found to shorten to a greater degree than the other fibre types (Klont *et al.*, 1998; Dingboom & Weijs, 2004). The small proportion of these fibres in springbok muscle may therefore contribute to its tenderness by reducing the shortening during rigor.

The apparently highly glycolytic nature of springbok meat may also be the cause of the rapid tenderization during ageing found in this study (Chapter 5 and 6). So-called 'white

muscles', or muscles with a large proportion of fast glycolytic fibres, have been reported to tenderize more rapidly than 'red muscles' (Sazili *et al.*, 2005). This is attributed to the higher calpain:calpastatin ratio found in glycolytic muscles (Sazili *et al.*, 2005), with significant, positive correlations between the proportion of slow-twitch MHC isoforms and the calpastatin activity being found in sheep muscle (Sazili *et al.*, 2005). The association between calpain and calpastatin activity and tenderization has been well established (Hopkins & Huff-Lonergan, 2004; Huff Lonergan, Zhang, & Lonergan, 2010). In addition to the differences in calpain and calpastatin activity, the rapid tenderization may also be the result of the greater susceptibility to proteolysis of the z-line proteins in fast-twitch fibres (Choi & Kim, 2009).

3.3.2 Fibre cross-sectional area (CSA)

The overall cross-sectional areas (CSA) found for springbok indicate that the species has much smaller muscle fibres than humans and relatively similar fibre CSA values to bovine LTL muscle (Vestergaard, Oksbjerg & Henckel, 2000; Kohn *et al.*, 2011). The CSA of type I and type IIA fibres was similar to those found in porcine LTL while the fast-twitch glycolytic fibres had considerably smaller surface areas (Henckel *et al.*, 1997). The CSA values found in this study were lower than those reported for springbok by Curry *et al.* (2012). This may be as a result of genetic differences in the springbok used. Alternatively the later sampling time used in this study (eight rather than four hours PM) may have resulted in the fibres shrinking somewhat prior to freezing (Curry *et al.*, 2012).

Female springbok had considerably larger fibres than males for all the fibre-types and both muscles, with this being most distinct in the type I fibres (Table 3.1, Fig. 3.3). This is in contrast to the finding in most species that male muscles have fibres with greater CSA values, reportedly as a result of the hypertrophy-stimulating effects of testosterone (Dingboom & Weijs, 2004; Choi & Kim, 2009). Similar results have however been found for pigs, with sows reported to have higher CSA values than boars (Dingboom & Weijs, 2004). Curry *et al.* (2012) found no significant difference in the CSA between genders for springbok or fallow deer.

As can be seen in Table 3.1 and Figure 3.3, no significant difference between the muscles ($p = 0.586$) or between the fibre-types ($p = 0.111$) was found in samples from female springbok. In contrast, while non-significant, some indication of interaction was found between muscles and fibre-types in male springbok ($p = 0.092$) and the fibre-types differed significantly ($p = 0.008$).

In male springbok the average CSA tended to increase with glycolytic capacity for both muscles, but the LTL had a lower CSA across all the fibre types (Table 3.1, Fig. 3.3). The difference in the CSA between the type IIX fibres and that of the other fibre-types was also

greater in male LTL than the male BF. The progressive increase in size from type I to IIA, IIAx and IIX is consistent with literature, and has been reported in a number of other species (Dingboom & Weijs, 2004; Choi & Kim, 2009; Kohn *et al.*, 2011). Few studies have compared the fibre size of the BF and LTL muscles; however, similar results were found in sheep by Barkawi, El-Asheeri, Hafez, Ibrahim & Ali (2009).

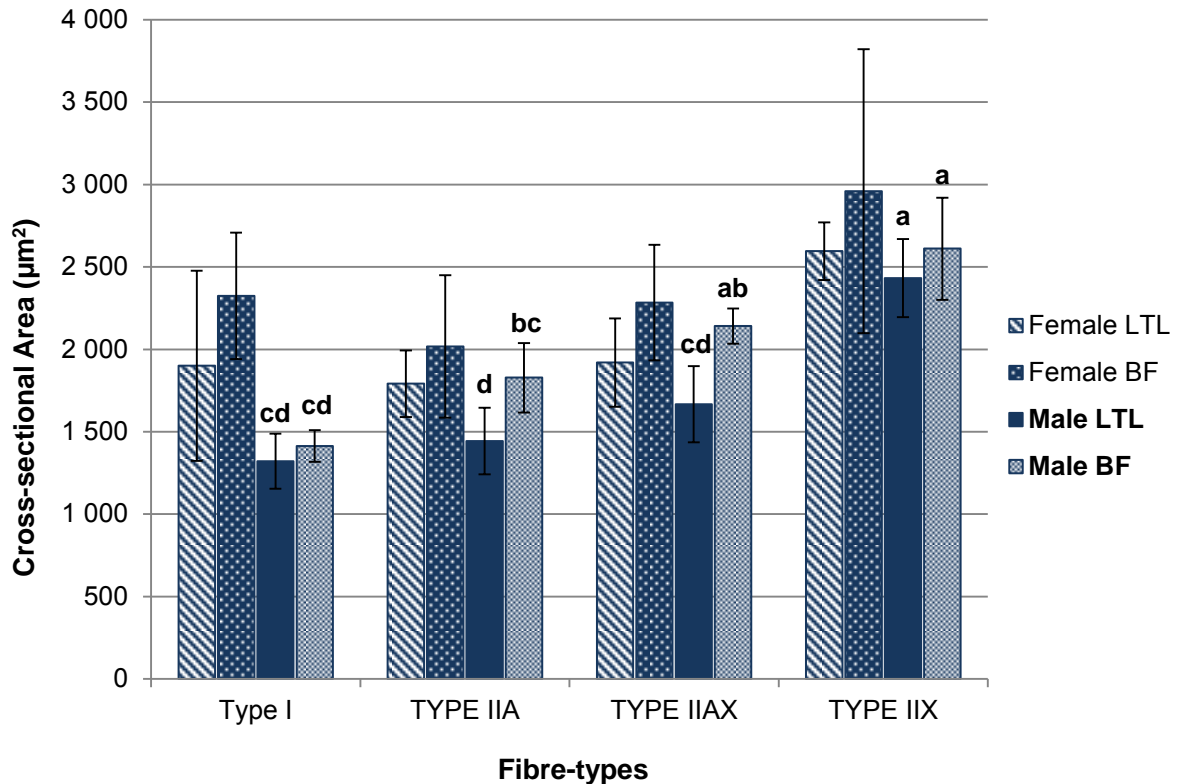


Figure 3.3 The cross-sectional area (CSA) of male and female springbok *Longissimus thoracis et lumborum* (LTL) and *Biceps femoris* (BF) muscle.

Different letters indicate significant differences ($p \leq 0.05$) between the CSA of each fibre type in each muscle. Significance is not compared across the genders. Error bars indicate the standard error of the mean for each group.

The average fibre cross-sectional area is most important in terms of meat quality as a result of its effect on muscle texture and tenderness. There is however considerable controversy regarding the effect of muscle fibre size on tenderness. Choi & Kim (2009) report that larger fibre CSA's (especially for type IIB or X) are related to increased toughness and hardness in cattle and pigs. In contrast, according to Dingboom & Weijs (2004) there is a positive relationship between tenderness and the fibre cross-sectional area. It thus seems likely that an optimal fineness of texture exists, and that this differs between species. The CSA found for springbok appears to be within the range of that reported for domesticated species however, and it is therefore unlikely to affect consumer liking of the meat to any great extent.

3.4 Conclusion

The results from this study suggest that male springbok may be at more risk of developing DFD meat than female springbok and that the use of separate handling procedures for the LTL and BF may be more necessary in male than female springbok. This is based on the slightly less glycolytic nature of male springbok muscle and the greater differences in the fibre-type composition between the muscles in this gender. However, the effect of factors such as *ante-mortem* stress and carcass handling protocols may have a greater effect on the meat quality than the fibre-type composition. Further research on the relationship between the contractile and metabolic nature of muscle fibres in springbok muscle is necessary.

The average cross-sectional area of the fibres was found to be low but within the range reported for other meat-producing species. The effect on consumer acceptance is thus likely to be relatively negligible.

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CHAPTER 4

The physico-chemical changes in springbok (*Antidorcas marsupialis*) *longissimus thoracis et lumborum* and *biceps femoris* muscles during the rigor period

Abstract

This study aimed to describe the changes taking place during rigor in springbok *Longissimus thoracis et lumborum* (LTL) and *Biceps femoris* (BF) muscles. The pH was determined at 2, 3, 5, 8, 12, 18, 24 and 30 hours *post-mortem* (PM) and the calpain and cathepsin activity was determined at 2, 18 and 30 and 2, 8, 18, 24 and 30 hours PM respectively for twelve (six male, six female) mature springbok. Non-linear regressions ($y = c + (a - c) (1 - m)^x$) were fitted to the pH and temperature data. Significant interaction was present between gender, muscle and time PM, with the female LTL cooling more rapidly and acidifying slower than the other samples. Both female LTL and BF muscles were at risk of developing cold-shortening and all the samples cooled more rapidly than recommended for cattle or sheep. Delayed chilling, alternative hanging methods or electrical stimulation may thus be necessary. Higher cathepsin B and BL activity was recorded in female springbok than male springbok and the LTL had higher cathepsin BL activity than the BF across both genders. There was an increase in cathepsin BL activity PM, most likely due to the release of the cathepsins from the lysosomes. Calpain activity was higher in male springbok than females and the BF muscle had higher calpain and calpastatin activity than the LTL across both genders. Calpain I, II and calpastatin activity declined during rigor, which is in agreement with the results of previous studies and supports the theory that the calpains are activated early PM. Correlations found between the pH decline rate and the change in calpastatin activity indicated that more rapid acidification results in a greater decrease in calpastatin activity.

Keywords: pH, temperature, calpain, cathepsin, cold-shortening, game meat, venison

4.1 Introduction

The game industry is one of the most rapid-growing in the agricultural sector in South Africa and contributed R7.7 billion to the economy in 2008 (Thomas, 2012). While the current contribution of meat sales to the total income is very small (Hoffman, 2007), it may in future play a role as not only an additional source of income for farmers but also in improving the food security of the country.

At present springbok are the most important game species for meat production, contributing over 80% of the game carcasses exported in 2005 and 66.5% of the total game harvested by Camdeboo Meat Processors in 2013 (Hoffman & Wiklund, 2006; personal communication with P. Neethling, Camdeboo). It is thus important that the quality of the game meat produced is optimised, and in order to do this the nature of the meat needs to be thoroughly understood.

The conversion of muscle to meat during the rigor period is a complex and dynamic process that involves a complete change in the physical and biochemical nature of the muscle (Warriss, 2000). Two of the most influential and drastic alterations are the decline in the pH and temperature.

The temperature of the carcass declines *post-mortem* (PM) as a result of heat dissipation to the surrounding air; with the rate of this decline depending on the size and fat level of the carcass as well as the temperature of the environment (Warriss, 2000). The temperature of the muscle in turn has a large influence on the rate at which any enzymes that still remain active can function, with the enzymes involved in glycolysis being no exception to this (Warriss, 2000). By influencing the activity of the glycolytic enzymes the temperature can accelerate or retard the conversion of glycogen to lactic acid and thus the decline in the pH of the muscle (Newbold & Harris, 1972; Warriss, 2000).

Meat colour, water-holding capacity and tenderness are all highly affected by the nature of the decline in pH and temperature during the rigor period (Hamoen, Vollebregt & Van der Sman, 2013; Kim, Warner & Rosenvold, 2014). Of these, tenderness has been found to be one of the most important attributes of meat quality for consumers (Bickerstaffe, Bekhit, Robertson, Roberts & Geesink, 2001), and is influenced by the effect of the changes in pH and temperature on muscle shortening during rigor and the proteolytic activity both during rigor and later during ageing (Warriss, 2000; Hwang & Thompson, 2001).

Two of the proteolytic enzymes thought to play a role in tenderization are the calpains and the cathepsins (Warriss, 2000). The calpains are thought to be activated relatively early PM, and are thus particularly vulnerable to changes in the pH and temperature decline (Dransfield, 1994; Huff Lonergan, Zhang, & Lonergan, 2010). The cathepsins are reported to be active later during conditioning; however, they are contained within the lysosomes in

living muscle, and the rate of the decline in pH is thought to influence the rate at which they are released into the sarcoplasm (Calkins & Seideman, 1988; O'Halloran, Troy, Buckley & Reville, 1997; Lawrie & Ledward, 2006). It is therefore important to understand what effect the observed declines in pH and temperature during rigor have on these two enzyme systems.

While studies on the changes during rigor in sheep and cattle have been done, previous research has found that the response of muscle to different rigor temperatures differs between species (Bekhit, Farouk, Cassidy & Gilbert, 2007). This may be as a result of the effect of variation in fibre-type composition on the metabolic and contractile nature of the muscle. It is therefore not appropriate to extrapolate findings for beef, lamb or other venison species to springbok meat (Bekhit *et al.*, 2007). Despite this, literature on the nature of the decline in pH and temperature in springbok is extremely limited (Marais, 2013), and no other research on the changes in proteolytic enzyme activity in springbok meat has been done.

The purpose of this study was to expand on the current knowledge on pH, temperature and calpain and cathepsin activity changes during the early PM period in springbok meat. By comparing these results to various set points developed for the optimisation of meat quality it can be determined whether the handling of springbok carcasses can be improved in any way.

4.2 Materials and methods

4.2.1 Harvesting and slaughter

Twelve mature springbok (six male, six female), were harvested according to standard operating procedure (SOP number SU-A CUM13-00034) at Elandsberg nature reserve near Wellington in the Western Cape of South Africa (33°25'08.0"S 19°01'12.8"E) following the same procedures as described in section 2.1 of Chapter 3.

4.2.2 Sampling and *in situ* measurements

The temperature decline of the carcasses, as well as the ambient temperature to which each carcass was exposed, was recorded using automatic temperature loggers (LogTag TREX-8 temperature recorder fitted with a ST100T-15 temperature probe; LogTag, Auckland, New Zealand). These were inserted within two hours PM, with the left LTL being monitored at approximately 2 cm from the spinous processes between the first and second lumbar vertebra and the BF at its approximate centre on both the horizontal and vertical planes. The loggers recorded the temperature of the muscle or air every minute for 31 hours or until sampling was complete. LogTag Analyzer version 2.3 software was used to download the data.

Samples for chemical analysis were taken at eight time periods PM, namely 2, 3, 5, 8, 12, 18, 24 and 30 hours. In order to maintain the degree of contraction and the rate of chilling as close to normal as possible the muscles were not excised from the carcass. Portions were removed from each muscle at each time period, with four portions being removed from the muscles on each side of the carcass and the position of the portion for each time period being randomly determined. The entire BF was used for sampling whereas the LTL was sampled from between the last lumbar vertebra caudally and the junction with the scapula cranially. As each time period was reached the assigned portion was removed from the carcass and divided into samples for each relevant chemical analysis. The samples were snap-frozen in liquid nitrogen, with the precise time of freezing being recorded. All samples were stored at -80°C until analysis.

4.2.3 Chemical analysis

4.2.3.1 pH

The muscle pH was determined using the sodium-iodoacetate method (Zhu, Ruusunen, Gusella, Zhou & Puolanne, 2011). This entailed homogenizing (Kinematica Polytron PT 2500 E; Lasec SA, Cape Town, South Africa) 0.5 g of the frozen meat sample in 5 ml of sodium-iodoacetate solution (5 mM iodoacetate and 150 mM potassium chloride in distilled water, adjusted to pH 7 using potassium hydroxide or hydrochloric acid) for 30 seconds at approximately 8000 rpm. The pH of the homogenate was subsequently determined using a desktop pH meter (Jenway 3510 pH meter; Lasec SA, Cape Town, South Africa; calibrated using pH 4.0 and pH 7.0 standard buffers). The samples and homogenates were kept on ice throughout the process.

4.2.4.2 Cathepsin activity

Enzyme extraction:

One gram of frozen muscle sample was homogenized at 7500 - 8500 rpm for 30 seconds (Kinematica Polytron PT 2500 E; Lasec SA, Cape Town, South Africa) in 2.5 ml of lysing buffer consisting of 50 mM sodium acetate, 100 mM sodium chloride (NaCl), 7.84 mM ethylenediaminetetraacetic acid (EDTA) and 0.2% Triton X-100 (adjusted to pH 5 with acetic acid). The homogenate was stirred for one hour at 4°C before being centrifuged at the same temperature for 40 min at 4024 g. The supernatant was subsequently filtered (Whatman number 1 filter paper). Samples were kept at either refrigerator temperatures or on ice throughout the extraction procedure.

Fluorescent assay:

The enzyme extract was assayed for cathepsin B, B and L, and H activity according to the procedure of Thomas, Gondoza, Hoffman, Oosthuizen and Naudé (2004), and Van Jaarsveld, Oelofsen and Naude (1998); with minor adjustments being made. Twenty-five microlitres of the supernatant, 50 μ l of assay buffer (340 mM sodium acetate, 60 mM acetic acid, 3.14 mM EDTA and 8 mM dithiothreitol; adjusted to pH 5.5 using sodium hydroxide) and 10 μ l 0.1% Brij were pipetted into wells in three black microplates (Greiner Cellstar 96 well black plates; Sigma-Aldrich, St Louis, USA), one for each enzyme, which were then incubated for 2 minutes at room temperature. The enzyme-specific substrates (B: Z-RR-AMC, 5 μ M; B and L: Z-FR-AMC, 5 μ M; H: R-AMC, 10 μ M; Bachem, Bubendorf, Switzerland) were pipetted into an adjacent lane of wells on each plate. The plates were subsequently incubated for 5 minutes at 40°C in order to preheat both the enzyme extract and the substrate to the correct temperature. The substrate was added to each well (25 μ l), with the contents of the well being thoroughly mixed in the process. The fluorescence (excitation 360 nm, emission 460 nm; Varioskan version 3.01.15, Thermoscientific, Waltham, Massachusetts, USA) was measured every 30 seconds for a total of 5 min in order to obtain a progress curve (fluorescence versus time).

The initial slope (linear portion) of the progress curve was used for calculating enzyme activity. In order to calculate the specific enzyme activity a subsample of each enzyme extract was analysed for protein content using the DC protein assay kit II (Bio-Rad catalogue number 500-0112, California, USA). Cathepsin specific activity is thus defined as the change in fluorescence measured (excitation 360 nm, emission 460 nm) per minute per mg of extractable protein (Δ fluorescence/min/mg).

Prior to enzyme determinations, a dilution series of the enzyme extracts was used to establish the concentration dependence of the three assays. It was found that the change in fluorescence per minute in the three assays increased with an increase in the enzyme concentration.

The joint activity of cathepsin B and L is reported in this study as BL activity, rather than the apparent effect of cathepsin L being determined as the difference in activity between cathepsin B and L, and B. This method of expressing the cathepsin activity was chosen as the enzymes have been reported to differ in their specificity for the different substrates (van Jaarsveld *et al.*, 1998). In addition, the calculation of cathepsin L activity requires the assumption that cathepsin B activity and cathepsin L activity levels are independent of each other.

4.2.3.3 Calpain activity

The calcium-activated proteases (calpain I and calpain II) and their inhibitor calpastatin were extracted from the meat samples as described by Dransfield (1996); with minor adaptations being made. A 3 g sample of frozen meat was homogenized in 15 ml extraction buffer (75 mM Tris pH 7.8, 10 mM EDTA, 0.05% [v/v] 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 1 µl/L pepstatin A) at 4°C. The homogenate was centrifuged at 10000 g and filtered. The volume of the filtrate was then made up to 20 ml with extraction buffer. The filtrate was adjusted to pH 7.5, with care being taken to prevent it from dropping below pH 7 at any point. The protein concentration of the filtrate was determined using the Biurette method (Gornall, Bardawill & David, 1949).

A Gilson apparatus (fraction collector, FC204; peristaltic pump, Minipuls 3; detector, 112UV; valve actuator, Valvemate II; system interface, 506C) in conjunction with the automated Unipoint LC system software (version 4.0) was used to separate the calpastatin and calpain I from calpain II by means of the two-step gradient ion-exchange chromatography-method (Geesink & Koochmarai, 1999). The sample was run through a 20 ml DEAE Sepharose (GE Healthcare Bio-sciences AB, C 10/10 column) packed column with 0.0 M, 0.175 M and 0.35 M NaCl-Tris buffers (pH 7.5) being run sequentially to separately elute the different fractions. Three protein peaks were obtained, one from each of the NaCl Tris buffers. Fractions containing these protein peaks were pooled. The fractions eluted with the 0.0 M buffer (fraction 1) contained calpastatin only while the 0.175 M NaCl buffer (fraction 2) typically contained both calpastatin and calpain I. The latter fraction is used for the indirect determination of calpain I activity. The third fraction (0.35 M NaCl-Tris buffer) contained only calpain II.

Calpain activity was determined using an azo-casein assay (7.5 µg/ml azo-casein in 0.1 M Tris buffer, 0.1 M calcium chloride and 0.05% [v/v] mercaptoethanol adjusted to pH 7.5 at 4°C), with the reaction being stopped after 1 hour using 10% trichloroacetic acid. The sample was subsequently centrifuged at 4000 g and the absorbance measured at 366 nm. The calpain II activity was determined directly on aliquots from fraction 3 without any prior treatment. The inhibitory activity of calpastatin was determined by assaying the proteolytic activity of the calpain II fraction (fraction 3) before and after the addition of heated aliquots of fraction 1 and 2. The heat process was used to inactivate the calpain I in the fractions. Calpain I activity was determined by assaying the total proteolytic activity of calpain I and II in combined aliquots of fraction 2 and 3 with the fraction 2 aliquot being either heated or unheated in order to inactivate calpain I (calpain I = [calpain I + calpain II – calpastatin] - [calpain II-calpastatin]). The heat treatment used in the determination of the calpastatin and calpain I activity entailed heating the aliquot to a temperature of 95°C for 15 min and then

cooling on ice prior to centrifuging at 4000 g for 15 min to precipitate the denatured proteins (Koochmaraie, 1990). The calpain and calpastatin determinations as described above are considered to be estimates and not exact determinations, as they are influenced by numerous factors such as protein extractability, the inseparability of calpastatin and calpain I and the stability of the enzymes.

One unit of calpain activity is defined as an increase in absorbance at 366 nm of 1.0 per hour at 25 °C. One unit of calpastatin activity is defined as the amount that inhibited one unit of calpain II activity. Data is expressed as units per milligram of extractable protein (specific activity).

4.2.3.4 Fibre-type composition

The fibre-type composition of the muscles used in this study was determined and discussed in Chapter 3.

4.2.4 Statistical analysis

The trial had a completely randomised design, with the three main effects - gender, muscle and time PM - being tested in a three-factor factorial experiment. Statistical analysis was performed using the statistical software program Statistica version 12 (StatSoft Inc, 2013). Initial processing of the data included testing for normality using normal probability plots and testing for homoscedasticity using Levene's test. Once these assumptions had been found to hold true, mixed model repeated measures of analysis of variance (ANOVA's) were performed using the VEPAC (variance estimation, precision and comparison) function in Statistica. In the event that the effect of time PM was significant, Fisher's LSD test was used to determine which of the individual time periods differed significantly from one another. Pearson's correlation coefficients (r) were calculated when appropriate to determine whether significant correlations between variables existed.

In addition to the ANOVA tests, non-linear regressions were also fitted to the pH and temperature data, with time PM as the independent variable. The equation for the regression had the following structure:

$$y = c + (a - c)(1 - m)^x$$

In this equation (y) represents the dependant variable (pH or temperature), (x) represents the independent variable (time PM), (c) gives an indication of the minimum y -value obtained, (a) is the intercept of the line with the y -axis and (m) is the decay constant.

The regressions were fitted to the data per treatment group and interaction in order to determine whether the main effects had a significant influence on the values of the equation constants. The regressions were also fitted per animal and per muscle, thereby generating a

decay constant for each sample. These were then compared to the change in the enzyme activity from 2 to 18 hours PM and 2 to 30 hours PM in order to determine whether there were significant correlations between the rate of change in the pH and temperature and the rate of change in the enzyme activity.

It must be noted that the regression curves are only fitted for data collected from two hours PM onwards. This is most important for the temperature data, as the carcasses were only skinned and placed in the cool room at approximately this time. The insulating presence of the skin and the higher ambient temperature prior to this would have changed the nature of the temperature decline. While indeed indicating the y-intercept of the regression, the (*a*) value therefore does not necessarily indicate the *in vivo* conditions in the animal at death.

Main effects, interactions and correlations with $p \leq 0.05$ were considered to be significant. Values are reported as the LSMean \pm the standard error of the mean (SEM).

4.3 Results

The initial statistical analysis of the pH and temperature data indicated that a third-order interaction between gender, muscle and hours PM was present ($p_{\text{temp}} = 0.002$; $p_{\text{pH}} = 0.019$; Table 4.1 and 4.2). Non-linear regressions were therefore fitted to each gender-muscle treatment group separately in order to determine the differences in the pattern of decline. The equation constants for each regression line are depicted in Table 4.3.

Table 4.1

The effect of gender, muscle and time PM on the temperature (°C) of springbok *Longissimus thoracis et lumborum* and *Biceps femoris* muscles (LSMean \pm SEM)

Main effects		Male		Female	
		LTL	BF	LTL	BF
Hours PM:	2	31.6 ^a \pm 0.86	31.7 ^a \pm 1.15	25.6 ^b \pm 1.43	30.8 ^a \pm 1.26
	3	22.4 ^c \pm 1.38	21.5 ^c \pm 1.52	17.7 ^d \pm 0.74	23.8 ^{bc} \pm 1.79
	5	11.0 ^e \pm 0.97	12.1 ^e \pm 1.13	8.1 ^f \pm 0.39	12.3 ^e \pm 1.24
	8	5.4 ^{fg} \pm 0.46	6.7 ^{fg} \pm 0.54	4.0 ^{hi} \pm 0.65	5.9 ^{fgh} \pm 0.44
	12	4.1 ^{gi} \pm 0.45	4.8 ^{gi} \pm 0.15	3.7 ^{hi} \pm 0.61	4.5 ^{gi} \pm 0.11
	18	3.9 ^{hi} \pm 0.43	4.2 ^{hi} \pm 0.12	3.6 ⁱ \pm 0.45	4.2 ^{hi} \pm 0.05
	24	3.8 ^{hi} \pm 0.42	4.2 ^{hi} \pm 0.11	3.7 ^{hi} \pm 0.43	4.2 ^{hi} \pm 0.10
	30	3.8 ^{hi} \pm 0.43	4.2 ^{hi} \pm 0.09	3.8 ^{hi} \pm 0.49	4.2 ^{hi} \pm 0.22

LSMean: least squares mean; SEM: standard error of the mean.

LTL: *Longissimus thoracis et lumborum*; BF: *Biceps femoris*; PM: *post-mortem*.

a, b, c, d, e, f, g, h, i Least square means with different superscripts differ significantly from one another ($p \leq 0.05$).

Table 4.2

The effect of gender, muscle and time PM on the pH (LSMean \pm SEM) of springbok *Longissimus thoracis et lumborum* and *Biceps femoris* muscles

Main effects		Male		Female	
		LTL	BF	LTL	BF
Hours PM:	2	6.32 ^{bc} \pm 0.121	6.37 ^{ab} \pm 0.070	6.38 ^b \pm 0.095	6.56 ^a \pm 0.083
	3	6.03 ^{ef} \pm 0.153	6.27 ^{bcd} \pm 0.131	6.39 ^{ab} \pm 0.087	6.39 ^b \pm 0.128
	5	6.03 ^{ef} \pm 0.090	5.95 ^{efi} \pm 0.102	6.11 ^{ec} \pm 0.094	6.28 ^b \pm 0.072
	8	5.80 ^{gikl} \pm 0.047	5.90 ^{efghij} \pm 0.094	6.10 ^{ed} \pm 0.079	6.04 ^e \pm 0.051
	12	5.75 ^{hkl} \pm 0.043	5.77 ^{gkl} \pm 0.062	5.96 ^{efg} \pm 0.067	5.96 ^{efgh} \pm 0.054
	18	5.67 ^{kl} \pm 0.034	5.73 ^{kl} \pm 0.029	5.75 ^{ikl} \pm 0.031	5.86 ^{fk} \pm 0.028
	24	5.63 ^l \pm 0.039	5.72 ^{kl} \pm 0.042	5.69 ^{jl} \pm 0.027	5.78 ^{ikl} \pm 0.037
	30	5.67 ^{kl} \pm 0.019	5.67 ^{kl} \pm 0.004	5.69 ^l \pm 0.030	5.77 ^{ikl} \pm 0.030

LSMean: least squares mean; SEM: standard error of the mean.

LTL: *Longissimus thoracis et lumborum*; BF: *Biceps femoris*; PM: *post-mortem*.

a, b, c, d, e, f, g, h, i Least square means with different superscripts differ significantly from one another ($p \leq 0.05$).

Table 4.3

The equation constants of the non-linear regression ($y = c + (a - c) (1 - m)^x$) fitted to the decline in the temperature and pH of springbok *Longissimus thoracis et lumborum* and *Biceps femoris* muscles during the rigor period

Main effects		Temperature			pH		
		<i>c</i>	<i>a</i>	<i>m</i>	<i>c</i>	<i>a</i>	<i>m</i>
Female	LTL	3.656	80.085	0.427 ^a	5.639	6.602	0.092 ^b
	BF	4.117	72.558	0.340 ^b	5.788	6.846	0.141 ^{ab}
Male	LTL	3.773	85.385	0.380 ^{ab}	5.668	6.671	0.205 ^a
	BF	4.340	80.409	0.369 ^{ab}	5.722	6.936	0.236 ^a

Temperature: female LTL $r^2 = 0.98$; female BF $r^2 = 0.98$; male LTL $r^2 = 0.99$; male BF $r^2 = 0.99$.

pH: female LTL $r^2 = 0.85$; female BF $r^2 = 0.87$; male LTL $r^2 = 0.76$; male BF $r^2 = 0.81$.

c: minimum y-value; *a*: y-intercept; *m*: decay rate.

LTL: *Longissimus thoracis et lumborum*; BF: *Biceps femoris*.

^{a, b} Least square means within the same column with different superscripts differ significantly from one another ($p \leq 0.05$).

The only aspect of the regressions for both the temperature and pH that differed ($p \leq 0.05$) between the gender-muscle groups was the decay rate. In both cases there were no differences between the muscles in male springbok, while the female LTL had a significantly higher temperature decay rate than the female BF and a significantly lower pH decay rate than either muscle in male springbok. These differences can be clearly seen in Figure 4.1 and 2.

The female LTL muscle's high temperature decay rate resulted in it reaching ambient temperature relatively quickly, with the statistical comparison of the temperatures at each time point indicating that it stabilised at around 8 hours PM. The female BF and male muscles only reached ambient temperature at approximately 12 to 18 hours PM (Table 4.1).

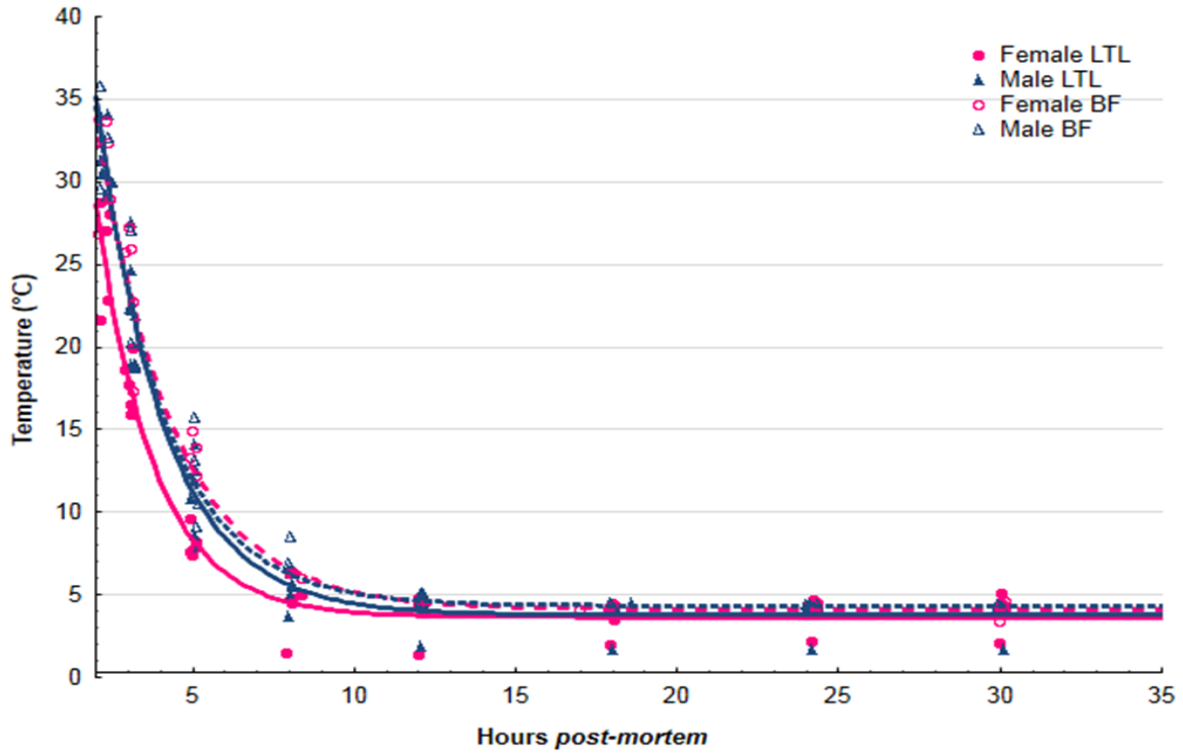


Figure 4.1 The decline in the temperature of the *longissimus thoracis et lumborum* (LTL) muscle in female (— ; $r^2 = 0.98$) and male (— ; $r^2 = 0.99$) springbok and *biceps femoris* (BF) muscle in female (- - ; $r^2 = 0.98$) and male (..... ; $r^2 = 0.99$) springbok during the rigor period.

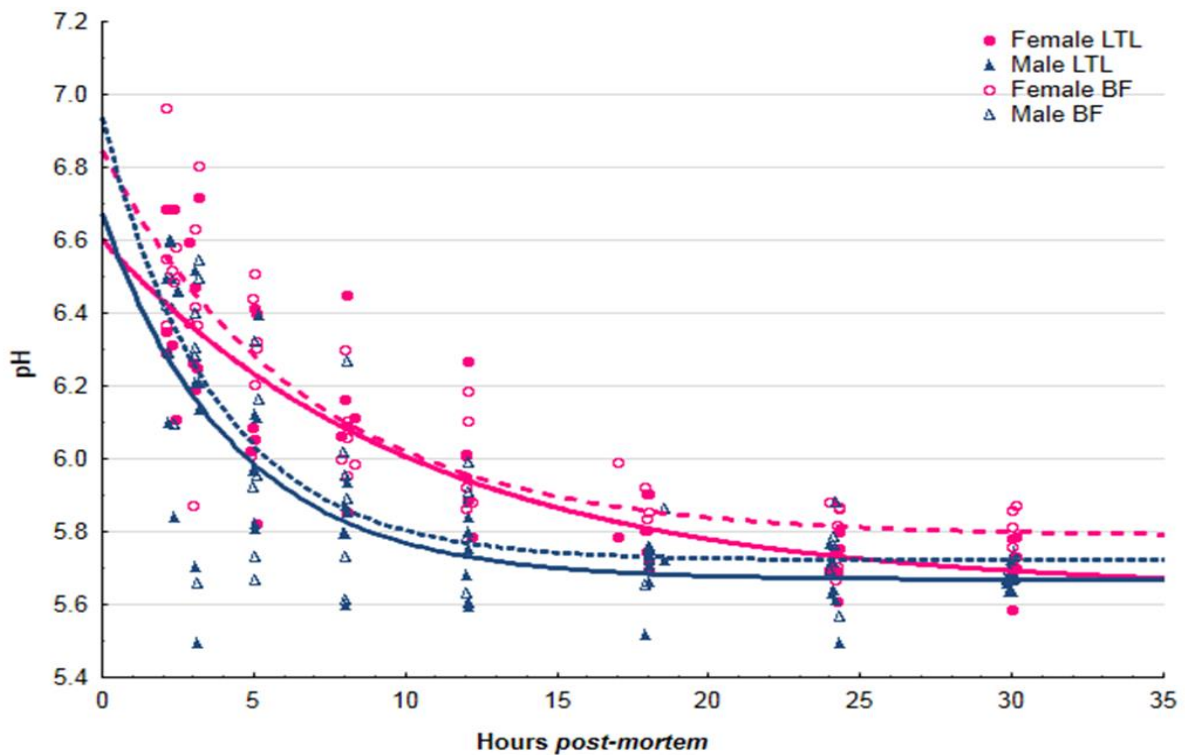


Figure 4.2 The decline in the pH of the *longissimus thoracis et lumborum* (LTL) muscle in female (— ; $r^2 = 0.85$) and male (— ; $r^2 = 0.76$) springbok and *biceps femoris* (BF) muscle in female (- - ; $r^2 = 0.87$) and male (..... ; $r^2 = 0.81$) springbok during the rigor period.

Neither gender nor muscle significantly influenced cathepsin H activity, whereas both the genders ($p = 0.020$) and muscles ($p = 0.026$) differed for cathepsin BL activity and the genders ($p = 0.015$) differed for cathepsin B activity (Table 4.4).

No significant change in cathepsin B or H activity was found during the rigor period, although there appeared to be a trend for the activity to increase. There was an increase ($p = 0.009$) in cathepsin BL activity from two to 30 hours PM.

Table 4.4

The cathepsin B, BL, and H activity (LSMean \pm SEM) in springbok muscle during the rigor period, as effected by gender, muscle and time PM

Main effects		Cathepsin specific activity (Δ fluorescence/min/mg protein)		
		B	BL	H
Gender:	Male	2.33 ^b \pm 0.147	2.92 ^b \pm 0.212	0.93 \pm 0.031
	Female	3.71 ^a \pm 0.180	4.77 ^a \pm 0.244	0.98 \pm 0.035
Muscle:	LTL	3.10 \pm 0.201	4.15 ^a \pm 0.276	0.96 \pm 0.033
	BF	2.93 \pm 0.172	3.55 ^b \pm 0.238	0.94 \pm 0.034
Hours PM:	2	2.74 \pm 0.292	2.90 ^b \pm 0.285	0.94 \pm 0.046
	8	2.69 \pm 0.261	3.90 ^a \pm 0.460	0.92 \pm 0.050
	18	2.85 \pm 0.277	3.68 ^{ab} \pm 0.419	0.93 \pm 0.063
	24	3.58 \pm 0.303	4.55 ^a \pm 0.437	1.00 \pm 0.054
	30	3.23 \pm 0.320	4.22 ^a \pm 0.381	0.98 \pm 0.050

LSMean: least squares mean; SEM: standard error of the mean.

LTL: *Longissimus thoracis et lumborum*; BF: *Biceps femoris*; PM: *post-mortem*.

^{a, b}Least square means in the same column (within main effect) with different superscripts differ significantly from each other ($p \leq 0.05$).

The calpain I and II activity was influenced by gender ($p_{\text{calpain I}} = 0.0496$; $p_{\text{calpain II}} = 0.0213$), with higher levels being found in male springbok. The BF also had higher activity levels of these enzymes than the LTL, as well as having higher calpastatin activity ($p_{\text{calpain I}} = 0.0012$; $p_{\text{calpain II}} < 0.0001$; $p_{\text{calpastatin}} = 0.0231$) (Table 4.5).

Calpain I and II as well as calpastatin all declined during the rigor period ($p_{\text{calpain I}} < 0.001$; $p_{\text{calpain II}} = 0.023$; $p_{\text{calpastatin}} = 0.003$); however no change in the calpastatin to calpain ratio was found (Table 4.5 and Fig. 4.3).

While no significant interaction between gender and time PM was found, there did appear to be a trend for the calpastatin activity to decline more rapidly in male springbok muscle ($p = 0.092$). This was most likely a direct response to the more rapid decline in the pH of the male muscles, as indicated by the correlations depicted in Table 4.6.

Table 4.5

The calpain I and II and calpastatin specific activity as well as the calpastatin to calpain ratio (LSMeans \pm SEM) in springbok muscle during the rigor period, as effected by gender, muscle and time PM

Main effects		Calpain specific activity (U/mg protein)			
		Calpain II	Calpain I	calpastatin	Calpastatin/(I+II)
Gender:	Male	0.0174 ^a \pm 0.00038	0.0179 ^a \pm 0.00085	0.027 \pm 0.0016	0.760 \pm 0.0362
	Female	0.0150 ^b \pm 0.00033	0.0141 ^b \pm 0.00069	0.021 \pm 0.0009	0.735 \pm 0.0290
Muscle:	LTL	0.0149 ^b \pm 0.00032	0.0143 ^b \pm 0.00088	0.021 ^b \pm 0.0010	0.728 \pm 0.0350
	BF	0.0174 ^a \pm 0.00037	0.0177 ^a \pm 0.00068	0.027 ^a \pm 0.0015	0.767 \pm 0.0303
Hours PM:	2	0.0166 ^a \pm 0.00055	0.0185 ^a \pm 0.00079	0.026 ^a \pm 0.0018	0.738 \pm 0.0359
	18	0.0163 ^{ab} \pm 0.00047	0.0161 ^b \pm 0.00099	0.024 ^b \pm 0.0017	0.739 \pm 0.0390
	30	0.0156 ^b \pm 0.00046	0.0134 ^c \pm 0.00100	0.022 ^b \pm 0.0015	0.765 \pm 0.0459

LSMean: least squares mean; SEM: standard error of the mean.

LTL: *longissimus thoracis et lumborum*; BF: *biceps femoris*; PM: *post-mortem*.

^{a, b} Least square means in the same column (within main effect) with different superscripts differ significantly from each other ($p \leq 0.05$).

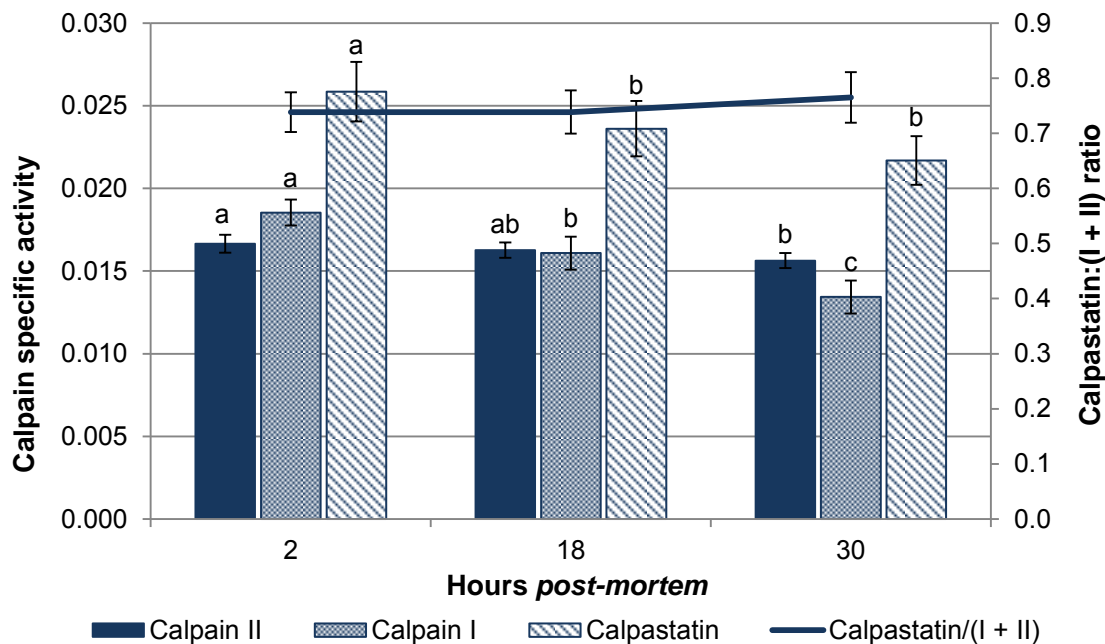


Figure 4.3 The change in calpain I, calpain II and calpastatin specific activity and the calpastatin:calpain I + II ratio over the first thirty hours PM.

Different letters indicate significant differences ($p \leq 0.05$) between the mean values for each time period per calpain. Error bars indicate the standard error of the mean for each group.

Significant negative correlations between the pH decay rate and the change in calpastatin activity from two to 18 hours and two to 30 hours were found, indicating that a more rapid change in the pH (higher decay constant) resulted in a greater decline in the

calpastatin activity (Table 4.6). A significant negative correlation between the pH decay rate and the change in the cathepsin H activity was also found (Table 4.6). However, considering the lack of any significant change in the cathepsin H activity during rigor this correlation is unlikely to be of much importance.

Table 4.6

A summary of the significant correlations between the pH decay rate and the change in enzyme activity during the rigor period

	r	p
Calpastatin (2 to 18 hours)	-0.634	< 0.01
Calpastatin (2 to 30 hours)	-0.640	< 0.01
Calpastatin:(m+μ) (2 to 30 hours)	-0.417	0.05
Cathepsin H (2 to 30 hours)	-0.577	< 0.01

r: Spearman's correlation coefficient

No significant correlations between the rate of change in the temperature and the change in enzyme activity were observed. This indicates that the rate of temperature decline had little direct effect on the change in enzyme activity. However, it is likely to have an indirect effect via its influence on the change in pH.

4.4 Discussion

4.4.1 The effect of gender and muscle

4.4.1.1 Temperature and pH

The significant effect of both gender and muscle on the rate of cooling is most likely a reflection of the differences in size between the muscles. Smaller muscles or carcasses have higher surface-area to volume ratios, which allows the more rapid dissipation of heat (Hoffman & Laubscher, 2009; Kim *et al.*, 2014). The higher warm carcass masses of the male springbok used in this study (male = 16.8 ± 0.95 kg; female = 13.1 ± 0.73 kg; $p = 0.018$) most likely resulted in the LTL muscles from female springbok being smaller and thus cooling faster. Similar results have been reported for heavy and light veal carcasses (Hamoen *et al.*, 2013) and larger impala carcasses have been reported to cool significantly slower than smaller ones (Hoffman & Laubscher, 2009).

On the other hand, the fairly similar temperature decay rates found for the female BF and the male muscles (Fig. 4.1) may be a reflection of the differential muscle development of male and female animals. Male animals are known to develop to a greater extent in the neck and thorax than female animals, which could have resulted in the difference in the mass of the LTL between the genders being greater than the difference between the mass of the BF

between the genders (Lawrie & Ledward, 2006). Slower cooling of the BF than the LTL muscle has been reported in previous studies (Marais, 2013).

The cooling of all the springbok carcasses was rapid relative to the values reported for beef (20 hours to reach 4°C) and similar to those of lamb (10 - 13 hours to reach 4°C) (O'Halloran *et al.*, 1997; McGeehin, Sheridan & Butler, 1999). This is most likely as a result of the small, relatively narrow structure of springbok carcasses. However, the general lack of subcutaneous fat on the springbok carcasses may also have played a role (Smith, Dutson, Hostetler & Carpenter, 1976). A 12th rib subcutaneous fat layer of at least 6.2 mm has been reported to be necessary for the prevention of cold-shortening in beef carcasses (Savell, Mueller & Baird, 2005).

High temperatures are known to stimulate glycolysis in PM muscle, thereby increasing the rate of lactic acid production and the decline in pH (Bekhit *et al.*, 2007; Hoffman & Laubscher, 2009). This relationship can be seen in the current results as the high temperature decay constant and low pH decay constant for the female LTL as well as in the difference between the two muscles in male springbok (Table 4.3). The only exception to this is the female BF, which has the lowest rate of temperature decline but an intermediate pH decline rate. It has however been reported that despite the large effect of temperature on the decline in pH the response can differ between muscles (Newbold & Harris, 1972).

The fibre-type composition determined for these samples (Chapter 3) indicated that the BF tended to contain greater proportions of type I fibres and fewer type IIA, IIX and IIX fibres. This may provide some explanation for the slower-than-expected decline in the pH of female BF muscles as muscles containing greater proportions of oxidative fibres have been reported to have slower rates of glycolysis and higher ultimate pH levels (Klont, Brocks, & Eikelenboom, 1998). This is supported by the correlations between the fibre-type composition and the pH decay rate found in this study, despite the lack of significance of these correlations (Pearson's $r_{\text{type I}} = -0.47$; $p = 0.13$). However, the difference in the fibre-type composition between the muscles was only significant in male springbok.

Apart from the relatively slow decline in the pH, the female BF muscle also had a higher ultimate pH (*c*-value) than the other muscles, although this difference was not significant ($p > 0.05$). A high ultimate pH can indicate a shortage of glycogen at death (Newbold & Harris, 1972; Kritzinger, Hoffman, & Ferreira, 2003), and similar differences in the pattern of pH decline for muscles with a high or low glycogen content have been reported for veal calves (Hamoen *et al.*, 2013). Why the BF muscle in female springbok should be more depleted of glycogen than the other muscles is uncertain.

Despite the relatively high ultimate pH of the female BF (5.77 ± 0.030), it was still below 5.8 (Table 4.2). This indicates that the meat is unlikely to exhibit DFD-like

characteristics, as DFD meat is defined as having an ultimate pH of above 6 (Warriss, 2000). It must however be noted that an ultimate pH of between 5.2 and 5.7 has been recommended by Meat Standards Australia, with meat with higher pH values being reported as dry and tough (Hamoen *et al.*, 2013). The values found for the other gender-muscle groups are within the range reported for impala (Hoffman & Laubscher, 2009) and springbok in other studies (Van Rensburg, 1997; Hoffman, Kroucamp & Manley, 2007).

A rapid rate of pH decline in conjunction with a relatively high muscle temperature is associated with the undesirable condition known as PSE (Savell *et al.*, 2005). This may seem to indicate that male springbok are at risk of developing this condition. However, despite the relatively fast rate of pH decline found in male springbok muscle the pH is still considerably above 6 at 45 minutes PM, indicating that PSE-type conditions are unlikely to occur (Warriss, 2000; Kim *et al.*, 2014).

In contrast, the muscles from female springbok were at considerable risk of cold-shortening. Cold-shortening is said to occur when a muscle reaches an internal temperature of 10°C to 15°C while the pH is still above 6.2 (Lawrie & Ledward, 2006). This is due to the cold triggering a release of calcium ions from the sarcoplasmic reticulum while considerable amounts of ATP are still present to enable muscle plasticity and power contraction (Newbold & Harris, 1972; Honikel, Roncales & Hamm, 1983; Hamoen *et al.*, 2013). Figure 4.1 indicates that in both the female BF and LTL these temperatures were attained at approximately four to five hours PM, at which point the pH was still at around 6.3 to 6.4 (Fig. 4.2). The occurrence of cold-shortening could result in female springbok having tougher meat than males, and may cause increased drip losses (Honikel *et al.*, 1983).

This is in contrast to the results of Marais (2013), who reported a considerably slower decline in temperature for springbok carcasses than found in this study, and concluded that cold-shortening was unlikely to occur. However, this may be as a result of the carcasses in that study only being exposed to refrigerator temperatures later PM (Marais, 2013).

Apart from the PSE and DFD specifications, a variety of recommendations with regard to the pH and/or temperature at certain times PM have been made. The temperature of the muscle at the time at which it reaches a pH of 6 has been found to have a considerable effect on meat quality, with higher temperatures thought to improve tenderness by stimulating proteolysis (O'Halloran *et al.*, 1997; Hopkins *et al.*, 2011). Thompson *et al.* (2005) found that lamb meat with the best sensory quality was yielded when the muscle was at 21°C at a pH of 6. On the other hand, O'Halloran *et al.* (1997) suggests that the pH should be at 6.1 by 3 hours PM in order to maximise tenderness.

Examination of the regression curves in Figure 4.1 and 4.2 indicate that the muscles from male animals were at approximately 11°C when they reached pH 6, while female LTL

and BF muscles were at around 4°C and 5°C respectively. This suggests that all the muscles cooled considerably more quickly than is optimal (Thompson *et al.*, 2005). In contrast, while the 3 hour pH of female springbok was considerably too high ($\text{pH}_3 \approx 6.4$), the pH decline recorded for male springbok was close to achieving the 3 hour pH of 6.1.

These comparisons seem to indicate that springbok carcasses cool more quickly than is desirable and that male springbok may have more tender meat. Delayed cooling, alternative hanging methods or electrical stimulation may therefore be necessary for female springbok in particular in order for optimum tenderness to be obtained (Newbold & Harris, 1972; Savell *et al.*, 2005; Toohey, Hopkins, McLeod, & Nielsen, 2006). However, considering the low shear force values reported in Chapter 5 and 6 the commercial necessity of this is uncertain. Previous research has also found that the electrical stimulation of springbok carcasses does not influence the decline in pH or the tenderness of springbok meat (Marais, 2013).

4.4.1.2 *Cathepsin activity*

Female springbok had higher cathepsin B ($p = 0.015$) and BL ($p = 0.020$) activity than males, and the LTL had higher cathepsin BL activity than the BF ($p = 0.026$) (Table 4.4). While the reason for this is uncertain, it may help compensate for the reduced tenderness caused by the high cooling rate and slow pH decline of the female BF if the meat is aged. Rapidly chilled muscles have been found to undergo considerable tenderization during ageing, although it does not fully compensate for the reduced sarcomere length due to cold shortening (Hwang & Thompson, 2001).

There appeared to be a trend for the cathepsin activity to increase during the rigor period; however this was only significant for cathepsin BL ($p = 0.009$). This is in contrast with the findings of Dransfield, Etherington, & Taylor (1992), who reported no change in cathepsin B and B+L activity from 45 min to 24 hours PM in beef. However, Thomas, Gondoza, Hoffman, Oosthuizen, & Naudé (2004) found significant changes in the cathepsin B, L and H activity of ostrich muscle, with a general increasing trend being observed from 2.5 to 16 hours. Cathepsin activity has also been observed to increase during later periods PM (Chapter 5), with this being attributed to the release of the cathepsins from the lysosomes as the structural integrity of the muscle weakens PM (Thomas *et al.*, 2004).

Despite the increase in cathepsin activity observed during the rigor period it is uncertain to what extent this activity could take place in the muscle itself. Cathepsins are acidic proteases with pH optima of below 6 (Hopkins & Huff-Lonergan, 2004), which indicates that they would have been inhibited until five to 10 hours PM, at which point the low temperatures would limit their activity. Unfortunately, very little research on cathepsin activity

during rigor has been done, and the comparison of results from different papers is often not ideal as a variety of different extraction and assay methods are used (Dransfield *et al.*, 1992; O'Halloran *et al.*, 1997).

4.4.1.3 Calpain activity

The higher levels of calpain and calpastatin activity found in the BF muscle and male springbok may be linked to the greater proportion of type I fibres in these muscles (Chapter 3) as primarily oxidative muscles have been found to have higher calpain and calpastatin activity levels than glycolytic muscles (Ouali & Talmant, 1990; Klont *et al.*, 1998). The lack of a significant difference in the calpastatin to calpain ratio is not entirely as expected however as glycolytic muscles are reported to have a higher level of calpain activity relative to the inhibitory effects of calpastatin (Lawrie & Ledward, 2006).

A decline in calpain and calpastatin activity was observed during the rigor period. This is in agreement with literature as similar trends were found by O'Halloran *et al.* (1997) and Dransfield *et al.* (1992). This decrease in activity is attributed to the increase in the calcium ion concentration that takes place PM as a result of the inability of the sarcoplasmic reticulum to pump calcium out of the sarcoplasm in the absence of ATP (Calkins & Seideman, 1988; Lawrie & Ledward, 2006). The increased calcium ion concentration both activates the calpains and results in the enzymes' autolysis, causing a decrease in the measurable activity (Huff Lonergan *et al.*, 2010). The activated calpain enzymes also proteolyse calpastatin, leading to the observed decline in the inhibitor's activity (Huff Lonergan *et al.*, 2010).

The greater decline in calpain II activity during the second half of the rigor period is in agreement with the higher calcium ion concentrations required for the activation of this enzyme, as this would result in its later activation and thus later autolysis (Dransfield, 1994; Huff Lonergan *et al.*, 2010). Calpain I was reported to be activated at approximately 6 hours PM and calpain II at closer to 16 hours PM in beef (Dransfield, 1994).

While no significant interaction between gender and time PM was found, there did appear to be a trend for the calpastatin activity to decline more rapidly in male springbok muscle ($p = 0.092$). This was most likely a direct response to the more rapid decline in the pH of the male muscles, as is indicated by the correlations given in Table 4.6.

Significant negative correlations between the change in calpastatin activity from both two to 18 and two to 30 hours and the pH decay rate were found, indicating that a more rapid change in the pH (higher decay constant) resulted in a greater decrease in the calpastatin activity. This effect of rapid glycolysis on calpastatin activity is in agreement with the findings of O'Halloran *et al.* (1997) and Hwang & Thompson (2001).

The effect of pH on calpastatin activity is often attributed to a greater activation of the calpain enzymes when rigor is entered at a higher temperature (Bekhit *et al.*, 2007). However, in this study the correlations between the rate of pH decline and the change in calpain activity were negligible. This suggests that other enzymes may have been responsible for the breakdown of calpastatin activity. The caspases have been reported to be capable of this (Kemp, Sensky, Bardsley, Buttery & Parr, 2010).

The lack of a correlation between the pH decay rate and the change in calpain activity found in this study may be as a result of the rapid chilling that took place. According to Hwang & Thompson (2001), the calpain activity in muscles that are chilled rapidly (15°C at pH 6) is less sensitive to changes in the pH.

4.4.2 The effect of *ante-mortem* stress

While efforts were made to avoid *ante-mortem* stress in the springbok used for this study, three of the twelve springbok harvested were considered to have experienced considerable stress prior to death. In order to determine whether the presence of stressed springbok in the data set had an effect on the results the significance of stress was initially tested as an additional main effect. While no significant effects were observed some interesting trends were found.

Stressed springbok had a higher muscle temperature than unstressed springbok for up to eight hours PM. They were also found to have more rapid initial declines in the pH, with values of below six being recorded by eight rather than 12 hours PM. This is in agreement with the results of Kritzinger *et al.* (2003), who suggested that the higher stress levels experienced by impala cropped during the day contributed to the more rapid decline in pH that was observed.

Stressed springbok also appeared to have more rapid declines in calpain I and II activity without there being a corresponding decline in calpastatin activity, possibly as a result of the higher muscle temperature in stressed springbok (Dransfield *et al.*, 1992). This resulted in a considerable difference ($p = 0.059$) in the change in the calpastatin to calpain ratio over the rigor period between stressed and unstressed springbok, with the ratio increasing from 0.67 to 0.83 in stressed springbok while in unstressed springbok the ratio did not change.

This suggests that *ante-mortem* stress may result in reduced tenderization in springbok as a result of the more rapid autolysis of the calpain enzymes. This is in agreement with the results of Warner, Greenwood, Pethick & Ferguson (2010), who found that the meat from cattle which experienced acute *ante-mortem* stress was rated as being

tougher than that from unstressed animals. In addition, stressed animals had meat with higher drip and cooking losses (Warner *et al.*, 2010).

4.4 Conclusion

The patterns of decline of the pH and temperature found for springbok indicate that the carcasses cool very rapidly PM, with the LTL muscles in smaller carcasses such as those of females having the highest chilling rate. This makes the meat susceptible to cold shortening and prevents it from attaining the pH and temperature conditions recommended for optimum tenderness.

The changes found in the proteolytic enzyme activity during the rigor period agreed with the current understanding of their mechanisms of action and with the results of existing literature. Although cathepsin activity was present and tended to increase from two to 30 hours PM it is uncertain whether the enzymes would have been active *in situ*. The declines in calpain I and II and calpastatin activity found indicate that the enzymes were activated relatively soon PM despite the sub-optimal conditions.

The use of techniques such as delayed chilling, alternative hanging methods or electrical stimulation may improve tenderness; however, considering the low shear force values reported for springbok in previous studies, the added cost of utilising these techniques may not be justified.

Although only a limited number of animals could be compared the results from this study also suggest that *ante-mortem* stress could have a considerable effect on springbok meat quality and the effectiveness of ageing. Further studies examining this factor specifically are recommended.

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CHAPTER 5

The physical and biochemical changes in springbok (*Antidorcas marsupialis*) *Longissimus thoracis et lumborum* and *Biceps femoris* muscle during conditioning

Abstract

This study aimed to determine the optimum ageing period for vacuum-packed springbok (*Antidorcas marsupialis*) *Longissimus thoracis et lumborum* (LTL) and *Biceps femoris* (BF) muscle. The muscles from 13 mature springbok (seven males, six females) were divided into six portions, with each portion being randomly assigned to age 1, 2, 5, 8, 14 or 21 days. The pH, water-holding capacity (WHC), cumulative purge loss, cooking loss, Warner-Bratzler shear force (WBSF), calpain I, II and calpastatin activity and cathepsin B, BL and H activity were determined for each ageing period. The total and soluble collagen content was determined per muscle. The WBSF decreased with ageing; however only the male LTL showed any further changes after five days *post-mortem*. Cumulative purge losses increased steadily until 14 days of ageing, where after little increase was found up to 21 days. Cooking loss increased from day eight onwards. The pH and WHC were significantly influenced by time *post-mortem* but no clear trend was apparent. Cathepsin B, BL and H activity increased during ageing while the calpain I, II and calpastatin activity declined up to five days *post-mortem*. Male springbok had a higher pH and higher cathepsin H activity but otherwise gender did not have an effect. The BF had a higher pH, lower purge loss, higher cooking loss, higher WBSF and higher calpain and calpastatin activity than the LTL. In female springbok the LTL had a higher WHC than the BF. No differences in either the total or soluble collagen between genders or muscles were found. It appears that there is no benefit of ageing springbok meat from these two muscles for more than five days, and that longer ageing results in increased moisture loss. The calpain enzymes appear to be primarily responsible for the tenderizing process.

Keywords: meat, ageing, calpains, cathepsins, tenderization

5.1 Introduction

The current global population is estimated at over 7.1 billion (United States Census Bureau, 2014, <http://www.census.gov/popclock/>), with this expected to increase to around 9 billion by 2050 (Godfray *et al.*, 2010). Despite commendable increases in food production, over 12% of the population is still considered to be undernourished (Godfray *et al.*, 2010; Food and Agricultural Organization of the United Nations, 2013). It is thus clear that international food production will need to be increased exponentially in order to keep up with demand (Wheeler & Von Braun, 2013). However, recent studies indicate that climate change will cause large-scale alterations in the weather conditions in many areas of the world (McMichael, Woodruff & Hales, 2006). These changes will range from increased droughts in the southern hemisphere to widespread flooding in other regions of the world, with an overall escalation in the occurrence of extreme weather conditions (McMichael *et al.*, 2006). In order for the agricultural industry to continue increasing food production despite these challenges innovative thinking in terms of not only precision farming, technological advances and intensification but also the optimisation of farming systems for individual locations and conditions will be required (Hoffman & Cawthorn, 2012).

The use of novel species that are better adapted to local conditions is one of the many possible methods of increasing protein production (Hoffman & Cawthorn, 2012). The development of region-specific industries for the production of unique food products in areas with otherwise poor economies also has the potential to increase employment and general well-being in rural areas. While not nearly at the level of the venison industries in the United States and New Zealand the game ranching industry in South Africa is one of the most promising examples of indigenous species usage at present (Hoffman & Cawthorn, 2012).

The Southern African game ranching industry contributed R7.7 billion to the South African economy in 2008, making it the sixth largest industry in the agricultural sector (Thomas, 2012). It is also one of the fastest growing industries in the sector, with an annual growth in revenue of 20.3% being recorded for the past 15 years (Thomas, 2012). At present a very small proportion of this income can be attributed to meat sales (3.7% of the total income in 2000) and the contribution of game meat to red meat production is very limited; with trophy and leisure hunting, ecotourism and live sales being more important earners (Hoffman, 2007; Van Zyl & Ferreira, 2004).

However, as the number of game farms and thus the supply of various game species has increased in recent years, the prices obtained at live game auctions for species such as springbok have declined (Hoffman, Muller, Schutte, & Crafford, 2004). The hunting and ecotourism industries are also both highly dependent upon global economics and can thus be unpredictable, with a decline in foreign leisure hunters being found from 2008 to 2012 as

a result of the economic recession (Thomas, 2012). The sale of game meat has the potential to provide farmers with a more reliable income. In addition, South Africa is currently far from self-sufficient in terms of meat production, with 10.99% of mutton and 5.59% of the beef consumed per annum being imported in 2011/2012 (DAFF Directorate Statistics and Economic Analysis, 2013). The use of 24.4% of the available grazing land for a game ranching industry that does not help reduce this supply-demand deficit for red meat therefore seems irresponsible (Thomas, 2012).

In order for game meat to become a standard product on supermarket shelves both supply and demand need to be improved (Hoffman *et al.*, 2004; Hoffman, Muller, Schutte, Calitz, & Crafford, 2005). The nature of game meat as low in fat, high in protein and high in iron, as well as both free-range and organic, should be used to promote game meat consumption (Schönfeldt, 1993; Field, 2004; Hoffman *et al.*, 2005; Hoffman & Wiklund, 2006). The misconceptions about game meat also need to be overcome, with the education of consumers on how to correctly prepare it likely playing a role (Hoffman *et al.*, 2005). This alone will not be enough to build a robust and sustained industry however. In order to ensure a continued demand for game meat, producers need to be able to ensure the supply of a product of consistent and high quality (Hutchison, Mulley, Wiklund, & Flesch, 2010). This indicates the necessity of extensive research into game meat from all relevant species so that correct handling protocols can be developed for each species, rather than just extrapolating pre-existing standards from the beef and sheep industries.

Tenderness has been found to be one of the most important factors determining consumer approval of meat (Bickerstaffe, Bekhit, Robertson, Roberts, & Geesink, 2001; Hutchison *et al.*, 2010). Considering that one of the most prevalent misconceptions about game meat is its tenderness, or lack thereof (Hoffman *et al.*, 2004; Du Buisson, 2006), it is clear that this is a quality of game meat that needs to be studied.

This study was carried out to determine the optimum ageing period for vacuum-packed springbok meat from two muscles forming part of high-value cuts in the carcass, the *Longissimus thoracis et lumborum* and the *Biceps femoris* muscle. Although recommendations for ageing periods are found in literature, these are scarce and not always backed by good science (Field, 2004; van Rensburg & Zondagh, 1993). Much of the information is also for carcasses aged whole, whereas the ageing of vacuum-packed individual muscles or cuts is becoming increasingly popular as a way to save on the costs of ageing and decrease the risk of microbial growth (Hodges, Cahill, & Ockerman, 1974; Buys, Nortjé, & van Rensburg, 1997). The biochemical basis of the observed physical changes was also considered in this study in order to allow the full comparison of springbok meat with other farmed species such as beef.

Springbok was chosen for this study as they contribute the largest amount to game meat production, export (80% of all game carcasses exported in 2005) and local consumption (Hoffman *et al.*, 2005; Hoffman & Wiklund, 2006). Van Rensburg (1997) also found that South African game farmers preferred springbok to other game species, and that they are harvested in greater numbers than any other game species in South Africa.

5.2 Materials and methods

5.2.1 Harvesting and slaughtering

Seven mature male and six mature female springbok (*Antidorcas marsupialis*) were harvested in August 2013 at Brakkekui farm near Witsand in the Western Cape Province of South Africa (34°18'24.0" S; 20°49'3.9" E; altitude of 93 m). Harvesting was done at night according to standard operating procedure (SOP number SU-A CUM13-00034), with a spotlight being used to locate and temporarily immobilise the springbok prior to killing with a shot to the head with a 30-06 or .270 calibre rifle. Exsanguination was done in the field immediately after harvesting each animal. All springbok were killed with a single head-shot and none were observed to be stressed during the *ante-mortem* period. Ethical clearance for this study was issued by the Stellenbosch University Animal Care and Use Committee (Ethical clearance number SU-ACUM13-0034).

Once the required number of springbok had been harvested the exsanguinated carcasses were transported to a nearby slaughtering facility. The bled mass of each carcass was recorded prior to skinning and gutting, where after the warm carcass mass was recorded and the carcasses were placed in a cool room (0 - 5°C) to undergo rigor. All carcasses were suspended by both Achilles tendons in order to ensure even shortening of muscles in both sides of the carcass.

5.2.2 Sampling

Carcasses were transported intact to the cool room of the meat processing facility at the Department of Animal Sciences, Stellenbosch University, for further sampling. The cold carcass mass was recorded prior to the commencement of sampling at approximately 36 hours *post-mortem* (PM). The *Longissimus thoracis et lumborum* (LTL) and the *Biceps femoris* (BF) muscles were excised from both sides of the carcass, with the entire BF being removed and the LTL being removed from between the last lumbar vertebra caudally and the natural termination of the muscle at the cervical vertebra cranially. Any epimysium remaining on the muscle after excision was removed. Each LTL and BF was subsequently cut perpendicularly to the longitudinal axis of the muscle to give three approximately equal portions, resulting in six portions per muscle per carcass. Each portion was randomly

assigned to one of six ageing periods (Table 5.1). All portions were vacuum-packed and aged for the specified period at 0 - 5°C. A sample for the determination of the collagen content was removed from the cranial-caudal centre of each muscle prior to packaging. This sample was vacuum-packed separately, snap-frozen in liquid nitrogen and stored at -80°C for later analysis.

At the end of each ageing period the portions were removed from the vacuum packaging and samples were taken for the determination of water-holding capacity (WHC), cooking loss and Warner Bratzler shear force. The remainder of the portion was divided into separate samples that were vacuum-packed, snap-frozen in liquid nitrogen and stored at -80°C until analysis for cathepsin and calpain activity.

Portions assigned to the one day ageing period were processed identically to the rest of the portions despite same-day sampling.

Table 5.1

Summary of the experimental layout of the trial per main effect (gender, muscle and ageing period)

Gender	Number of animals	Muscle	Ageing periods (days <i>post-mortem</i>)					
Male	7	LTL	1	2	5	8	14	21
		BF	1	2	5	8	14	21
Female	6	LTL	1	2	5	8	14	21
		BF	1	2	5	8	14	21

LTL: *Longissimus thoracis et lumborum*; BF: *Biceps femoris*.

5.2.3 Physical analysis

5.2.3.1 pH

The ultimate pH (pH_u) of both the LTL and the BF was determined prior to muscle excision at approximately 35 hours PM in order to ensure the full resolution of rigor prior to measurement. The pH of the LTL was measured approximately 2 cm from the spinous processes between the first and second lumbar vertebra using a calibrated (pH standard buffers at pH 4.0 and pH 7.0) Crison 506 portable pH meter (Lasec SA, Cape Town, South Africa). The pH of the BF was measured at approximately the centre of the muscle on both the dorsal-ventral and cranial-caudal planes.

The pH of each portion at the completion of each specific ageing period was also measured in order to determine the change in the pH during ageing.

5.2.3.2 Cumulative purge loss

Portions were weighed prior to vacuum-packaging to determine their initial mass. They were subsequently weighed at the completion of ageing after being blotted dry with absorbent paper-towels to determine the moisture loss during ageing. This moisture loss was expressed as a percentage of the initial mass of each portion.

5.2.3.3 Cooking loss

At the end of each ageing period portions were cut perpendicularly to the direction of the muscle fibres to produce two steaks of approximately 2 cm thick. These steaks were weighed individually and placed in plastic bags and suspended in a water-bath (model 102 digital electrical bridge thermostat; model 132A 40 l water-bath; Scientific, Roodepoort, South Africa) set to 80°C. The steaks were cooked at this temperature for 30 minutes in order to ensure complete cooking. Steaks were then removed and placed in the refrigerator at 0 - 5°C to cool overnight. Once completely cooled they were removed from the bags, blotted dry with absorbent paper-towels and weighed. The cooking loss was determined as the difference between the raw and cooked mass of each steak and expressed as a percentage of the raw mass (Honikel, 1998).

5.2.3.4 Warner Bratzler shear force

The toughness of the meat was determined using a model 4444 Instron Universal Testing Machine (Apollo Scientific cc, Alberta, Canada) fitted with a Warner Bratzler blade. The Instron had a load cell of 2 kN and crosshead speed of 200 mm/min. The Warner Bratzler fitting was 1.2 mm thick and had a triangular opening with a base length of 13 mm and a perpendicular height of 15 mm.

A core-borer was used to cut cylindrical cores with a diameter of 1.27 cm from the cooking loss steaks. The longitudinal axis of each core was approximately parallel to the direction of the muscle fibres. A minimum of six cores was cut per sample, depending on the size of the steaks.

The force in Newton required to shear each core perpendicular to the long axis of the core and thus the direction of the muscle fibres was determined. The average of the values for all cores was used as the Warner Bratzler shear force of the sample (Honikel, 1998).

5.2.3.5 Water holding capacity (WHC)

A small sample was removed from approximately the centre of each aged portion. After being diced with a scalpel 0.500 g was weighed out onto a filter paper disc (Munktell paper filter 292 90 mm, Lasec SA, Cape Town, South Africa). This disc was placed between

two clear Perspex plates and compressed at 588 N for 60 seconds in a standardised clamp. The two plates with the filter paper in between were then removed from the clamp and photographed using a Canon Powershot SX240 HS fitted to a tripod. The areas of both the inner meat circle and the outer expressed-fluid circle were determined using Image J (version 1.47, <http://rsb.info.nih.gov/ij>) and the WHC was calculated as the ratio of the inner circle area to the outer circle area. The WHC for all samples was done in duplicate (Grau & Hamm, 1953).

5.2.4 Chemical analysis

5.2.4.1 Collagen determination

For the determination of the total collagen content 1 g samples taken from the centre of each muscle were homogenized (Kinematica Polytron PT 2500 E; Lasec SA, Cape Town, South Africa) in 10 ml of distilled water, after which 10 ml of 37% hydrochloric acid was added and the homogenate was mixed thoroughly. This was then incubated at 120°C for three hours to ensure complete protein hydrolysis. After hydrolysis the sample was filtered to remove particulates using Whatman number 1 filter paper. Ten microlitres of the filtrate from each sample was subsequently transferred to a clear 96 well microplate (Greiner Cellstar 96 well flatbottom plate, Sigma-Aldrich, St Louis, USA). The content of the wells was evaporated overnight at 60°C.

The soluble collagen content was determined using an adapted version of the method described by Christensen *et al.* (2011). A second 1 g sample was taken from the same initial sample as for the total collagen content. This was placed in 10 ml 0.3% sodium chloride solution and incubated at 90°C for two hours. The sample was subsequently homogenized for approximately 30 seconds at 9000 - 10000 rpm, after which it was centrifuged (Sigma 2-16 K, Wirsam scientific, Cape Town, SA) for 12 min at 4500 g at room temperature. A 500 µl sample of the supernatant was withdrawn and placed in a cryovial (Lasec SA, Cape Town, South Africa) to which 500 µl of 37% HCl was subsequently added. This was thoroughly mixed prior to incubation at 120°C for three hours. Forty microlitres of each hydrolysed sample was subsequently transferred to a clear 96 well microplate (Greiner Cellstar 96 well flatbottom plate, Sigma-Aldrich, St Louis USA) and the content of the wells was evaporated overnight at 60°C.

Once the content of the wells had been evaporated the hydroxyproline content of each well was determined using the Hydroxyproline Assay Kit (catalogue nr MAK008, Sigma-Aldrich, St Louis USA). This involved a reaction with first a chloramine T/oxidation buffer mixture for 5 min at room temperature followed by a diluted DMAB reagent for 90 minutes at 60°C. The absorbance at 560 nm was then determined using a microplate reader

(Spectrostar Nano, BMG Labtech, Ortenberg, Germany). This absorbance value was converted to a hydroxyproline content using a standard curve and from there to collagen concentration by multiplying by a factor of eight (based on the hydroxyproline content of collagen) (Kolar, 1990).

5.2.4.2 *Cathepsin activity*

The activity of cathepsin B, B and L (BL) and H were determined according to the methods of Thomas, Gondoza, Hoffman, Oosthuizen and Naudé (2004) and Van Jaarsveld, Oelofsen and Naude (1998), with minor adjustments. The full description of the enzyme extraction and fluorescent assay procedure is given in the materials and methods of Chapter 4. Cathepsin activity is defined as the change in fluorescence measured (excitation 360 nm, emission 460 nm) per minute per mg of extractable protein (Δ fluorescence/min/mg).

With regards to the determination of cathepsin activity it must be noted that while enzyme-specific substrates were used for the assay, literature has mentioned that this specificity may not be complete (Van Jaarsveld, Naude & Oelofsen, 1998). It is therefore possible that other enzymes present in the crude extract may have contributed to the measured activity (Calkins & Seideman, 1988). The crude extract would also contain cathepsin inhibitors (cystatins), the concentration of which was not determined due to the complexity of this procedure (Van Jaarsveld *et al.*, 1998; Sentandreu, Coulis, & Ouali, 2002). The measured activity therefor also takes into account the possible inhibitory effect of the cystatins. The affinity of the enzymes for each substrate has also been reported to vary (Van Jaarsveld *et al.*, 1998); and for this reason the joint rather than individual activity for cathepsins B and L is reported. Despite these limitations it is felt that the results obtained do supply an indication of the proteolytic potential of cathepsins in springbok meat and allow the comparison of treatments.

5.2.4.3 *Calpain activity*

The calpain activity was determined according to the methods of Dransfield (1996) and Geesink and Koohmaraie (1999), with adaptations. The methodology is described in full in the materials and methods of Chapter 4. One unit of calpain activity (U) is defined as a 1.0 increase in the absorbance at 366 nm per hour at 25°C. One unit of calpastatin activity is defined as the amount that inhibited one unit of calpain II activity. Specific activity levels are expressed per mg of extractable protein (U/mg protein).

5.2.5 Statistical analysis

The trial was designed as a three-factor factorial experiment in a completely randomised design. The main effects tested were gender, muscle and ageing period (days PM) and their interactions, with the following model being used:

$$y_{ijk} = \mu + g_i + m_j + a_k + (gm)_{ij} + (ga)_{ik} + (ma)_{jk} + (gma)_{ijk} + \varepsilon_{ijk}$$

The terms within the model are defined as (μ) the overall mean, (g) the effect of gender, (m) the effect of muscle and (a) the effect of the ageing period, with (gm), (ga) and (ma), the respective second order interactions, as well as (gma) the third order interaction, and the overall error (ε) associated with the main effects and the various interactions.

Statistical analysis was performed using Statistica version 12 (StatSoft Inc, 2013) software. Prior to determining whether differences existed between treatments the data was tested for normality using normal probability plots and the homogeneity of variances was tested using Levene's test. Outliers that resulted in the data not being normally distributed were removed. This was only necessary for the cooking loss data. The mixed model repeated measures of analyses of variance (ANOVA's) were then performed using the VEPAC function in Statistica to determine whether differences existed between the treatments. Fisher's LSD test was used to determine which individual treatments differed from one another. Pearson's correlation coefficient (r) was calculated to determine the significance of correlations between specific variables when appropriate.

Main effects, interactions and correlations with $p \leq 0.05$ were considered to be significant. Values are reported as the LSMean \pm the standard error of the mean (SEM).

5.3 Results

5.3.1 Physical analysis

No significant 2nd or 3rd order interactions were observed for the pH. While it did appear that some 2nd order interaction between muscle and days PM may be present ($p = 0.077$), both muscles showed a similar trend during ageing and the main effects were thus considered.

The change in the pH during ageing was significant ($p < 0.001$) but inconsistent (Fig. 5.1). The pH of the genders differed ($p = 0.006$), with males having a higher mean pH than females (Table 5.2). There was also a difference between the muscles ($p = 0.013$), with a higher pH being found in the BF.

Table 5.2

Springbok muscle pH, cumulative purge loss, cooking loss, Warner Bratzler shear force and water-holding capacity as per gender, muscle and ageing period (LSMean \pm SEM)

Main effects		pH	Cumulative purge loss (%)	Cooking loss (%)	Water-holding capacity (inner:outer area)	Warner Bratzler shear force (N)
Gender:	Male	5.69 ^a \pm 0.009	3.31 ^a \pm 0.212	28.79 ^a \pm 0.313		30.34 ^a \pm 1.125
	Female	5.60 ^b \pm 0.008	3.10 ^a \pm 0.237	28.24 ^a \pm 0.386		30.11 ^a \pm 1.195
Muscle:	LTL	5.63 ^b \pm 0.011	3.87 ^a \pm 0.237	26.14 ^b \pm 0.160		27.21 ^b \pm 1.003
	BF	5.66 ^a \pm 0.009	2.54 ^b \pm 0.180	30.89 ^a \pm 0.248		33.23 ^a \pm 1.201
Ageing period (days PM):	1	5.66 ^a \pm 0.015	0.69 ^e \pm 0.079	28.12 ^c \pm 0.640	0.760 ^b \pm 0.0264	40.75 ^a \pm 2.041
	2	5.59 ^b \pm 0.019	1.61 ^d \pm 0.158	28.31 ^{cb} \pm 0.660	0.637 ^d \pm 0.0157	37.28 ^a \pm 2.187
	5	5.66 ^a \pm 0.019	3.00 ^c \pm 0.233	28.04 ^c \pm 0.548	0.696 ^c \pm 0.0169	26.96 ^b \pm 1.473
	8	5.67 ^a \pm 0.016	3.93 ^b \pm 0.274	27.96 ^c \pm 0.556	0.824 ^a \pm 0.0201	25.67 ^b \pm 1.069
	14	5.68 ^a \pm 0.015	4.88 ^a \pm 0.258	29.01 ^{ab} \pm 0.558	0.746 ^b \pm 0.0240	26.63 ^b \pm 1.156
	21	5.62 ^b \pm 0.015	5.11 ^a \pm 0.265	29.63 ^a \pm 0.610	0.652 ^{cd} \pm 0.0191	24.04 ^b \pm 1.209

LSMean: least squares mean; SEM: standard error of the mean.

LTL: *Longissimus thoracis et lumborum*; BF: *Biceps femoris*; PM: *post-mortem*.

^{a, b, c, d, e} Least square means in the same column (within main effect) with different superscripts differ significantly from each other ($p \leq 0.05$).

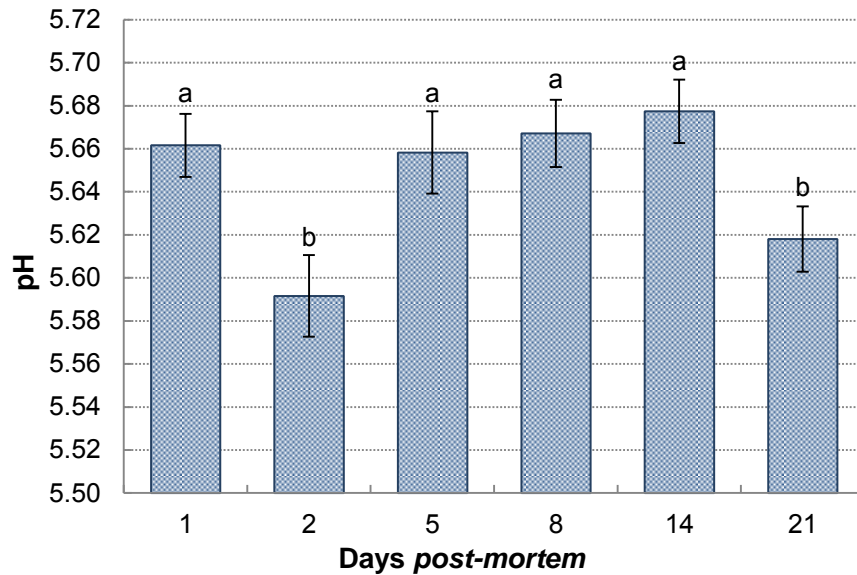


Figure 5.1 The change in pH of springbok muscle during ageing up to 21 days *post-mortem*. Different letters indicate significant differences ($p \leq 0.05$) between the mean values for each ageing period. Error bars indicate standard error of the mean of each group.

Interactions were found in the purge loss for both muscle by days PM ($p = 0.019$) and muscle by gender ($p = 0.041$) (Fig. 5.2 and 5.3 respectively). Despite the interaction, the main effect data was still representative of the effects of gender, muscle and ageing individually. No difference was observed between genders ($p = 0.417$); however the BF had lower ($p < 0.001$) levels of moisture loss than the LTL. Purge loss increased in a linear manner from day one to day 14 ($p < 0.001$), at which point it plateaued, with no significant difference being present between day 14 and day 21.

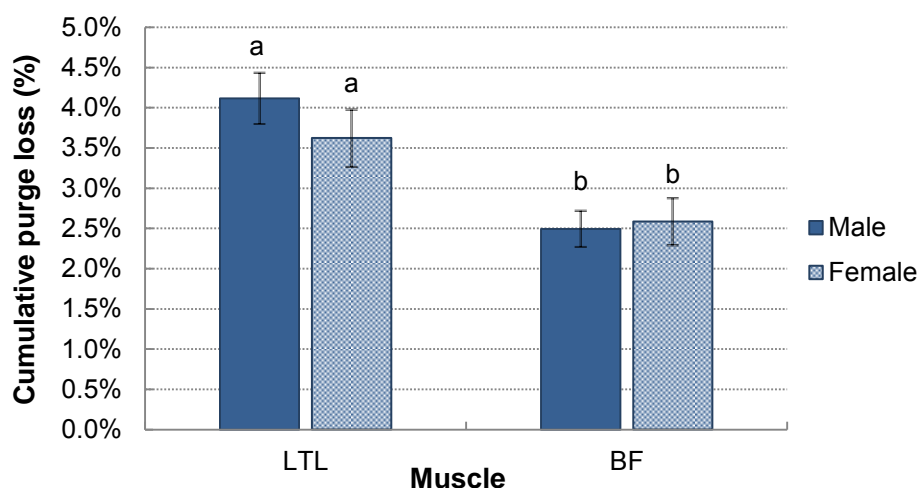


Figure 5.2 The average purge loss during ageing of the *Longissimus thoracis et lumborum* (LTL) and *Biceps femoris* (BF) muscles from male and female springbok. Different letters indicate significant differences ($p \leq 0.05$) between the mean values for each group. Error bars indicate the standard error of the mean of each group.

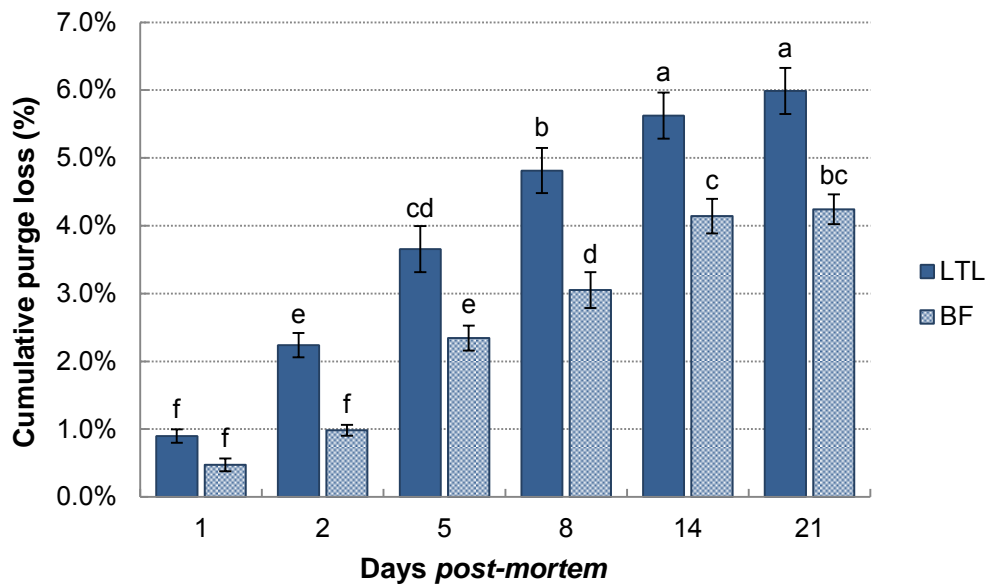


Figure 5.3 The purge loss of springbok *Longissimus thoracis et lumborum* (LTL) and *Biceps femoris* (BF) muscles during ageing up to 21 days *post-mortem*. Different letters indicate significant ($p \leq 0.05$) differences between the mean values of each interaction group. Error bars indicate the standard error of the mean of each group.

No significant interactions were observed for cooking loss and no difference was found between the genders ($p = 0.315$). The BF had higher ($p < 0.001$) levels of cooking loss than the LTL, and ageing period also had an effect ($p < 0.001$). This was only apparent from day eight onwards however, with the first four ageing periods not showing any differences in cooking loss (Fig. 5.4).

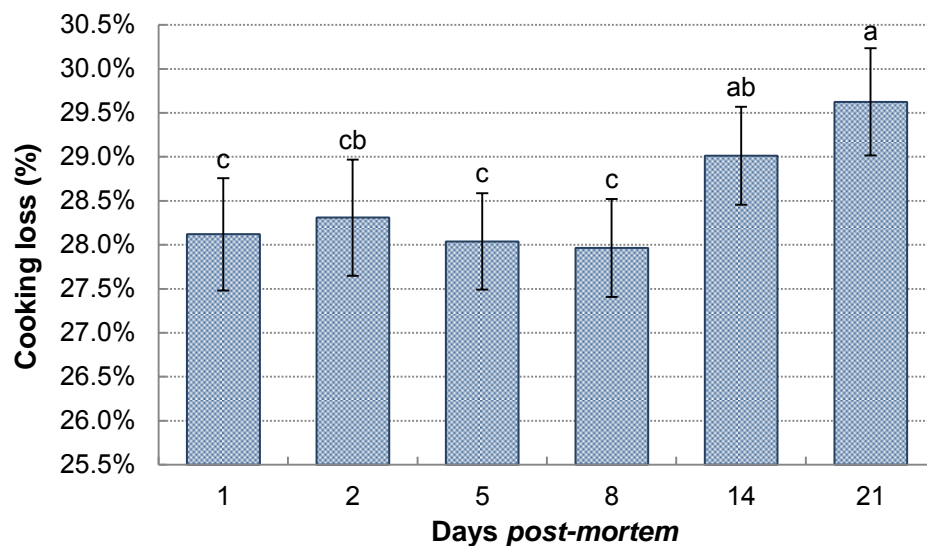


Figure 5.4 The change in the cooking loss of springbok muscle during ageing for up to 21 days *post-mortem*. Different letters indicate significant differences ($p \leq 0.05$) between the mean values for each ageing period. Error bars indicate the standard error of the mean for each group.

Water-holding capacity (WHC) showed 2nd order interactions for gender by days PM ($p = 0.031$) and gender by muscle ($p = 0.001$). However the gender by days PM interaction is not considered here as the general trend in the change in WHC is the same for the two genders. In contrast the gender by muscle interaction did influence the main effects data as it involved a change of rank between the genders for each muscle. This resulted in both the gender and muscle main effects being insignificant. As can be seen in Figure 5.5, in males the difference between the muscles is insignificant, with the BF tending to have a higher WHC than the LTL. In females this trend is reversed, with the LTL having a significantly ($p \leq 0.05$) higher WHC than the BF.

With regards to the effect of ageing on the WHC, a change was apparent ($p < 0.001$); however no single trend could be identified (Fig. 5.6).

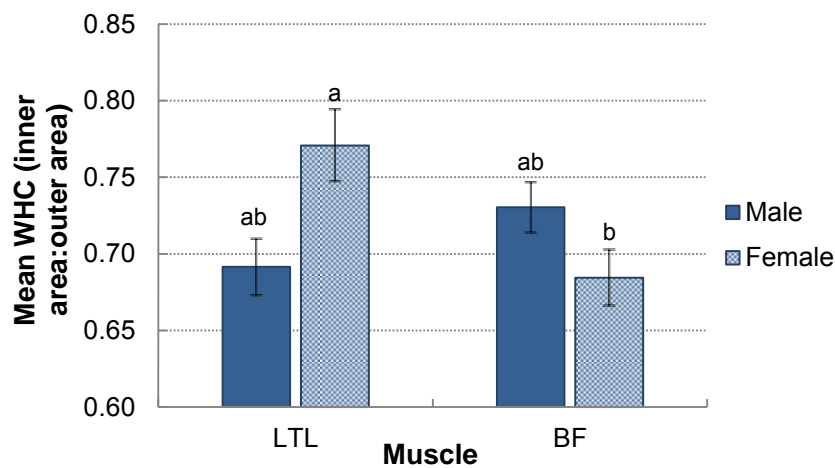


Figure 5.5 The mean water-holding capacity of the *Longissimus thoracis et lumborum* (LTL) and *Biceps femoris* (BF) muscles from male and female springbok. Different letters indicate significant differences ($p \leq 0.05$) between the mean values of each gender-muscle interaction group. Error bars indicate the standard error of the mean of each group.

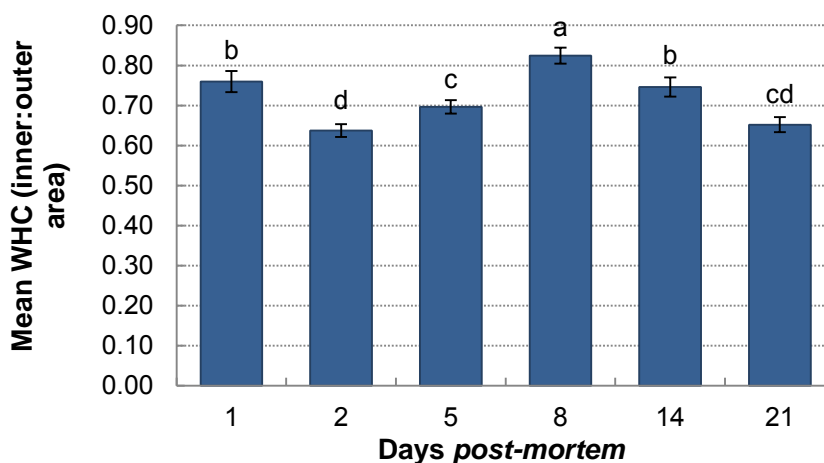


Figure 5.6 The change in the water-holding capacity of springbok muscle during ageing up to 21 days post-mortem.

Different letters indicate significant differences ($p \leq 0.05$) between the mean values for each ageing period. Error bars indicate the standard error of the mean of each group.

The Warner Bratzler shear force (WBSF) 3rd order interaction of gender by muscle by days PM was significant at the 5% level. While all the interaction groups showed a decline in shear force with ageing (Fig. 5.7 and 5.8), it can be seen that the trend was far more consistent in the LTL than the BF. The WBSF of both muscles also seemed to plateau earlier in the female animals than in the male animals, with no significant increase in tenderness being apparent from five days of ageing onwards. While the shear force of the BF showed a similar plateau in both genders, in the male LTL it appeared to still be declining at 21 days PM.

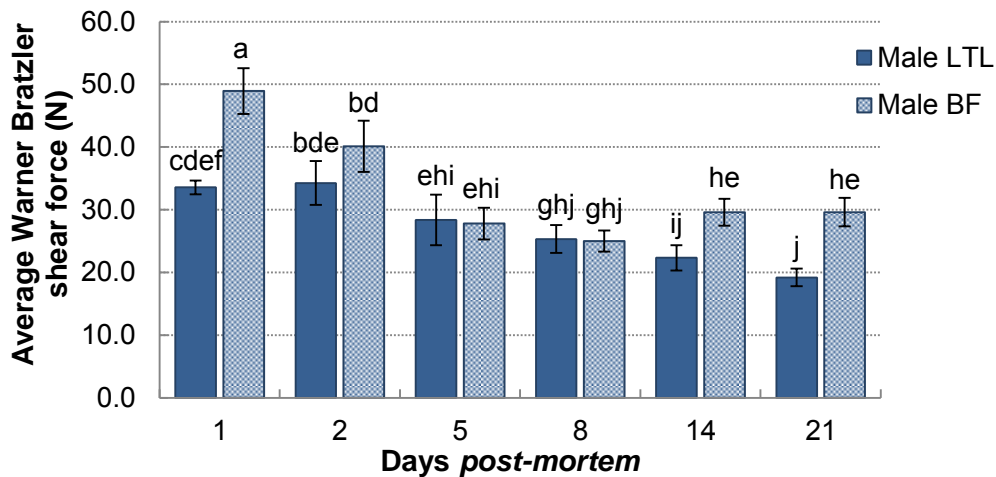


Figure 5.7 The Warner Bratzler shear force of male *Longissimus thoracis et lumborum* (LTL) and *Biceps femoris* (BF) muscles during ageing up to 21 days *post-mortem*. Different letters indicate significant differences ($p \leq 0.05$) between the mean values of each gender-muscle-days PM interaction group. Error bars indicate the standard error of the mean of each group.

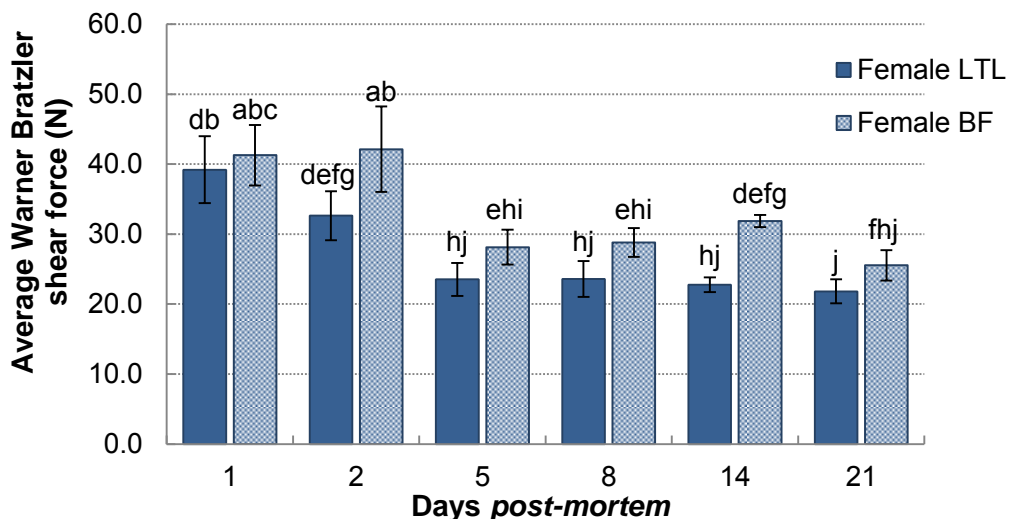


Figure 5.8 The Warner Bratzler shear force of female *Longissimus thoracis et lumborum* (LTL) and *Biceps femoris* (BF) muscles during ageing up to 21 days *post-mortem*. Different letters indicate significant differences ($p \leq 0.05$) between the mean values of each gender-muscle-days PM interaction group. Error bars indicate the standard error of the mean of each group.

Despite the significant 3rd order interaction present in the WBSF the least square means and standard errors for the main effects are given in Table 5.2. These values can still be interpreted as despite the interaction the general trends for the main effects were unaltered. It can be seen that no difference was found in shear force between the genders ($p = 0.926$) and the BF was significantly tougher than the LTL ($p < 0.001$), confirming the trends seen in Figure 5.7 and 5.8. A decline in shear force with ageing is apparent ($p < 0.001$); however significant pairwise differences were only present between day 1 and 2, and day 5, 8, 14 and 21, indicating that no further changes in tenderness occurred after five days of ageing.

5.3.2 Chemical analysis

No interactions between any of the main effects were found for cathepsin B, B and L (BL) or H activity. There was also no significant difference between either the genders or the muscles for either cathepsin B or BL (Table 5.3). However male springbok were found to have higher ($p = 0.027$) levels of cathepsin H activity than females. The levels of activity of all the cathepsins increased during ageing ($p_B < 0.001$, $p_{BL} < 0.001$, $p_H = 0.001$). Cathepsin B and BL showed similar trends (Fig. 5.9), while cathepsin H showed very little change over time, with only day 21 differing significantly from the rest of the ageing periods.

Table 5.3

The cathepsin B, BL, and H activity of springbok meat as per gender, muscle and ageing period (days PM)
(LSMeans \pm SEM)

Main effects		Cathepsin specific activity (Δ fluorescence/min/mg protein)		
		B	BL	H
Gender:	Male	5.45 ^a \pm 0.230	5.64 ^a \pm 0.422	1.39 ^a \pm 0.063
	Female	5.91 ^a \pm 0.307	5.61 ^a \pm 0.412	1.11 ^b \pm 0.053
Muscle:	LTL	5.91 ^a \pm 0.292	6.19 ^a \pm 0.490	1.31 ^a \pm 0.062
	BF	5.46 ^a \pm 0.314	5.05 ^a \pm 0.311	1.19 ^a \pm 0.058
Ageing period (days):	1	4.14 ^b \pm 0.353	4.44 ^c \pm 0.743	1.08 ^b \pm 0.087
	2	4.42 ^b \pm 0.396	3.98 ^c \pm 0.404	1.08 ^b \pm 0.089
	8	6.20 ^a \pm 0.428	5.36 ^{cb} \pm 0.498	1.25 ^b \pm 0.093
	14	6.61 ^a \pm 0.387	6.56 ^{ab} \pm 0.638	1.29 ^{ab} \pm 0.074
	21	7.04 ^a \pm 0.522	7.78 ^a \pm 0.664	1.55 ^a \pm 0.105

LSMean: least squares mean; SEM: standard error of the mean.

LTL: *Longissimus thoracis et lumborum*; BF: *Biceps femoris*; PM: *post-mortem*.

^{a, b, c} Least square means in the same column (within main effect) with different superscripts differ significantly from each other ($p \leq 0.05$).

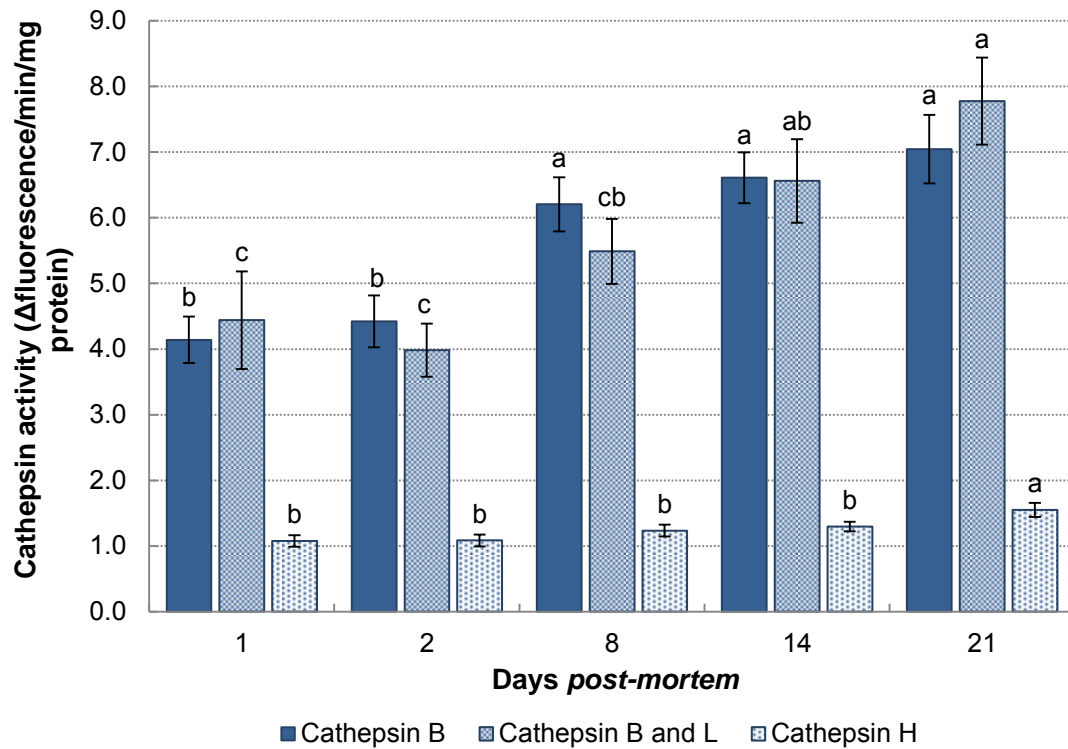


Figure 5.9 The mean cathepsin B, BL, and H activity in springbok meat during ageing up to 21 days *post-mortem*.

Different letters indicate significant differences ($p \leq 0.05$) between the mean values for each ageing period per cathepsin. Error bars indicate the standard error of the mean for each group.

There were no significant interactions between any of the main effects for calpain I, calpain II, calpastatin or the calpastatin to calpain ratio (Table 5.4). There was also no significant effect of gender on the calpain or calpastatin activities. The BF had higher ($p_{\text{calpain II}} = 0.002$, $p_{\text{calpain I}} = 0.016$, $p_{\text{calpastatin}} = 0.003$) levels of activity than the LTL for both the calpain enzymes as well as calpastatin. However there was no effect of muscle on the calpastatin to calpain ratio ($p = 0.414$).

Table 5.4

The calpain II, calpain I and calpastatin activity, as well as the calpastatin to calpain ratio of springbok meat as per gender, muscle and ageing period (days PM) (LSMeans \pm SEM)

Main effects		Calpain specific activity (U/mg protein)			
		Calpain II	Calpain I	Calpastatin	Calpastatin: (I+II)
Gender:	Male	0.0144 ^a \pm 0.00055	0.0079 ^a \pm 0.00077	0.0157 ^a \pm 0.00106	0.719 ^a \pm 0.0422
	Female	0.0141 ^a \pm 0.00070	0.0067 ^a \pm 0.00071	0.0133 ^a \pm 0.00114	0.634 ^a \pm 0.0402
Muscle:	LTL	0.0125 ^b \pm 0.00052	0.0060 ^b \pm 0.00068	0.0120 ^b \pm 0.00097	0.656 ^a \pm 0.0476
	BF	0.0160 ^a \pm 0.00055	0.0086 ^a \pm 0.00079	0.0170 ^a \pm 0.00110	0.696 ^a \pm 0.0369
Ageing period (days):	1	0.0159 ^a \pm 0.00065	0.0096 ^a \pm 0.00108	0.0169 ^a \pm 0.00150	0.664 ^a \pm 0.0489
	2	0.0150 ^a \pm 0.00079	0.0073 ^b \pm 0.00078	0.0157 ^a \pm 0.00127	0.711 ^a \pm 0.0475
	5	0.0118 ^b \pm 0.00050	0.0050 ^c \pm 0.00066	0.0108 ^b \pm 0.00101	0.653 ^a \pm 0.0599

LSMean: least squares mean; SEM: standard error of the mean.

LTL: *Longissimus thoracis et lumborum*; BF: *Biceps femoris*; PM: *post-mortem*.

^{a, b, c} Least square means in the same column (within main effect) with different superscripts differ significantly from each other ($p \leq 0.05$).

A similar situation was found for the effect of ageing on the calpain and calpastatin activities (Fig. 5.10). There was a change ($p_{\text{calpain II}} < 0.001$, $p_{\text{calpain I}} = 0.001$, $p_{\text{calpastatin}} < 0.001$) in activity over ageing for calpain I and II as well as calpastatin, with the activity of all three decreasing from one to five days PM. However these changes cancelled each other out, resulting in no change ($p = 0.554$) in the calpastatin to calpain ratio during ageing being apparent.

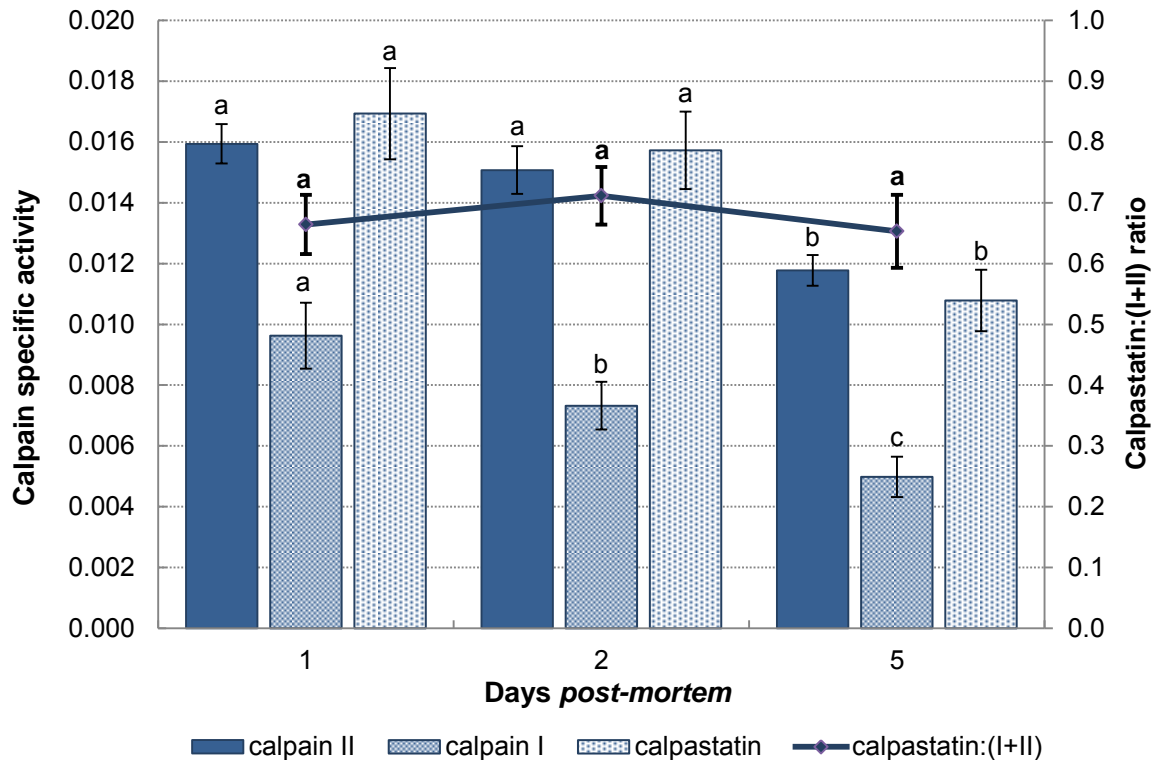


Figure 5.10 The mean calpain I, calpain II, calpastatin activity and calpastatin:calpain ratio in springbok meat during ageing up to 21 days *post-mortem*. Different letters indicate significant differences ($p \leq 0.05$) between the mean values for each ageing period per calpain. Error bars indicate the standard error of the mean for each group.

When interpreting the values given in Table 5.4 one must take cognisance of the fact that calpain specific activity levels of below 0.01 U/mg protein are considered extremely low (personal communication with Dr Lorinda Frylinck, Agricultural Research Council, Irene, on 07 July 2014). The reliability of such values is thus questionable as the proportion of experimental error increases as the total recorded activity decreases.

5.4 Discussion

The factors contributing to the toughness of meat can be divided into those making up the connective tissue (primarily the total and insoluble collagen content) and those attributable to other structural components of the meat (the myofibrillar, cytoskeletal and sarcoplasmic proteins) (Purslow, 2005; Koohmaraie, Kent, Shackelford, Veiseth & Wheeler, 2002). The collagen content determines the baseline or background toughness of the meat, as measured using the Warner Bratzler shear force (Sentandreu *et al.*, 2002; Purslow, 2005). The physical contribution of the remaining structural proteins acts additively, resulting in the variation in the toughness of meat beyond that contributed by the connective tissue (Koohmaraie *et al.*, 2002). These proteins can be broken down PM, resulting in the observed

increase in the tenderness of meat during ageing (Ouali, 1992). The proportion of the total shear force of fresh meat that is due to the contribution of factors not broken down during ageing will influence the degree of tenderization that is observed, with this influence increasing concomitantly with the collagen content (Kerry & Ledward, 2009). The contribution of collagen to the background shear force value can be seen in the correlation ($r = 0.51$, $p = 0.011$) found between the total collagen content and the shear force after 21 days of ageing. This correlation is not significant at any of the other ageing periods.

As seen in Figure 5.7 and 5.8, little change in the shear force of springbok meat is observed past five days of ageing. It has been found that the same plateau in tenderization is only observed after eleven to fourteen days of ageing in beef (Koochmaraie, Whipple, Kretchmar, Crouse & Mersmann, 1991; Smith, Culp & Carpenter, 1978; Smith *et al.*, 1978; Sentandreu *et al.*, 2002; Nowak, 2011). This may indicate that in springbok meat the proportion of the total structural integrity that is due to the collagen content is relatively high, as the baseline toughness is reached very rapidly during ageing. However, this hypothesis is not supported by the values for the total collagen content or collagen solubility found in this study.

The total collagen content of the LTL was found to be 1.29 mg/g meat (wet mass), with a solubility of 27.6%. In contrast the collagen content of beef LTL has been reported at 4 - 6 mg/g, with a solubility of 25 - 31% (Torrescano, Sánchez-Escalante, Giménez, Roncalés & Beltrán, 2003; Seideman, 1986; Von Seggern, Calkins, Johnson, Brickler & Gwartney, 2005). This indicates that the collagen contributing to the shear force of cooked meat (insoluble collagen) is considerably higher in beef than in springbok. This is to be expected considering the shear force measured for un-aged springbok LTL (male - 33.57; female - 39.2 N; Fig. 5.7 & 5.8), relative to that of beef (40 - 60 N) (Crouse & Koochmaraie, 1990; Shackelford, Wheeler & Koochmaraie, 1997). These values are consistent with findings that game meat/venison is more tender than beef, and in some species does not require any ageing to reach acceptable levels of tenderness (Hutchison *et al.*, 2010). However, considering that despite the higher collagen content, beef takes longer to reach final tenderness than springbok, it seems likely that the rapid plateau of the shear force found in springbok is more likely an indication of a faster rate of PM proteolysis than a restriction on tenderization due to the collagen content.

The shear force of female springbok LTL was found to decline by a total of 17.4 N over the full 21 day ageing period (Fig. 5.8). Of the total decrease in shear force over 21 days of ageing 90.2% was completed by the fifth day of ageing. Beef LTL is commonly aged for a period of 14 days, with a decline of 10 - 16 N shear force being observed during this time (Crouse & Koochmaraie, 1990; Shackelford, Wheeler & Koochmaraie, 1997). Approximately

the same extent of ageing was thus found in five days in female springbok as is normally found in 14 days in beef, indicating a more than two fold higher rate of tenderization in the former. Similar patterns of decline were found in the BF from both male and female springbok.

The rate of tenderization in meat has been correlated with the activity of the proteolytic enzymes involved in ageing and the overall efficiency of these proteolytic systems, as indicated by the ratio of the activity of the enzymes to that of their inhibitor or *vice versa* (Kemp, Sensky, Bardsley, Buttery & Parr, 2010; Nowak, 2011). This has been used to explain the high rate of tenderization found in some venison, where the activity of the proteolytic enzymes has been found to be high relative to that of beef (Hutchison *et al.*, 2010). Although the roles of less understood systems such as the caspases and the proteome are currently being investigated, the cathepsins and particularly the calpains are still regarded as being responsible for many of the changes found during ageing (Huff-Lonergan, Zhang & Lonergan, 2010; Nowak, 2011; van Jaarsveld, Naudé & Oelofsen, 1997) and were thus focussed on in this study.

The activities of calpain I, calpain II and calpastatin were found to decrease from day one to day five PM. This decline is indicative of the enzymes being activated in the meat and thus contributing to tenderization (Huff Lonergan *et al.*, 2010; Dransfield, 1993; Dransfield, Wakefield & Parkman 1992). The activation of calpain enzymes requires an increase in the calcium ion concentration in the sarcoplasm to 5 - 65 μM for calpain I and 300 - 1000 μM for calpain II (Huff Lonergan *et al.*, 2010). This calcium ion concentration also results in both brief and extended autolysis. While brief autolysis (calpain I 80 kDa subunit to 76 kDa, calpain II 80 kDa subunit to 78 kDa, calpain I and II 28 kDa subunit to 18 kDa) activates the enzyme and reduces the calcium ion threshold for its activation, further autolysis is also initiated and results in the enzymes' inactivation (Huff Lonergan *et al.*, 2010). A lack of decline in activity is thus indicative of a lack of autolysis, indicating that the conditions present in the meat at that point were likely not favourable for enzyme activity. The findings of this study therefore support the conclusion that calpains had an influence on the change in shear force, as was reported in Kemp & Parr (2012); Sentandreu *et al.* (2002) and Dransfield *et al.* (1992). The concomitant decrease in the rate of tenderization after five days PM could thus be due to the lack of residual calpain activity after this time.

The decline in calpain I activity is in agreement with the results found in literature for other species (Kemp *et al.*, 2010; Nowak, 2011; Dransfield, 1993). However, calpain II is normally reported as being more stable, with little change in activity during even extended ageing being found (Pomponio & Ertbjerg, 2012; Dransfield, 1993). The decline found in springbok meat suggests that calpain II played a greater role in tenderization than is

normally found in beef. This may partially explain the more rapid tenderization found. Whether this increased calpain II activity is as a result of differences in the nature of the enzyme or in the calcium ion concentration in the meat are matters for future study. Calpastatin activity was also found to decline PM, which is consistent with literature (Kemp & Parr, 2012).

As a result of the cropping method used to harvest the springbok in this study, stress levels prior to death were kept very low in comparison to those of livestock sent to commercial abattoirs. This may also have favoured more rapid ageing, as positive correlations between adrenalin levels and calpastatin activity have been reported in literature (Kemp *et al.*, 2010).

The determination as well as interpretation of cathepsin activity data in literature varies from paper to paper. Calkins and Seideman (1988) and Johnson, Calkins, Huffman, Johnson and Hargrove (1990) determined the cathepsin activity at one hour PM, correlating these values to the shear force measured after different ageing periods. While correlations between the shear force and the measured cathepsin activity were found, this interpretation was based on the assumption that equal rates of activation took place in all the samples and that the enzymes were in fact activated *in situ* PM (Calkins & Seideman, 1988; Johnson *et al.*, 1990). Alternatively, Thomas, Gondoza, Hoffman, Oosthuizen and Naudé (2004) found that cathepsin activity in ostrich meat remained stable or increased during ageing, and concluded from this that the lysosomal enzymes did play a role in tenderization. However, no changes in shear force were found during the period for which the activity was measured, casting doubt on this interpretation.

In this study the activities of cathepsins B, B and L, and H were all found to increase during ageing. When interpreted according to the 'autolysis indicates activation' theory described for calpains, this increase in activity suggests that the cathepsin enzymes contributed little to the tenderization process. This finding is in agreement with the conclusions of Koochmaraie (1994), van Jaarsveld *et al.* (1997) and Sentandreu *et al.* (2002). The lack of tenderization after day five, despite the high level of cathepsin activity during this time, further supports this interpretation. It is however possible that further myofibrillar degradation may have taken place during this period but was masked by the effect of the collagen content on shear force. The increase in cathepsin activity during ageing found in this study and others has been suggested to be the result of the gradual degradation of the lysosomes during ageing (Thomas *et al.*, 2004).

The BF was found to have significantly higher shear force values than the LTL throughout the ageing period. This was most likely as a result of the higher insoluble collagen content of the BF, as indicated by the higher total collagen and lower collagen

solubility found in this muscle ($p_{\text{total}} = 0.107$, $p_{\% \text{solubility}} = 0.187$), despite the lack of significance of these differences. However, the rate of decline in the shear force over five days of ageing was greater for the BF than the LTL (17.14 N vs 10.44 N), and this represented a greater proportion of the total change over the 21 days of ageing (99% vs 64%).

The BF was also found to have significantly higher levels of calpain I and II activity than the LTL. On the other hand the calpastatin activity was also significantly higher, resulting in there being no difference between the muscles in the calpastatin to calpain ratio. This ratio has been reported as being a better indicator of the efficiency of the calpain system than the activities of the enzymes alone (Sentandreu *et al.*, 2002; Kemp & Parr, 2012; Ouali, 1992). It must however be noted that the ratio does not necessarily give an accurate representation of the proteolytic potential of the system. If one considers the absolute values for the calpains and calpastatin in the BF relative to the LTL one can see that the BF could still potentially contain a greater number of uninhibited calpains. This would explain the greater rate of tenderization in this muscle.

The rate of decline in shear force, in conjunction with the absolute WBSF values obtained for the BF, suggest that connective tissue contributes a larger proportion of the total structural integrity of this muscle relative to the LTL, and that the myofibrillar fraction of this muscle is relatively susceptible to proteolytic degradation.

As seen in Figures 5.7 and 5.8, the shear force of the LTL muscle from male springbok did not plateau at five days PM as was found for the other gender-muscle groups. The continued decline in the shear force past five days of ageing could indicate that the male LTL had a lower baseline toughness than the other samples. This could be as a result of the higher collagen solubility found for these samples, despite the lack of significance ($p = 0.395$) of the 2nd order interaction for this variable. No other noticeable or significant differences between male LTL muscles and the other gender-muscle groups were found for any of the other variables.

An undesirable side-effect of ageing meat is the loss of mass due to the purge of moisture from the meat. In this study over 5% of the initial mass of each portion was lost over the 21 day ageing period. This degree of moisture loss is high relative to values reported for vacuum-aged beef (Johnson, 1991; Hodges *et al.*, 1974; Lagerstedt, Enfält, Johansson & Lundström, 2008). Of this moisture loss 61% was lost by the fifth day of ageing, and 13% was solely due to the vacuum packing of the portions. The effect of vacuum packaging on purge loss has been commented on in literature, with it being attributed to the physical compression of the meat during packaging (Payne, Durham, Scott & Devine, 1998). The decrease in the rate of moisture loss towards the end of the ageing

period is in agreement with literature (Fig. 5.3) (Hodges *et al.*, 1974). This is as a result of there being a finite volume of water available in the meat to be released.

Significantly lower levels of moisture loss were found for the BF than the LTL throughout the ageing period (Fig. 5.3). This could have been due to differences in the trimming of the two muscles, which may have resulted in the LTL portions having a greater cut surface area (Johnson, 1991). However, the BF was also found to have a significantly higher pH. The positive association between the pH and the retention of water has been observed under a number of different situations and is well explained in literature (Huff-Lonergan & Lonergan, 2005; Warriss, 2010; Lawrie & Ledward, 2006; Bouton, Carroll, Fisher, Harris & Shorthose, 1973). This hypothesis is not however supported when examining the differences between the genders, as male springbok had significantly higher pH values throughout the ageing period and yet were found to have higher (non-significant) moisture losses during ageing. There were also no significant correlations ($p \leq 0.05$) between the pH and WHC at any of the ageing periods found in this study. The more rapid proteolysis found in the BF may have also contributed to the lower moisture loss, as this can decrease the movement of entrapped water out of the cell (Huff-Lonergan & Lonergan, 2005).

Despite the significant difference between the muscles in both the moisture loss and the pH, this trend was not supported by the WHC data. A significant interaction between gender and muscle involving a change of rank was present for the WHC, resulting in there being no significant difference between the muscles for this variable. However, on examination of the interaction data it can be seen that in female animals the BF had a significantly lower WHC than the LTL, whereas in males no difference between the muscles was found. This is in direct contrast with the lower moisture losses found for the BF during ageing, as a negative correlation between WHC and purge loss is expected.

There was also no correlation found between the WHC and the moisture loss during ageing, with no single trend in the WHC being observed PM (Fig. 5.6). This is in contrast with literature and the common belief that the WHC increases with ageing (Lawrie & Ledward, 2006). However, by comparing Figure 5.1 to Figure 5.6 it can be seen that the pH and WHC follow the same pattern during ageing. It therefore seems likely that the observed change in the WHC is as a result of the changes in the pH.

There is little information on the change in pH during ageing available in literature, with Ruiz de Huidobro, Miguel, Onega and Blázquez (2003) reporting no change in the pH of beef *Longissimus dorsi* (LD) muscles aged up to six days. Boakye and Mittal (1993) reported an overall increase in the pH up to 16 days of ageing and accounted this to changes in the charge of the meat proteins due to the activity of proteolytic enzymes. This could provide an

explanation for the decline and subsequent increase in the pH from day one to day five found in this study but fails to explain the decrease at 21 days. The biochemical mechanism of this explanation is also unknown.

Ageing had a significant effect on the cooking loss, with an increase being found from day eight to day 21, while no change was found prior to this period. Conflicting reports on the effect of ageing on cooking loss are found in literature, with Straadt, Rasmussen, Andersen and Bertram (2007) finding an increase from day one to four followed by no further change in pork LD. In contrast, Abdullah & Qudsieh (2009) found a decrease in the cooking loss of LD muscles after ageing for seven days and Ruiz de Huidobro *et al.* (2003) found no change for beef LD muscles during ageing for up to five days PM. It is therefore difficult to explain the particular pattern of cooking loss during ageing found in this study. It is possible that the increase at the end of the ageing period was as a result of the proteolytic changes taking place PM reaching a critical point at which the bound water in the meat could be released during cooking.

In contrast with the lower purge loss found in the BF, the cooking loss in this muscle was found to be significantly higher than that in the LTL (30.8% and 26.2% respectively). As there was only a 1.4% difference in the purge loss between the two muscles this more than made up for the lower moisture loss during ageing. It seems likely that the higher cooking loss found for the BF is directly linked to the lower purge loss for this muscle, as more moisture was therefore available to be lost during the cooking process.

5.5 Conclusion

When comparing the shear force values obtained in this study for springbok LTL and BF muscle to those reported for beef it can be seen that springbok meat is at least as tender, if not more so, than equivalent beef muscles. The trends obtained for the change in shear force during ageing also indicate that ageing for more than five days does not result in any further improvement in the shear force. The tenderization process itself appeared to be mainly as a result of the activity of calpain enzymes, with the cathepsins not appearing to be active in the meat PM. Longer ageing periods resulted in substantial increases in mass loss as purge as well as increased cooking losses. Based on these results it is recommended that vacuum-packed springbok LTL and BF muscles are not aged longer than five days at 0 - 5°C. However, in order to more accurately determine the ideal ageing period for springbok meat it is necessary to not only assess the instrumentally determined changes but also the textural changes as detected by the human palate. Alterations in other sensory attributes such as flavour and aroma will also further define the interval *post-mortem* during which optimal quality is obtained.

5.6 References

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CHAPTER 6

Changes in the aroma, flavour and texture of springbok (*Antidorcas marsupialis*) *Longissimus thoracis et lumborum* muscle during conditioning as assessed by physical analysis and a trained sensory panel

Abstract

This study determined the effect of ageing at 4°C on the sensory quality of springbok *Longissimus thoracis et lumborum* (LTL) muscle. The LTL muscles from 12 mature springbok (six female, six male) were divided into four equal portions and aged for 1, 3, 8 or 28 days, after which they were blast frozen. The myofibrillar fragment length (MFL) and pH were determined for raw samples and descriptive sensory analysis (trained panel), Warner Bratzler shear force (WBSF) and proximate chemical composition were determined for cooked samples. There was an increase in gamey, metallic, liver-like, sour/aged and off/manure attributes and a decline in beef-like aroma during ageing. These changes are ascribed to the combined effects of oxidation and proteolysis. Increases in tenderness and sustained juiciness and decreases in residue and MFL were observed; however there was no significant change in the WBSF or the moisture content of the cooked meat over aging time. The shear force was extremely low for all ageing periods. Significant gender effects were only present for metallic aroma (female > male), residue (male > female) and cooking loss (male > female). It was concluded that springbok meat should be aged for a maximum of eight days in order to reduce the risk of undesirable changes in aroma and flavour.

Keywords: descriptive sensory analysis, ageing, tenderization, game meat

6.1 Introduction

Despite improvements in food security since 1990 it still persists as a global problem, with Africa presenting one of the greatest challenges (FAO, 2013). In order to meet food requirements developing countries need to maximise both local food production and national buying power through improved per capita income and employment rates (FAO, 2013). Unfortunately, in many cases either natural resources are limited or the capital required for the development of the infrastructure necessary for the utilisation of the natural resources is not available (Godfray *et al.*, 2010). There is also increasing conflict between the development of agriculture and the conservation of endangered indigenous species and natural ecosystems (Godfray *et al.*, 2010). The potential exacerbating effect of global warming on the incidence of extreme weather conditions poses an additional challenge (Godfray *et al.*, 2010). The game ranching industry has the potential to aid in overcoming all of these challenges (Lindsey, 2011).

Indigenous species are better adapted to the climatic conditions, forage availability, diseases and predation present in a particular environment than imported domesticated species (Du Buisson, 2006). This allows their survival and production with fewer inputs and thus costs, as well as reducing the impact on the environment if managed properly (Mossman & Mossman, 1976; Lindsey, 2011). They also represent a relatively untouched source of genetic variation which can be exploited through both breeding and genetic modification (Godfray *et al.*, 2010). Game ranching supplies a wider range of sources of income than traditional farming; with ecotourism, trophy and recreational hunting and live sales all increasing profits (Lindsey, 2011). The conversion of livestock farming to game ranching has also been found to increase both the number of jobs created as well as the average income of employees (Lindsey, 2011). The wider-reaching benefits of game ranching thus include the preservation of biodiversity, job creation and the fuelling of a nation-wide tourism industry (Mossman & Mossman, 1976; Lindsey, 2011).

In the South African context, game species form an integral part of the country's international image. Tourists from around the world visit the country for the African safari experience, and in many cases the consumption of game meat forms an integral part of this (Hoffman, Crafford, Muller, & Schutte, 2003; Hoffman & Wiklund, 2006). However, in order for the demand for game meat by both tourists and local consumers to continue the experience must be a pleasant one.

The optimisation of the quality of meat from domesticated species has been extensively studied and is still a topic of much research, with meat scientists quantifying the effect of breed, age, rearing method and diet, among others. In contrast, when handling and processing game meat many producers simply follow operating procedures developed more

for logistical than meat quality optimisation (personal communication with Piet Neethling, director of Camdeboo Meat Processors and Charl de Villiers, from Amatola/Mosstrich). This, along with the general perception of game meat as being tough, dry and requiring special preparation (Radder & Le Roux, 2005; Hoffman & Wiklund, 2006), leads to extensive ageing periods being used, often resulting in loss of flavour and textural quality as well as increased costs to the producer for holding meat under chilled conditions.

While instrumental measures can give a basic indication of meat quality they lack the ability to completely replicate the eating experience. A low Warner Bratzler shear force does not fully reflect the textural experience of eating a perfectly prepared fillet steak, and measures of fatty acids and volatile compounds cannot replace a full description of the flavour of a grass-fed beef roast (Warriss, 2010). The use of descriptive sensory analysis thus forms a vital part of the assessment and comparison of meat quality.

Meat is primarily aged in an effort to improve textural attributes, with a focus on increased tenderness (Warriss, 2010). However, over-ageing can also cause detrimental changes, leading to an optimum point of ageing being found (Lawrie & Ledward, 2006). While texture was the focus of this study, meat quality is determined by a wide array of aroma, flavour and textural characteristics, all of which undergo both desirable and undesirable changes during ageing. The descriptive sensory analysis performed thus considered all these facets in order to determine how the overall sensory quality of the meat changed during ageing.

6.2 Materials and methods

6.2.1 Harvesting and slaughtering

Eighteen (nine male, nine female) mature springbok were harvested according to standard operating procedure (SOP number SU-A CUM13-00034) over two nights in March of 2014. They were obtained from Brakkekuil farm, located near Witsand in the Western Province of South Africa (34°18'24.0" S; 20°49'3.9" E; altitude of 93 m). Harvesting was done at night using a spotlight to locate and temporarily immobilise the springbok (Hoffman & Laubscher, 2010). They were killed with a single shot to the head from a 30-06, .308 or .270 calibre rifle and exsanguinated immediately thereafter by cutting the jugular vein and carotid arteries. Observed stress levels were recorded if necessary. Once the required number of animals had been harvested the bled carcasses were transported to a nearby slaughtering facility where they were skinned and eviscerated. All the carcasses were dressed within three hours of death. The dressed carcasses were placed in a cool room (< 7°C) to undergo rigor. Carcasses were hung by both Achilles tendons in order to ensure

equal contraction of the muscles in both sides of the carcass during development of rigor mortis.

Ethical clearance for this study was issued by the Stellenbosch University Animal Care and Use Committee (ethical clearance number SU-ACUM13-0034).

6.2.2 Sampling

Carcasses were transported whole to the meat processing facility at the Department of Animal Sciences at the University of Stellenbosch for further sampling. The *Longissimus thoracis et lumborum* (LTL) muscle was excised from both sides of the carcass from its natural termination at the cervical vertebra cranially to the last lumbar vertebra caudally. Each muscle was cut perpendicularly to its longitudinal axis to produce two portions of approximately equal mass, resulting in four portions per carcass. Each of these portions was randomly assigned to one of four ageing periods (1, 3, 8 or 28 days) and vacuum-packed. The portions were aged at refrigerator temperatures (3 - 6°C), with care being taken to avoid stacking of the portions which could have resulted in different responses to ageing due to variable compression of the meat.

On the completion of each ageing period the portions were frozen to a temperature of -30°C in a blast freezer (Marcold refrigeration co. (PTY) LTD, Cape Town, South Africa). All samples were completely frozen within one hour. Once frozen the portions were stored at -20°C for two to seven weeks until sensory analysis.

6.2.3 Sample preparation

Vacuum-packed samples were thawed at 0 - 5°C for approximately 24 hours before each of the sensory sessions. Once thawed they were removed from the vacuum bags, blotted dry with absorbent paper and placed in individually marked oven bags (Spar® or Glad®). No seasonings or other additives were used when preparing the samples. Thermocouple probes attached to handheld digital temperature monitors (Major Tech model MT645², Johannesburg, South Africa) were placed in the centre of each portion (AMSA, 1995) and the oven bags were closed using the supplied bag-ties. Two conventional Defy ovens (model 835) were preheated to a temperature of 160°C, with the temperature being controlled using temperature probes installed in the ovens and a computerised monitoring system (Viljoen, Muller, De Swardt, Sadie & Vosloo, 2001). Portions were placed on stainless steel grids fitted on oven roasting pans and placed in the ovens to cook to an internal temperature of 72°C (as indicated by the thermocouple probes). They were allowed to rest five minutes after removal from the oven, where after they were removed from the oven bags and blotted dry using absorbent paper towelling. Each portion was cut into

approximately 1cm³ blocks, each of which was individually wrapped in tin-foil. The wrapped blocks were placed in ramekins marked with randomly generated sample codes. The blocks were reheated in a preheated industrial oven (Hobart, France) at 70°C for seven minutes prior to serving and were placed in water-baths set at 70°C for the duration of the training/testing session in order to maintain them at the correct temperature.

6.2.4 Descriptive sensory analysis

Descriptive sensory analysis (DSA) of the samples was performed by nine panel members with previous experience of the sensory analysis of meat.

Prior to the testing phase, portions from six of the 18 harvested springbok were used to train the panel in six training sessions. Training was done according to the guidelines and recommendations of AMSA (1995) and Lawless & Heymann (2010). During the training period seven aroma attributes (overall intensity, beef-like, gamey, liver-like, metallic, sour/aged, off/manure), six flavour attributes (gamey, beef-like, liver-like, metallic, sour/aged, off/manure) and five textural attributes (sustained juiciness, tenderness, residue, mealiness, visual assessment of grain) were decided upon and elucidated (Table 6.1). Reference samples were used during the training period to help define the attributes. These reference samples were: >40 day aged beef rump steak (sour aroma/flavour), <14 day aged beef rump steak (beef aroma/flavour, coarse grain for visual assessment), blesbok (*Damaliscus pygargus phillipsi*) loin (gamey aroma/flavour), 30 day aged springbok loin (mealiness), Egyptian goose (*Alopochen aegyptiacus*) breast (gamey aroma, metallic aroma, texture), chicken breast (mealiness) and pan-fried ox liver (liver-like aroma/flavour, mealiness). Reference samples used for the training period were prepared in a similar manner to the sample portions, apart from the ox-liver, which was pan-fried in canola oil. During the training period each panel member received three blocks from each springbok portion and reference sample.

Portions from six male and six female springbok that had not undergone any *ante-mortem* stress were selected for the testing period. Each animal was randomly assigned to a testing session and twelve testing sessions were performed (twelve replications).

Table 6.1

Description and scales of assessment for each attribute used during descriptive sensory analysis

Sensory attribute	Description	Scale
Aromas		
Overall aroma intensity	Intensity of aroma in first few sniffs	0=extremely bland, 100=extremely intense
Beef-like aroma	Aroma associated with cooked beef loin	0=extremely bland, 100=extremely intense
Gamey aroma	Aroma associated with the meat from wild animal species - combination of liver-like & metallic	0=extremely bland, 100=extremely intense
Liver-like aroma	Aroma associated with pan-fried beef liver	0=extremely bland, 100=extremely intense
Metallic aroma	Aroma associated with metal/iron/blood	0=extremely bland, 100=extremely intense
Sour aroma	Aroma associated with vacuum-packed, aged meat/off milk	0=extremely bland, 100=extremely intense
Off/Manure aroma	Unpleasant aroma associated with farm-yard/contamination/off meat	0=extremely bland, 100=extremely intense
Flavours		
Gamey flavour	Flavour associated with the meat from wild animal species	0=extremely bland, 100=extremely intense
Beef-like flavour	Flavour associated with cooked beef loin	0=extremely bland, 100=extremely intense
Liver-like flavour	Flavour associated with that of pan-fried beef liver	0=extremely bland, 100=extremely intense
Metallic flavour	Flavour associated with metal/iron/blood	0=extremely bland, 100=extremely intense
Sour flavour	Flavour associated with off milk	0=extremely bland, 100=extremely intense
Off/Manure flavour	Unpleasant flavour associated with farm-yard/contamination/off meat	0=extremely bland, 100=extremely intense
Textural attributes		
Sustained juiciness	Amount of moisture perceived during mastication	0=dry, 100=extremely juicy
Tenderness	Impression of tenderness after mastication	0=tough, 100=extremely tender
Residue	Residual tissue remaining after mastication (difficult to chew through)	0=none, 100=abundant
Mealiness	Extremely fine texture. Disintegration of muscle fibre into very small particles that are retained on the tongue (perception within first few chews)	0=none, 100=abundant
Visual assessment of grain	Fineness/coarseness of the fibres present in the meat (look at cross-sectional surface)	0=extremely fine, 100=very coarse

The DSA was performed using the test re-test method. Panel members sat in individual tasting booths and assessed the four samples using an unstructured line scale extending from zero (minimum intensity/abundance) to 100 (maximum intensity/abundance) (AMSA, 1995). The samples were identified using random, numerical three-digit codes generated by Compusense Five (Compusense, Guelph, Canada) and were assessed in a completely random order. The room was maintained at 21°C and lit with artificial daylight. Panel members were supplied with distilled water (at room temperature), apple slices (Royal gala apples) and water-biscuits (Carr, UK) to cleanse their palettes between samples (AMSA, 1995).

6.2.5 Physical analyses

6.2.5.1 pH

Samples for the determination of the pH were taken from each portion after thawing and prior to cooking. These were frozen in liquid nitrogen and stored at -80°C until the pH could be determined using the sodium-iodoacetate method (Zhu, Ruusunen, Gusella, Zhou, & Puolanne, 2011) as described in Chapter 4 (section 2.3.1).

6.2.5.2 Cumulative purge and freeze/thaw loss

Portions were weighed at sampling (Mettler PC 4400 Delta range, Cape Scientific, South Africa), prior to vacuum-packaging, in order to determine their initial weight. After thawing the vacuum-packed portions at 0 - 5°C for approximately 24 hours they were blotted dry using absorbent paper towelling and weighed to determine the weight remaining after purge and thaw moisture losses. This moisture loss was expressed as a percentage of the initial weight of each portion.

6.2.5.3 Cooking loss

After the removal of the samples for the pH and myofibrillar fragment length determination the portions were weighed (Mettler PC 4400 Delta range, Cape Scientific, South Africa) to obtain an initial raw weight. After cooking and the five minute rest period the portions were removed from the oven bags, blotted dry using paper towelling and reweighed to determine a cooked weight. The moisture loss during cooking was expressed as a percentage of the initial weight of each portion (AMSA, 1995).

6.2.5.4 Warner Bratzler shear force (WBSF)

The instrumental 'toughness' of the samples was determined using the Warner Bratzler shear force test (Honikel, 1998).

Once the blocks required for the descriptive sensory analysis had been cut, the remaining cooked meat from each portion was wrapped in foil, placed in a vacuum-bag and left at refrigerator temperatures (0 - 5°C) for 24 hours to cool completely. Two 2 cm steaks were cut from each portion perpendicular to the direction of the muscle fibres. A minimum of six 1 cm x 1 cm x 2 cm blocks were cut from these steaks such that the muscle fibres ran parallel to the longitudinal axis of the blocks. The force in Newton required to shear each block perpendicular to its longitudinal axis was determined using an Instron Universal Testing Machine (Instron UTM, Model 2519-107) fitted with a Warner Bratzler blade. The Instron had a load cell of 2 kN and crosshead speed of 200 mm/min. The Warner Bratzler fitting was 1 mm thick and had a triangular opening with a semi-circular cutting edge (radius of 0.508 mm). The average of the values for all blocks (six to eight) was used as the Warner Bratzler shear force of the sample.

6.2.5.5 Myofibrillar fragment length (MFL)

Samples (*circa*. 6 g) for the determination of the myofibrillar fragment length (MFL) were taken from each raw portion after thawing and prior to cooking. They were snap-frozen in liquid nitrogen and stored at -80°C until analysis. The analysis was performed according to the method of Culler, Parrish, Smith and Cross (1978), with adaptations being made according to Heinze and Bruggemann (1994).

A portion of the frozen muscle sample was finely cut, with care being taken to avoid obvious fat and connective tissue. Of this, 3 g was weighed off and added to 30 ml of 4°C extraction buffer (0.02 M potassium phosphate buffer containing 100 mM potassium chloride, 1 mM magnesium chloride, 1 mM ethylenediaminetetraacetic acid and 1 mM sodium azide). The sample was allowed to thaw in the buffer for 60 seconds, where after it was homogenized at 20000 rpm for 30 seconds using a Bühler HO₄ homogenizer. The blade of the homogenizer was inverted so that the myofibrils were fragmented rather than sliced.

The homogenized samples were centrifuged at 4°C at 3000 rpm for 15 minutes. The supernatant was subsequently discarded and the pellet re-suspended in another 30 ml of MFL extraction buffer and the centrifuge process repeated. The supernatant was again discarded and the pellet re-suspended in 10 ml of extraction buffer. The suspension was filtered under vacuum through a 1000 µm polyethylene strainer, with 5 ml of extraction buffer being used to wash the myofibrils through the strainer. The resulting filtrate was filtered further through a 250 µm polyethylene strainer.

The final filtrate was mounted on a microscope slide and examined using an Olympus BX40 microscope under 400X magnification. The fragments were measured using the

analySIS software package from Life Science, with 100 fragments being measured per sample. Myofibrillar fragment length is given in μm .

6.2.5.6 Proximate composition

The proximate composition (moisture, protein, fat and ash) of all the cooked samples tested in the descriptive sensory analysis was determined according to the official methodology of the Association of Official Analytical Chemists (AOAC, 2002). These samples were kept at 0 - 5°C for 24 hours after cooking, where after they were homogenized and stored at -20°C until analysis. All analyses were done in duplicate.

The moisture content was determined according to AOAC official method 934.01. Samples of 2.5 g of homogenized meat were placed in dry, marked, crucibles, the weights of which had been recorded. The samples were allowed to dry at 100 - 105°C for a minimum of 24 hours. They were subsequently removed from the oven and placed in a desiccator to cool. Once completely cool the crucibles and dried samples were weighed. The moisture content of the samples was calculated as the difference between the sample weight before and after drying.

The moisture-free samples were used for the determination of the ash content. The crucibles were placed in a furnace at 500°C for a minimum of six hours. They were then allowed to cool, first in the furnace and finally in a desiccator, before weighing. The ash content was determined as the remaining mass of the sample after incineration and expressed as a percentage of the original wet weight of the sample (AOAC, 2002 method 942.05).

The fat content was determined on 5 g of homogenized sample using a 1:2 chloroform/methanol solution for fat extraction. This ratio was chosen as springbok meat is known to have a low fat content (< 5% intramuscular fat) (Hoffman, Kroucamp, & Manley, 2007a). A rapid solvent extraction method was used, as described in Lee, Trevino and Chaiyawat (1996).

The defatted sample obtained as a by-product of the fat determination was used to determine the protein content of the sample. This was done using the LECO combustion or Dumas method. The defatted samples were dried completely and ground to a fine powder, 0.5 g of which was weighed off into LECO™ foil cups and analysed for nitrogen content. This nitrogen content was multiplied by a factor of 6.25 in order to obtain the protein content of the sample, which was subsequently converted to a value per gram wet meat (AOAC, 2002, method 992.15). The LECO was recalibrated after every ten test samples using an EDTA calibration sample (LECO Corporation, St Joseph, MI, USA).

6.2.6 Statistical analysis

The experimental design was a completely random split plot with gender as the main plot factor and ageing periods as subplot factors. Six male and six female springbok were selected at random, resulting in a total of 12 springbok ($n = 12$) being used in the trial. Each animal was randomly assigned to a testing session and twelve testing sessions were performed, evaluating samples from the four ageing periods for each springbok within each session.

Univariate analysis of variance was performed on all variables accessed using the GLM (General Linear Models) Procedure of SAS™ statistical software (Statistical Analysis System, Version 9.2, SAS Institute Inc., Cary, NC, USA). The model for the statistical design is indicated by the following equation:

$$Y_{ijk} = \mu + g_i + (gr)_{ik} + a_j + (ga)_{ij} + \varepsilon_{ijk}$$

The terms within the model are defined as; the overall mean (μ), the effect of gender (g_i), the correct error term for testing the gender main plot effect ($(gr)_{ik}$), the effect of ageing period (a_j), the effect of the interaction ($(ga)_{ij}$) and the correct error term for testing the ageing period subplot effect and finally the interaction between the former and latter (ε_{ijk}).

Pre-analysis of the data included the visual assessment of panel consistency using the software program PanelCheck (Version 1.3.2, www.panelcheck.com). The residuals of the sensory, instrumental and chemical data were also checked for normal distribution using the Shapiro-Wilk test (Shapiro & Wilk, 1965). In the event that the p-value for the Shapiro-Wilk statistic was less than 0.05 the residuals were examined and values with residuals with an absolute value greater than 3 were removed.

Fisher's Least Significant Differences (LSD's) were calculated at a 5% significance level to elucidate treatment differences. A probability level of 5% was considered significant for all significance tests.

The closeness of the linear relationship between the measured variables was determined using Pearson's correlation coefficient (r) (Snedecor & Cochran, 1980). Discriminate analysis (DA) was performed to determine whether the sensory, instrumental and chemical variables allow discrimination between springbok meat from different genders, aged for different periods, and to visualize the observations on a 2-dimensional map that shows how separated the groups are. Discriminate analysis was performed using XLStat software (Version 2012, Addinsoft, New York, USA).

6.3 Results and Discussion

The DA plot of the observations (Fig. 6.1) indicates that while the gender-ageing period treatment groups were clustered to some extent the within-group variation was very high, resulting in little significant separation. In female springbok the one and three day aged samples differed significantly ($p \leq 0.05$) from the 28 day aged samples but in males the only separation was found between one and 28 days. This lack of segregation can also be seen in the ANOVA results, with few of the sensory and instrumental variables differing significantly between the different ageing periods (Tables 6.2 & 6.3).

The DA loadings plot (Fig. 6.2) shows a bilateral grouping of the variables explaining 57.2% of the variation on the horizontal plane, with the vast majority of both the texture and aroma/flavour attributes being clustered in the top-left quadrant. Overall aroma intensity, sour aroma and flavour, metallic aroma and flavour, liver-like aroma and flavour, gamey aroma and flavour and off/manure aroma and flavour were clustered and associated with eight and 28 day aged samples. In contrast, beef-like aroma and flavour associated with one and three day aged samples. This demonstrated an increase in undesirable and a decrease in desirable aroma and flavour attributes with ageing, which is in agreement with findings in literature (Spanier, Vercellotti, & James, 1992; Brewer, 2006; Maughan, Tansawat, Cornforth, Ward, & Martini, 2012). Sustained juiciness, purge loss and tenderness also associated with the more aged samples; whereas residue, mealiness, cooking loss, average MFL and WBSF associated with the one and three day aged samples.

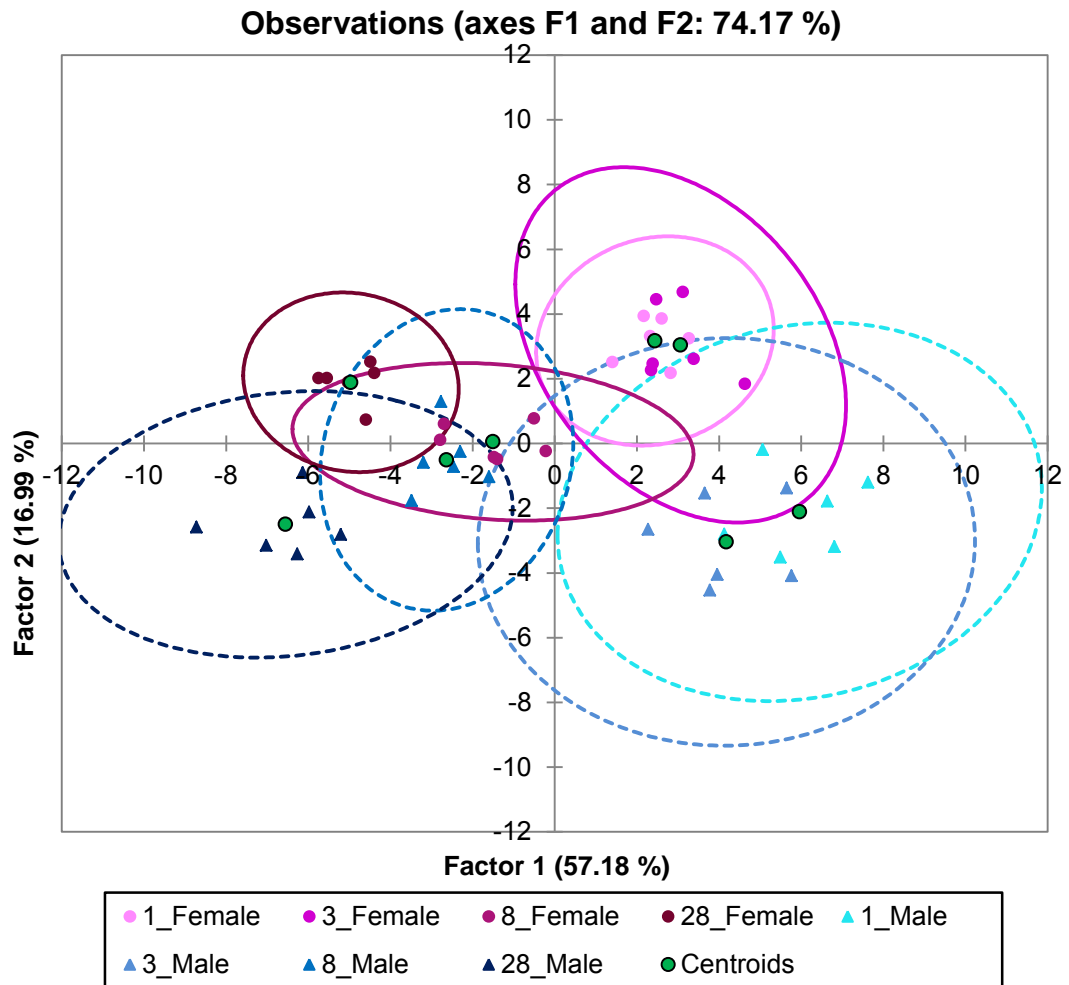


Figure 6.1 DA plot illustrating the classification of the gender-ageing treatment groups based on both sensory and instrumental variables.

Ellipses indicate the 95% confidence intervals of the treatments.

The separation between the genders appeared to decrease with increased ageing; which is in agreement with reports in literature of the levelling effect of ageing on meat quality (Monsón, Sañudo, & Sierra, 2005). However, even at one day PM the confidence ellipses of the genders overlapped, indicating a lack of gender effect. The results of the ANOVA indicate that significant gender effects were only present for metallic aroma, residue (Table 6.2) and cooking loss (Table 6.3).

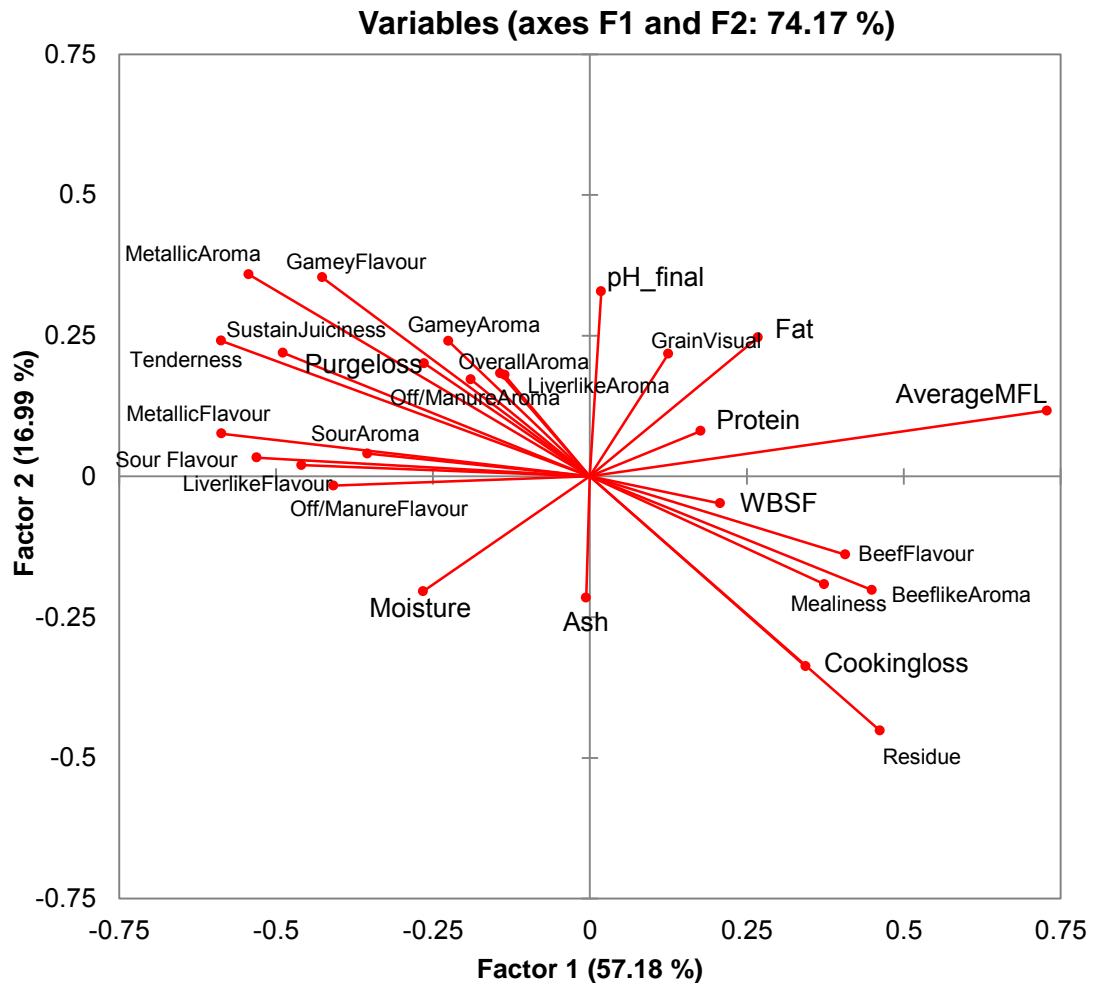


Figure 6.2 DA loadings plot of the sensory and instrumental variables indicating the correlation between the variables.

6.3.1 Flavour and aroma attributes

Gamey aroma was the most important contributor to the overall aroma intensity of springbok meat at all ageing periods (Table 6.2). This is indicated by not only the high absolute intensity of gamey aroma (58.05) relative to that of the overall aroma intensity (67.05) and beef-like aroma (35.70) but also the strong correlation found between the overall aroma and gamey aroma ($r = 0.634$, $p < 0.0001$). However no significant change in either the overall aroma intensity or the gamey aroma intensity with ageing was found.

A similarly high intensity was found for gamey flavour in all the springbok samples. This was expected and supports previous findings of a positive correlation between the intensity of gamey attributes and the polyunsaturated fatty acid (PUFA) content of the meat (Geldenhuys, Hoffman & Muller, 2014). Springbok meat has been found in previous studies to have a relatively high PUFA content (Hoffman, Kroucamp & Manley, 2007b). This is most

likely partially as a result of the low fat content of the meat, as the neutral storage lipids tend to have a diluting effect on the predominantly unsaturated structural phospholipids (Lawrie & Ledward, 2006). In addition, the forage-based nature of their diet may also be partly responsible. Pasture feeding has been reported to increase the PUFA content of meat in a variety of species such as red deer (Wiklund, Manley, Littlejohn & Stevenson-Barry, 2003), sheep (Fisher *et al.*, 2000) and cattle (French *et al.*, 2000), and the intensity of gamey flavour in beef has been found to be reduced by grain feeding (Maruri & Larick, 1992; Brewer, 2006). Alternatively, the presence of gamey attributes in meat has been linked to the diterpenoid content (Brewer, 2006), which is most likely also diet-related as higher concentrations of diterpenoids in grass- rather than grain-fed cattle have been reported (Maruri & Larick, 1992; Watanabe, Ueda, Higuchi, & Shiba, 2008).

Gamey flavour increased significantly during ageing, with the intensity of one and three day samples being significantly lower than that of 28 day samples ($p = 0.040$). This suggests that the link between PUFA and gamey flavour is a result of the greater susceptibility of polyunsaturated than saturated fatty acids to oxidation, resulting in an increased concentration of lipid oxidation products in fresh meat with a high PUFA content as well as an accumulation of these compounds in meat during ageing.

Gamey flavour has been associated with metallic and liver-like attributes in previous studies (Maughan *et al.*, 2012; Geldenhuys *et al.*, 2014). This is in agreement with the significant correlations between metallic aroma and gamey aroma ($r = 0.593$, $p < 0.0001$) and metallic flavour and gamey flavour ($r = 0.704$, $p < 0.0001$) found in this study. A moderate correlation between liver-like flavour and gamey flavour was also found ($r = 0.505$, $p < 0.001$). These correlations suggest that a similar underlying process may be responsible for the development of all these attributes during ageing.

The intensity of metallic and liver-like attributes in meat has been correlated with the free iron, haem-iron and myoglobin content (Stetzer, Cadwallader, Singh, Mckeith & Brewer, 2008; Geldenhuys *et al.*, 2014). Considering the relatively high iron content of springbok meat (Hoffman *et al.*, 2007a), it was expected that the intensity of these attributes would be higher than normally found for beef. The intensity of liver-like aroma and flavour in all the springbok samples was however low relative to those reported by Campo, Sañudo, Panea, Alberti and Santolaria (1999) for beef (Table 6.2). Intensity ratings for metallic aroma and flavour were considerably higher than those for liver-like attributes but were still lower than those reported for beef (Rødbotten, Kubberød, Lea & Ueland, 2004). However, it must be noted that the comparison of sensory ratings obtained during different studies is problematic considering the variation in both the preparation methods used and the scale used for assessment (Rødbotten *et al.*, 2004).

Both metallic aroma and flavour ($p = 0.004$ and $p = 0.003$ respectively) and liver-like flavour ($p < 0.001$) increased during ageing (Table 6.2). This is in agreement with studies performed on aged beef (Campo *et al.*, 1999; Brewer, 2006; Yancey *et al.*, 2006; Stetzer *et al.*, 2008).

Apart from the association with the free-iron, haem-iron and myoglobin content, various volatile compounds have also been suggested to play a part in the development of these attributes. Ethanal and 2-propanone have been linked to both liver-like and bloody attributes, while 2-pentyl furan has been correlated with metallic attributes (Brewer, 2006; Stetzer *et al.*, 2008). Additional volatile compounds that have been related to liver-like flavour and/or aroma are pentanal, hexanal, 3-hydroxy-2-butanone and hexanoic acid among others (Stetzer *et al.*, 2008). It has also been suggested that the interaction between sulphur-containing compounds and carbonyls produces liver-like aromas and flavours (Brewer, 2006).

Three possible causes of the observed increase in the intensity of gamey, liver-like and metallic attributes with ageing can therefore be identified:

Lipid oxidation during chilled storage could cause an accumulation of oxidation products which would subsequently result in an increase in the intensity of liver-like and metallic attributes. While this hypothesis seems likely considering the high PUFA content of game meat the lack of a correlation between liver-like aroma/flavour and TBARS values found by Yancey *et al.* (2006), as well as the lack of a significant change in the TBARS value during ageing found by Spanier, Flores, McMillin and Bidner (1997) cast doubt on this theory.

The second possibility is that protein degradation during conditioning results in the production of sulphur-containing compounds such as thiols, sulphides, thiazoles and sulphur-substituted furans from cysteine, methionine and other organic compounds. These compounds have been found to produce liver-like aromas and flavours through reactions with carbonyl-containing compounds (Brewer, 2006; Yancey *et al.*, 2006).

It is also possible that proteolysis and denaturation during ageing and cooking resulted in an increased release of iron from myoglobin and haem-complexes, increasing the concentration of free iron in the meat. Considering the link found between free-iron concentration and metallic and liver-like attributes (Geldenhuys *et al.*, 2014) this could directly increase the intensity of metallic attributes as well as promoting oxidation and thus the development of other undesirable aromas and flavours (Yancey *et al.*, 2006; Geldenhuys *et al.*, 2014).

The significantly higher metallic aroma found in female springbok ($p = 0.005$) could be as a result of differences in the fatty acid composition and/or fat content between the

genders and thus the concentration of volatile flavour precursors. The total fat content has been reported to be higher, and the MUFA content lower, in meat from female springbok (Hoffman *et al.*, 2007a; Hoffman, Kroucamp & Manley, 2007c). A similar trend in the IMF content was found in this study, although the difference was not significant ($p = 0.484$).

The sour/aged aroma and flavour followed a similar trend during ageing as the other undesirable attributes, with 28 day samples having significantly higher intensities ($p_{\text{aroma}} = 0.004$ and $p_{\text{flavour}} = 0.003$ respectively). Similar results were reported by Campo *et al.* (1999), Stetzer *et al.* (2008) and Spanier *et al.* (1997). Both amino acids and organic acids are thought to contribute a sour or acidic flavour to meat, with aspartic acid (and its amide asparagine) and histidine, and succinic, lactic, inosinic, ortho-phosphoric, pyrrolidone and carboxylic acid thought to be the most important amino acids and organic acids respectively (Brewer, 2006; Koutsidis *et al.*, 2008). The concentration of free amino acids is increased by PM degradation and calpain I and II activity has been found to correlate with increases in the intensity of sour attributes (Brewer, 2006). However, considering that samples were wet-aged (aged in moisture- and gas-impermeable vacuum-bags), it seems more likely that the sour flavour developed as a result of the production of lactic acid in the meat by lactic acid bacteria. Lactic acid bacteria have been found to be favoured under anaerobic vacuum-packaged conditions (Li, Babol, Wallby, & Lundström, 2013). Nevertheless it must be noted that even in the 28 day samples sour flavour was only rated an intensity of 6.7 on a 100 point scale.

An increase in off/manure flavour during ageing was found, with day 28 having significantly higher intensities than the other ageing periods ($p < 0.001$). However, a maximum rating of only 2.8 was found. This low average rating is more a reflection of the intermittence of the occurrence of off flavours than an indication of its intensity when it was detected, as the taint was extremely strong in some blocks, as indicated by the high maximum value (90) and standard error of the mean. During both the training and testing phases it was found that occasional samples, even occasional blocks within samples, were strongly tainted with an odour and flavour reminiscent of manure or contamination. The lack of consistency within samples makes explaining this taint difficult. It is possible, if unlikely, that the affected blocks had somehow come into contact with rumen fluid during slaughter. An alternative explanation is that the rate of lipid and protein oxidation within the muscle varied, resulting in only isolated sections having the taint. This is supported by the findings of Spanier *et al.* (1992), who reported that micro-flavour environments exist in meat due to the complex organisation of meat structure resulting in variable rates of heat and oxygen penetration during cooking and storage.

Table 6.2

The effect of conditioning and gender on the sensory attributes of springbok *Longissimus thoracis et lumborum* muscle (LSMeans \pm SEM*)

	Ageing period (days post-mortem)				Gender	
	1	3	8	28	Male	Female
Overall aroma intensity	65.9 \pm 1.01	67.7 \pm 0.94	66.2 \pm 1.03	68.5 \pm 1.08	66.7 \pm 0.640	67.4 \pm 0.815
Gamey aroma	56.8 \pm 1.14	57.7 \pm 0.90	58.8 \pm 1.21	58.9 \pm 0.94	57.5 \pm 0.82	58.6 \pm 0.66
Liver-like aroma	2.6 \pm 0.55	1.9 \pm 0.42	2.7 \pm 0.28	2.4 \pm 0.52	2.1 \pm 0.30	2.7 \pm 0.34
Metallic aroma	7.7 ^{bc} \pm 0.98	6.1 ^c \pm 0.50	10.1 ^{ab} \pm 1.05	11.3 ^a \pm 1.12	7.7 ^b \pm 0.89	9.9 ^a \pm 0.62
Sour/Aged aroma	0.2 ^b \pm 0.13	0.7 ^b \pm 0.26	0.4 ^b \pm 0.16	1.4 ^a \pm 0.35	0.5 \pm 0.19	0.8 \pm 0.19
Off/Manure aroma	0 \pm 0	0.3 \pm 0.20	0 \pm 0	0.5 \pm 0.26	0.1 \pm 0.10	0.3 \pm 0.13
Gamey flavour	60.1 ^b \pm 0.96	61.1 ^b \pm 1.44	61.7 ^{ab} \pm 1.18	64.6 ^a \pm 0.97	60.9 \pm 0.93	62.9 \pm 0.75
Beef flavour	39.8 \pm 0.87	40.1 \pm 1.18	38.6 \pm 0.81	36.6 \pm 1.16	39.2 \pm 0.89	38.4 \pm 0.60
Liver-like flavour	1.5 ^b \pm 0.46	1.2 ^b \pm 0.30	1.5 ^b \pm 0.26	3.6 ^a \pm 0.55	1.8 \pm 0.30	2.1 \pm 0.39
Metallic flavour	7.6 ^{bc} \pm 0.79	6.6 ^c \pm 0.91	10.8 ^{ab} \pm 1.39	13.0 ^a \pm 1.30	9.1 \pm 1.15	9.9 \pm 0.66
Sour/aged flavour	2.9 ^b \pm 0.66	3.3 ^b \pm 0.60	4.6 ^b \pm 0.79	6.7 ^a \pm 0.72	4.3 \pm 0.57	4.4 \pm 0.57
Off/Manure flavour	0.8 ^b \pm 0.39	0.9 ^b \pm 0.36	0.5 ^b \pm 0.32	2.8 ^a \pm 0.53	1.3 \pm 0.33	1.2 \pm 0.35
Sustained juiciness	60.9 ^{bc} \pm 2.36	58.6 ^c \pm 2.09	66.1 ^{ab} \pm 2.43	68.0 ^a \pm 1.99	61.6 \pm 1.93	65.2 \pm 1.41
Tenderness	69.4 ^b \pm 1.77	69.1 ^b \pm 1.57	74.3 ^a \pm 1.24	77.3 ^a \pm 1.75	70.8 \pm 1.42	74.2 \pm 1.10
Residue	2.0 ^a \pm 0.55	2.3 ^a \pm 0.62	0.99 ^b \pm 0.31	0.6 ^b \pm 0.39	2.3 ^a \pm 0.41	0.7 ^b \pm 0.20
Mealiness	11.4 \pm 1.20	13.4 \pm 1.18	9.8 \pm 1.32	9.6 \pm 1.13	11.7 \pm 0.94	10.4 \pm 0.84
Visual Grain	18.3 \pm 1.07	16.8 \pm 1.11	15.9 \pm 0.59	17.1 \pm 1.00	16.4 \pm 0.55	17.7 \pm 0.78

^{a, b, c} Least square means in the same row (within main effect) with different superscripts differ significantly from each other ($p < 0.05$).

*SEM: Standard error of the mean.

The only aroma attribute associated with the day one and three samples was beef-like aroma, with significant gender by ageing-period interaction being present for this attribute ($p = 0.004$). Beef-like aroma was the second highest contributor to the overall aroma, and has

been linked to compounds such as the beefy-meaty peptide (BMP - Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala), which occurs naturally in meat (Brewer, 2006). The reaction of cysteine and/or ribose with phospholipids is also reported to produce compounds with slight beefy aromas (Brewer, 2006).

A steady decline in beef-like aroma intensity from one to 28 days was found in samples from male springbok (Fig. 6.3). This is in agreement with findings reported in literature (Spanier *et al.*, 1997; Monsón *et al.*, 2005; Stetzer *et al.*, 2008); however in beef an initial increase during ageing is generally expected (Monsón *et al.*, 2005). It is possible that an initial increase may be the result of the formation of BMP through protein degradation, while the subsequent decline is due to continued proteolysis of the peptide. This stage may not have been detected in springbok due to the more rapid ageing process found in this species (Chapter 5). Beef-like attributes have also been correlated with the concentration of inosine monophosphate (IMP) in the meat (Brewer, 2006). The IMP content of bison meat and beef has been found to decrease significantly with aging, which likely also contributed to the decline in beef-like aroma (Koutsidis *et al.*, 2008; Williamson, Ryland, Suh, & Aliani, 2014). Significant negative correlations found between beef-like attributes and the pentanal concentration in the meat has led to suggestions that the decline observed is a result of oxidation (Spanier *et al.*, 1992; Stetzer *et al.*, 2008). However it is not certain whether this is a direct effect of the oxidation of beef-like aroma/flavour volatiles or a masking effect by the lipid oxidation products. Negative correlations ($p < 0.05$) between beef-like attributes and off/manure ($r_{\text{aroma}} = -0.462$, $r_{\text{flavour}} = -0.496$), metallic ($r_{\text{aroma}} = -0.315$, $r_{\text{flavour}} = -0.607$) and gamey attributes ($r_{\text{aroma}} = -0.293$, $r_{\text{flavour}} = -0.499$) were found in this study.

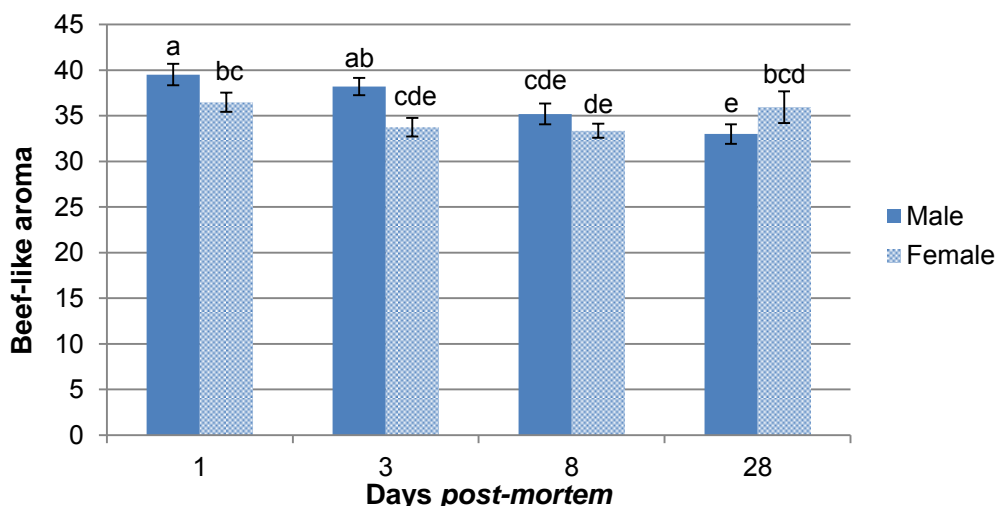


Figure 6.3 The change in beef-like aroma during ageing for meat from male and female springbok. Different letters indicate significant ($p < 0.05$) differences between the means of the treatments. Error bars indicate the standard error of the mean of each treatment.

The cause of the increase in beef-like aroma from eight to 28 days found in meat from female springbok is unknown. Heifers have been found to have stronger beef-like attributes than bulls; however differences during ageing have not been reported (Stetzer *et al.*, 2008). No significant change in beef flavour during ageing was found. This was unexpected considering the change in the beef-like aroma.

6.3.2 Textural attributes

Tenderness and sustained juiciness improved during ageing, as expected according to literature (Campo *et al.*, 1999; Laville *et al.*, 2009; Williamson *et al.*, 2014). However, while the tenderness of day one and three samples was significantly ($p = 0.001$) lower than that of day eight and 28, no significant increase in tenderness from eight to 28 days was found. This is in agreement with the results in Chapter 5, which indicated that no further ageing took place from five days onwards. A similar decline in the effect of ageing on tenderness has been reported for beef after 14 days of ageing (Monsón *et al.*, 2005).

However, contrary to expectations, no significant change in the Warner Bratzler shear force (WBSF) was found (Table 6.3). This can be seen in the DA loadings plot (Fig. 6.2) and is confirmed by the p-value generated during multivariate analysis ($p = 0.134$). While there did appear to be a trend for the shear force to decline during ageing the variation within each ageing group was such that the trend was not significant. The lack of a significant decline in the shear force also resulted in there only being a weak negative correlation between the shear force and the tenderness rating by the sensory panel ($r = -0.404$, $p = 0.004$). This is in contrast to the strong correlations found by Hoffman *et al.* (2007b) and Culler *et al.* (1978) ($r = -0.7$ and $r = -0.9$, respectively). The shear force was generally extremely low however, with meat from all four ageing periods falling in either the 'tender' category or very marginally above the 'tender' category as typically used to classify meat (Miller, Carr, Ramsey, Crockett, & Hoover, 2001; Sullivan & Calkins, 2011). The 'tender' category (WBSF < 22.2 N) was defined by Miller *et al.* (2001) as the WBSF threshold class for 100% consumer acceptability for tenderness, with the 'intermediate' category (22.2 N < WBSF < 34.0 N) having a 93% consumer acceptability.

Table 6.3

The effect of conditioning and gender on the physical and chemical attributes of springbok *Longissimus thoracis et lumborum* muscle (LSMeans \pm SEM*)

	Ageing period (days <i>post-mortem</i>)				Gender	
	1	3	8	28	Male	Female
Moisture (%)	65.0 \pm 0.63	64.8 \pm 0.83	66.2 \pm 0.47	66.2 \pm 0.63	65.8 \pm 0.39	65.3 \pm 0.54
Protein (%)	31.1 \pm 0.45	31.0 \pm 0.62	30.3 \pm 0.47	30.4 \pm 0.63	30.8 \pm 0.41	30.6 \pm 0.36
IM Fat (%)	3.1 ^{ab} \pm 0.30	3.2 ^a \pm 0.26	2.5 ^{bc} \pm 0.28	2.5 ^c \pm 0.31	2.6 \pm 0.20	3.0 \pm 0.21
Ash (%)	1.3 \pm 0.07	1.3 \pm 0.08	1.3 \pm 0.08	1.4 \pm 0.09	1.4 \pm 0.06	1.3 \pm 0.05
Cooking loss (%)	25.8 \pm 1.49	26.8 \pm 1.31	23.9 \pm 1.43	22.6 \pm 1.47	26.8 ^a \pm 0.93	22.7 ^b \pm 0.98
pH _u	5.49 \pm 0.019	5.50 \pm 0.016	5.50 \pm 0.016	5.49 \pm 0.020	5.47 \pm 0.0078	5.52 \pm 0.0145
WBSF (N)	23.26 \pm 1.056	22.16 \pm 0.787	21.45 \pm 1.026	20.15 \pm 1.030	21.20 \pm 1.075	22.31 \pm 0.877
MFL (μ m)	23.64 ^a \pm 0.905	22.29 ^a \pm 0.870	18.69 ^b \pm 0.721	16.33 ^c \pm 0.779	20.32 \pm 0.836	20.16 \pm 0.821

^{a, b, c} Least square means in the same row (within main effect) with different superscripts differ significantly from each other ($p < 0.05$).

*SEM : Standard error of the mean

It is notable that the WBSF recorded for even the one day aged samples in this trial were similar to the 14 day aged samples from Chapter 5. This discrepancy could be as a result of the different cooking methods used in the two studies, as positive correlations between shear force and degree of cooking have been reported in literature (Yancey, Wharton, & Apple, 2011). Alternatively, the increased tenderness and lack of variation in shear force between ageing periods may be as a result of the freezing process. Lagerstedt, Enfält, Johansson and Lundström (2008) found no difference in WBSF between two day aged beef that had been frozen and thawed and beef aged for seven days and not frozen. A decline in the effect of freezing on tenderness with increased ageing prior to freezing has also been found, which would decrease the perceived effect of ageing (Lagerstedt *et al.*, 2008). However, the limitations of the WBSF as a predictor of sensory tenderness must also be acknowledged (Shackelford, Wheeler, & Koochmarai, 1995; Lagerstedt *et al.*, 2008). It is also possible that the perceived improvement in the tenderness of eight and 28 day aged meat may have been as a result of the increased sustained juiciness reported (Table 6.2) for these samples (Lagerstedt *et al.*, 2008).

The increase in sustained juiciness from three to 28 days ($p = 0.023$) was also in agreement with findings in literature (Campo *et al.*, 1999; Williamson *et al.*, 2014). Hoffman *et al.* (2007b) suggests that this is caused by the loosening of the structure of the meat by proteolytic degradation, resulting in the more rapid release of moisture during mastication. The high correlation between tenderness and sustained juiciness found in this study ($r = 0.858$, $p < 0.001$) supports this hypothesis. However the increase in sustained juiciness may also be as a result of the non-significant ($p = 0.132$) but notable decline in cooking loss found with ageing.

In addition to tenderization and myofibrillar fragmentation, the proteolytic degradation during conditioning has been suggested to reduce the binding between the myofibrils and the sarcolemma by degrading linking structures known as costameres (Huff-Lonergan & Lonergan, 2005; Laville *et al.*, 2009). The contraction of the fibrous proteins of the myofibrils during cooking decreases the space between the myofilaments and forces water out (Tornberg, 2005). In fresh, un-aged meat the protein links between the myofibril and the sarcomere result in a contraction of the myofibrils causing the contraction of the whole cell and thus the movement of water into the extracellular space (Huff-Lonergan & Lonergan, 2005; Tornberg, 2005). Subsequent contraction of the collagen proteins of the endo- and perimysium with further heating forces this water out of the spaces between the sarcomere and endomysium and into channels between the fibres and fibre bundles that lead directly out of the muscle as a whole (Huff-Lonergan & Lonergan, 2005; Tornberg, 2005). However, in aged meat the degree of contraction of the muscle cell as a whole is reduced due to the

destruction of the costameres. This allows a larger proportion of the water to remain within the cell during initial heating and reduces the movement of the water out of the cell and out of the muscle, leading to lower cooking losses. This may have led to the perceived improvement in sustained juiciness in the aged meat. While no significant change in the moisture content of the meat during ageing was found (Table 6.3), there was a tendency for it to increase with ageing, supporting this hypothesis. The lack of significance in the effect of ageing on cooking losses may be as a result of the lack of repeatability of this parameter, as reported by Yancey *et al.* (2011).

Meat with higher levels of sustained juiciness was also found to have higher metallic and gamey aromas and flavours and lower beef-like attributes. This is seen in the very high correlation of sustained juiciness with metallic flavour ($r = 0.817$, $p < 0.001$) as well as high correlations with gamey flavour ($r = 0.672$, $p < 0.001$) and metallic aroma ($r = 0.738$, $p < 0.001$). While various oxidative and proteolytic processes most likely influenced the nature of the volatile compounds available in the aged meat it is possible that the link between sustained juiciness and flavour and aroma is more than a coincidence. The fluid lost during cooking contains soluble proteins and melted fatty acids (Lawrie & Ledward, 2006; Oillic, Lemoine, Gros, & Kondjoyan, 2011), and a decline in the loss of these compounds from the meat will increase their concentration and thus the intensity of the attributes with which they are associated. The significant negative correlations found between the cooking loss and these attributes (metallic aroma: $r = -0.627$, $p < 0.001$; metallic flavour: $r = -0.712$, $p < 0.001$; gamey flavour: $r = -0.512$, $p < 0.001$) also supports this observation. This could also provide an alternative explanation for the significantly higher metallic flavour found in female springbok, despite the lack of significance of the higher sustained juiciness found in females.

Significant gender by ageing period interaction was found for the cumulative purge loss ($p = 0.047$). As can be seen in Figure 6.4 an increase in cumulative purge loss with ageing was found for male but not female springbok. The results for male springbok were in agreement with the results in Chapter 5 and the general consensus found in literature (Hodges, Cahill, & Ockerman, 1974). In contrast, no significant change in purge loss was found in female animals. Assuming that the trend for ageing loss was as normal, this indicated that the losses due to freeze-thaw were greater for female animals and resulted in the masking of the effect of ageing on the cumulative purge loss. This is supported by the results of Ahnström, Hunt and Lundström (2012), who found that freeze-thaw losses tended to be higher for cows and heifers than for bulls. It may also indicate that, in female springbok at least, the moisture loss due to freeze-thaw decreased with ageing. This is supported by the finding that the effect of freezing decreases as the period of ageing prior to freezing increases (Lagerstedt *et al.*, 2008).

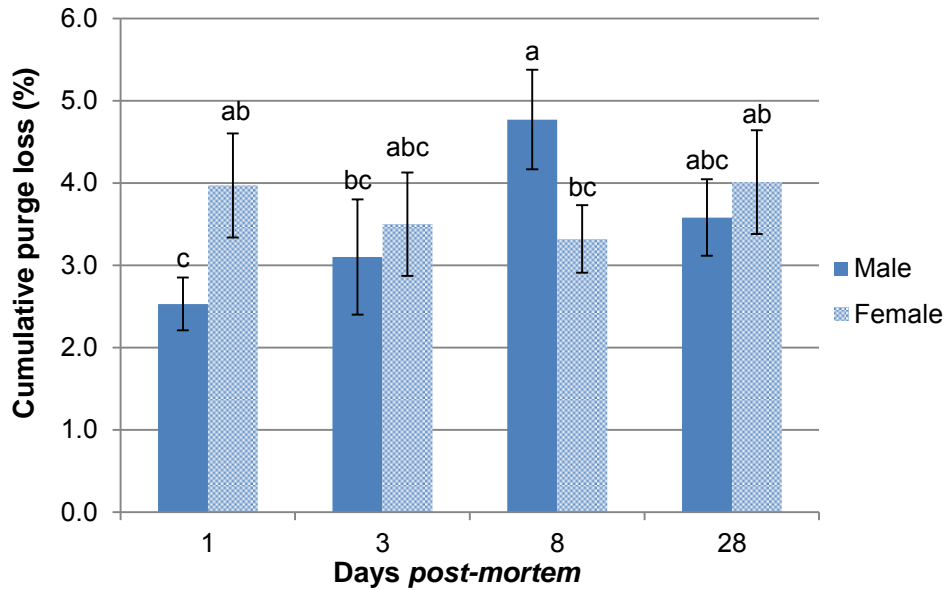


Figure 6.4 The change in cumulative purge loss during ageing for meat from male and female springbok. Different letters indicate significant ($p < 0.05$) differences between the means of the treatments. Error bars indicate the SEM of each treatment.

The lack of significant change in cooking loss during ageing is in contrast with the findings in Chapter 5 and may be as a result of the differences in how the meat was handled in the two studies. In this study the samples were frozen prior to cooking and were cooked to a lesser extent than in the previous trial. It has been reported that the freeze-thaw process can influence not only purge losses but also cooking losses (Lagerstedt *et al.*, 2008), and that the degree and method of cooking can have a significant effect on cooking losses (Lagerstedt *et al.*, 2008; Yancey *et al.*, 2011). A strong negative correlation between cooking loss and sustained juiciness was found ($r = -0.785$, $p < 0.001$). This was as expected as an increase in the cooking loss would suggest a dryer cooked product. A similar correlation was found by Toscas, Shaw and Beilken (1999) but not by Hoffman, Kroucamp and Manley (2007b).

The cooking loss (Table 6.3) was significantly higher for male than female springbok ($p = 0.016$). This may be as a result of the marginally higher purge loss found for female than male springbok (3.70% and 3.49%, respectively), with more water remaining available to be lost during cooking in males. However, the difference in ageing/freeze-thaw loss does not fully explain the magnitude of the difference found in the cooking loss. An alternative explanation for the gender differences in both the cumulative purge loss and the cooking loss is that the distribution of water in the meat differs between the genders. The water content of meat is found in three locations or states, namely free water, entrapped or immobilised water and bound water (Huff-Lonergan & Lonergan, 2005). Varying proportions

of water in each state could result in variable patterns of moisture loss while not necessarily influencing the final moisture content of the meat.

The effect of ageing on purge loss and cooking loss may also be confounded by the variation in the length of frozen storage between the ageing periods. The samples aged for the shortest periods were frozen for the longest time, and vice versa. While it is thought that the process of freezing itself has a larger influence on meat than the length of time for which the meat is frozen, it is possible that this may have had some influence (Vieira, Diaz, Martínez, & García-Cachán, 2009).

The abundance of residue decreased during ageing, which is in agreement with the increase in tenderness found. It is also in agreement with findings in literature (Campo *et al.*, 1999; Monsón *et al.*, 2005). Residue was rated as significantly more abundant in day one and three samples than in day eight and 28 samples ($p < 0.001$), with no significant differences present within these two subgroups (Table 6.2). The decline in residue is not entirely as expected, as it is generally considered to be more a reflection of the connective tissue content than of other contributors to toughness (Culler *et al.*, 1978; Lawrie & Ledward, 2006). However, as collagen has generally been found not to degrade during ageing (Lawrie & Ledward, 2006) the decline in the abundance of residue suggests that the myofibrillar fraction also played a role, as suggested by Campo *et al.*, (1999). This is supported to some extent by the correlation found between the abundance of residue and the live weight ($r = 0.449$, $p = 0.001$), which while present and significant is only moderate. The correlation between residue and overall tenderness is however negative and strongly significant, as expected ($r = -0.724$, $p < 0.001$).

A significantly ($p = 0.042$) higher abundance of residue was found in male than female springbok (Table 6.2). This may be related to the maturity of the animals as the male springbok used in this study had significantly higher live weights than the females (male: 32.1 ± 0.53 kg; female: 24.2 ± 1.16 kg; $p < 0.001$). While a direct gender effect on live mass has been previously reported for springbok (Kroucamp, 2004), it is possible that the males harvested were more mature than the females. The link between collagen solubility and animal age has been well established (Lawrie & Ledward, 2006), and may have contributed to the significant difference found in the abundance of residue. It is also likely that the lower tenderness found for male springbok, although non-significant, is linked to the higher residue rating for meat from male springbok. It must nevertheless be noted that despite the significant difference, the abundance of residue in males was still only rated as 2.3 on a 100 point scale, which is extremely low (Table 6.2).

A significant decrease in the average MFL with ageing was found ($p < 0.001$), indicating that myofibrillar degradation did take place (Table 6.3). Correlations of the MFL

with WBSF ($r = 0.288$, $p = 0.047$) and tenderness ($r = -0.291$, $p = 0.044$) were weaker than expected.

Contrary to expectations the abundance of mealiness was found to decrease with ageing, although this decline was non-significant ($p = 0.117$). It was thought that the decrease in MFL with ageing would result in increased mealiness as a result of the finer particle size of the aged meat. However, considering the significant increase in sustained juiciness with ageing it seems likely that this masked any effect of myofibrillar degradation. This is supported by the strong, significant and negative correlation found between the sustained juiciness and the abundance of mealiness ($r = -0.745$, $p < 0.001$).

6.3.3 Chemical composition

The effect of ageing on the proximate chemical composition of the meat was very limited (Table 6.3), with the only significant variation being found for the fat content of the cooked meat ($p = 0.024$). This was unexpected as portions were assigned to each ageing period from each animal and the position of the portion in the muscle (left or right, cranial or caudal) was randomised.

As the fat content was determined on the cooked and aged rather than fresh meat there are three potential sources for the observed decline with ageing.

It is possible that despite their random selection the distribution of portions from the cranial and caudal sections of the LTL were skewed for the different ageing periods. The intramuscular fat content has been found to vary within the LTL muscle (in pigs; Faucitano, Rivest, Daigle, Lévesque, & Gariépy, 2004), so an uneven division of portions may be responsible for the apparent effect of ageing on the fat content. However, it seems unlikely that this would result in the trend as observed. Also, inspection of the record of muscle randomisation seems to indicate that the first two ageing periods received a greater proportion of the caudal portions (primarily from the lumbar region) of the LTL. This contradicts the findings that the portions from one and three days of ageing had higher fat contents, as Faucitano *et al.* (2004) found that the fat content of the LTL was generally higher in the thoracic than lumbar regions.

The second possibility is that the change in the intramuscular fat (IMF) content is in fact as a result of the ageing process. As it seems unlikely that the intramuscular fat could be lost as drip during ageing this implies a change in the chemical nature of the lipids which resulted in decreased extractability. Unfortunately no other studies in which the intramuscular fat content as a whole was tested before and after ageing could be found. To the knowledge of this author a link between lipid oxidation and lipid extractability by

chloroform-methanol has also not been investigated thus far. This is therefore a matter requiring further research.

There could also be an indirect effect of ageing on the IMF content of the cooked meat. As the physical structure of the meat is degraded PM the restriction on the movement of molten fat out of the meat during cooking could be reduced. This would result in more fat being lost from the meat during cooking, and thus a lower fat content in the cooked meat after ageing. This seems to be the most viable hypothesis; even though the cooking loss data shows a non-significant decline with ageing. Nevertheless it is possible that the increased loss of fat during cooking was simply so small that it was masked by the effect of the water-content of the cooking losses (Lawrie & Ledward, 2006).

6.5 Conclusion

Ageing had a beneficial effect on the textural quality of springbok meat with increases in tenderness and juiciness and decreases in residue and mealiness. However, these changes were minor, with less than ten-point increases in both tenderness and juiciness being found. Textural improvements were also not significant when assessed using the Warner Bratzler shear force, and all the samples were found to be extremely tender when compared to categories developed for the assessment of beef.

In contrast, significant detrimental changes in flavour and aroma attributes with aging were found, with the favourable beef-like attributes declining and the occurrence of liver-like, metallic, sour and off/manure taints increasing.

Taking into consideration all the results as discussed it is recommended that springbok meat is not aged for more than eight days *post-mortem*, as no further improvements in texture were found from eight to 28 days.

6.6 References

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CHAPTER 7

7.1 General discussion and recommendations

The South African game industry is the sixth largest in the agricultural sector and has grown an average of 20.3% per annum over the past 15 years (Thomas, 2012). The production and sale of meat forms one of the many sources of income for game farmers, with springbok (*Antidorcas marsupialis*) being the most important game species for meat production (Hoffman & Wiklund, 2006; Hoffman, 2007). The development of the game meat market could potentially increase the contribution of this source of income to farmers as well as contributing to the tourism industry and possibly the nation's food security. However, in order for a stable and sustainable game meat industry to be developed farmers and meat processors need to be able to ensure that the products produced are of a high quality and that this quality is consistent (Hutchison, Mulley, Wiklund & Flesch, 2010). This can only be achieved through the optimisation and standardisation of the carcass and meat-handling protocols used, which is currently hampered by a lack of research into the nature of game meat. Tenderness is one of the most important facets of meat quality for the consumer as well as being one of the most variable attributes of meat (Bailey, 1972; King, Wheeler, Shackelford & Koohmaraie, 2009). It was therefore the goal of this study to add to the existing literature on the tenderness of springbok meat as well as examining the various factors that influence it.

The first part of this study (Chapter 3) determined the fibre-type composition of springbok muscle. This was important as many of the changes that take place during rigor and conditioning have been linked to the contractile and metabolic properties of the muscle (Klont, Brocks & Eikelenboom, 1998; Lefaucheur, 2010). Springbok muscle was found to consist of primarily (64 - 78%) type IIX fibres, which is in agreement with the sprinting ability of the species and suggests a muscle dominated by glycolytic metabolic mechanisms (Curry, Hohl, Noakes & Kohn, 2012). However, other aspects of springbok meat, such as its high iron content and highly active oxidative pathways, indicate that the relationship between the contractile (myosin heavy chain isoform type) and metabolic nature of springbok meat may differ from that of cattle, pigs or humans (Hoffman, Kroucamp, & Manley, 2007; Curry *et al.*, 2012).

Although the *Biceps femoris* (BF) muscle contained significantly more type I and fewer type IIA fibres than the *Longissimus thoracis et lumborum* (LTL) muscle in male springbok, the effect of this on the quality of the meat may be negligible as both these fibre-types are considered as oxidative (Lefaucheur, 2010; Curry *et al.*, 2012). While statistical significance could not be determined, female springbok appeared to contain a greater proportion of type

IIX fibres than males. This is in agreement with findings in sheep (Greenwood, Harden & Hopkins, 2007) and suggests that male springbok may be more prone to producing DFD (dark, firm and dry) meat than females (Dingboom & Weijs, 2004).

An increase in the cross-sectional area (CSA) with the glycolytic capacity of the fibre-type (I < IIA < IIX < IIX) was found in males but not in females. Such an increase is in agreement with findings in literature (Dingboom & Weijs, 2004; Choi & Kim, 2009; Kohn, Burroughs, Hartman & Noakes, 2011). The CSA values of springbok muscle (1367 - 2777 μm^2) were similar to the range reported for domesticated species (cattle LTL: 2092 - 2481 μm^2 ; Vestergaard, Oksbjerg, & Henckel, 2000), and it thus seems likely that the effect on the sensory quality of the meat will be negligible when comparing springbok meat to that from domesticated species.

The change in the muscle during rigor has a large effect on the nature of the fresh meat as well as the tenderization of the meat during ageing (Warriss, 2000; Hwang & Thompson, 2001). It in turn is influenced by not only the fibre-type composition of the muscle but also the bulk and fat cover of the carcass and the cooling regime used (Warriss, 2000). It was therefore necessary to quantify the changes in springbok meat during rigor in order to make recommendations for the handling of springbok carcasses during this period.

Springbok LTL and BF muscles cooled relatively rapidly, reaching ambient temperature at 8 - 12 hours *post-mortem* (PM). This was most likely as a result of the small size of the carcasses and the limited subcutaneous fat layer (Smith, Dutson, Hostetler, & Carpenter, 1976; Hoffman & Laubscher, 2009; Kim *et al.*, 2014). The rapid cooling resulted in the pH declining relatively slowly and pH/temperature set-points as recommended for domestic species (e.g. 21°C at pH 6) not being achieved. The cooling was most rapid and acidification slowest in the female LTL, most likely due to the lack of bulk of this muscle. Both the female LTL and BF appeared to be at risk of cold-shortening. This suggests that springbok meat may benefit from delayed chilling, alternative hanging methods or electrical stimulation.

Cathepsin activity tended to increase during rigor (most likely due to the degradation of the lysosomes: Thomas, Gondoza, Hoffman, Oosthuizen, & Naudé, 2004), with higher activity levels being found in females than males and in the LTL than the BF. This may help compensate for the more rapid chilling of these muscles if the meat is aged. However, it is not conclusive as to whether the cathepsins were active *in situ* during the rigor period. In contrast, calpain and calpastatin activity declined PM, indicating that they were activated *in situ*. The differences in the patterns of decline found for calpain I and calpain II support the current theory that calpain I is activated earlier PM than calpain II (Dransfield, 1994; Huff Lonergan, Zhang, & Lonergan, 2010). Significant negative correlations between the rate of

decline of the pH and the change in the calpastatin activity appeared to indicate that calpains and other enzymes capable of degrading calpastatin were activated to a greater extent in rapidly glycolysing muscles. The higher levels of calpain and calpastatin activity found in male springbok and the BF are in agreement with their apparently more oxidative nature (Ouali & Talmant, 1990; Klont *et al.*, 1998).

One of the oldest and most common methods used for the improvement of meat tenderness is ageing or conditioning (Dransfield, 1994). The tenderization observed during conditioning primarily takes place due to the action of intrinsic proteolytic enzymes (Klont *et al.*, 1998; Nowak, 2011), with the calpains and the cathepsins being two of the most extensively investigated (Warriss, 2000).

The instrumental assessment of the changes in springbok meat during ageing indicated that a minimum Warner Bratzler shear force (WBSF) of 27 N was obtained by five days PM (Chapter 5). A decrease in calpain I and II and calpastatin activity from one to five days PM was also found, which indicates that the enzymes were active *in situ* and suggests that they were responsible for the observed tenderization. While the decline in calpain I activity was in agreement with findings for beef, the concomitant decline in calpain II activity was unexpected as it has previously been found to be more stable than calpain I PM (Dransfield, 1993; Pomponio & Ertbjerg, 2012). This suggests that calpain II may have also contributed to tenderization in springbok muscle, which may explain the more rapid tenderization than is found in beef. Cathepsin activity increased during ageing, even past the point of no further improvements in WBSF. This suggests that the cathepsins did not contribute to tenderization to any great extent. The BF muscle had higher WBSF values than the LTL throughout the ageing period despite having higher calpain and calpastatin activity and a greater decline in shear force during ageing. This suggests that the greater toughness of the BF may be due to the effects of connective tissue, despite the lack of a significant difference between the muscles in the collagen content.

While instrumental methods are useful and convenient, the use of a trained sensory panel and descriptive sensory analysis is still the most appropriate method of assessing meat quality. The effect of ageing springbok LTL for four different periods was thus assessed using a sensory panel in conjunction with instrumental methods (Chapter 6). The sensory assessment of texture found that meat aged for eight days was significantly more tender and juicy than that aged for one or three days, but that there was no further textural improvement after ageing for 28 days. In contrast, a number of undesirable attributes, such as gamey, metallic, liver-like or sour aromas and/or flavours, tended to increase with ageing, particularly from eight to 28 days. Beef-like attributes on the other hand tended to decrease with ageing. These changes in flavour and aroma were most likely as a result of proteolytic changes to

the pool of volatile compounds available for flavour formation as well as the production of lactic acid by lactic acid bacteria (Brewer, 2006; Yancey *et al.*, 2006; Koutsidis *et al.*, 2008; Stetzer, Cadwallader, Singh, Mckeith & Brewer, 2008; Li, Babol, Wallby & Lundström, 2013). These results indicate that springbok meat from the LTL should be aged for a maximum of eight days in order to avoid detrimental changes in flavour and aroma.

It was interesting to note that despite the significant increase in the tenderness rating with ageing no correlating decline in the WBSF was found. In addition, the day one values recorded for the LTL in Chapter 6 (23.26 N) were considerably lower than those found in Chapter 5 (36.39 N). This discrepancy was ascribed to the effect of freezing (Lagerstedt, Enfält, Johansson & Lundström, 2008), as the samples used in Chapter 6 were frozen prior to assessment whereas those used in Chapter 5 were not. Further studies on the effect of freezing on springbok meat should be done, as the shear force values found in this study suggest that springbok meat destined for freezing may not require any ageing at all.

Additional research into the relationship between the metabolic and contractile nature of muscle fibres in different species is also required, as it appears that the extrapolation of the findings for other species may not be appropriate (Curry *et al.*, 2012). Much of the effect of fibre-type on meat quality is as a result of its influence on the oxidative and glycolytic capacity of the muscle. In the event that the relationship between the myosin heavy chain isoform and the metabolic nature of the fibre differs between species the use of immunohistochemical methods of fibre-typing when wishing to relate the fibre characteristics of a muscle to meat quality may not be ideal.

In addition, the discrepancy between the low shear force values found for springbok (Chapter 5 and 6) and the apparent likelihood of cold-shortening (Chapter 4) should be looked into. A study focussing on the changes in sarcomere length and the muscle contraction during rigor may be valuable to determine whether cold-shortening is actually taking place. It is possible that the pH and temperature thresholds for cold-shortening in game meat may differ from those found for domestic species as a result of the high proportions of type IIX fibres found in the muscle. Fast-twitch fibres have been reported to contract less during rigor than slow-twitch, oxidative fibres (Huff Lonergan *et al.*, 2010; Kim, Warner & Rosenvold, 2014).

While the numbers of springbok used in this study were acceptable by statistical standards a more extensive project involving the monitoring of shear force values for springbok harvested commercially may be valuable. In comparison to domestic species there is high level of genetic variation as well as variation in the rearing environment and harvesting method in game species (Van Aswegen, Labuschagne & Grobler, 2012). This increases the risk that a study performed using a single method of harvesting and a single

population of springbok may not be fully representative of the meat actually being made available for commercial sale.

The results of this study suggest that springbok meat is tender relative to beef and requires little ageing, with between five and eight days being recommended. They also indicate that prolonged ageing may have a detrimental effect on the quality of the meat. Springbok meat producers should therefore focus on shortening as well as standardizing the period from slaughter to sale.

7.2 References

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