The Biochemical Basis for Toughness in Beef

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning

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Abstract

The aim of this thesis was to elucidate role of small heat shock proteins (sHSPs) in the development of tenderness in beef. Data in this thesis were predominantly obtained from bull *M. longissimus dorsi* aged at -1.5°C for up to 28 days *post mortem*.

High (pH $\geq$ 6.2) and low ($\leq$ 5.79) ultimate pH (pH_u) meat were acceptably tender within 1 and 7 days *post mortem*, respectively. Tenderness in these pH_u groups is attributed to the extensive degradation of titin, nebulin, filamin and desmin by endogenous proteases. Rapid μ-calpain autolysis in high pH_u meat was concurrent with the faster degradation of titin, nebulin and filamin in this pH_u group. Conversely, faster degradation of desmin was concomitant with increasing cathepsin B activity in low pH_u meat. It is hypothesised that meat tenderness is pH_u compartmentalised by the variable degradation rates of myofibrillar proteins, which are in turn regulated by μ-calpain and cathepsin B activities.

Intermediate pH_u ($5.8 \leq pH_u \leq 6.19$) meat attained acceptable tenderness at 28 days *post mortem*. However, due to the high variance of shear force in this group, some intermediate pH_u meat will be unacceptably tough even after ageing. With up to 19% of bull beef attaining intermediate pH_u, understanding the mechanisms for the inconsistent tenderness in this group is advantageous to the meat industry.

Myofibrillar protein degradation was less extensive in intermediate pH_u meat, partly owing to the sub-optimal activities of μ-calpain and cathepsin B in this group. Exogenous αβ-crystallin reduced the extent of desmin and titin degradation induced by μ-calpain *in vitro*. Furthermore, αβ-crystallin inhibited μ-calpain activity, possibly by acting an alternative substrate for the enzyme.

In *post mortem* muscle, the increasing association of αβ-crystallin to muscle myofibrils was concomitant with the declining levels of HSP20, HSP27 and αβ-crystallin in muscle sarcoplasm as pH declined. This suggests the translocation of sHSPs from the sarcoplasm to the myofibrils and optimal binding of sHSP to the myofibrils in intermediate pH_u meat. It is hypothesised that the high chaperoning potential and μ-calpain inhibition of sHSP combined with the low proteolytic activity in intermediate pH_u meat results in the less extensive degradation of myofibrillar proteins leading to meat toughness as observed in intermediate pH_u meat.

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Paper I Lomiwes, D., Wiklund, E., Farouk, M. M., Frost, D. A., Dobbie, P. M. & Young, O. A. (2012). The status and seasonal variation of meat quality attributes in New Zealand beef. (manuscript in preparation)

Paper II Lomiwes, D., Farouk, M. M., Wu, G. & Young, O. A. (2012). The aetiology of meat tenderness is likely to be compartmentalised by ultimate pH. *Meat Science* (submitted)

Paper III Lomiwes, D., Farouk, M. M., Wiklund, E. & Young, O. A. (2012). Small heat shock proteins and their role in meat tenderness: A review. (manuscript in preparation)

Paper IV Lomiwes, D., Hurst, S. M., Dobbie, P., Frost, D. A., Hurst, R. D., Young, O. A. & Farouk, M. M. (2012). The protection of bovine skeletal myofibrils from proteolytic damage *post mortem* by small heat shock proteins. *Meat Science* (submitted)

Paper V Lomiwes, D., Farouk, M. M., Frost, D. A., Dobbie, P. M. & Young, O. A. (2012). Small heat shock proteins and toughness in intermediate pH_u beef. *Meat Science* (submitted)

Introduction

An introduction to New Zealand beef

In 1882, the *Dunedin* departed from Port Chalmers carrying New Zealand's first consignment of frozen lamb carcasses destined for the United Kingdom. Over 125 years since this historical event, meat has now become the country's second largest commodity export accounting for approximately 13 percent of New Zealand's total exports. World-leading research and technology, infrastructures and rigorous biosecurity standards has seen the meat industry thrive into a significant contributor to the New Zealand economy. In the year ending June 2009 alone, the value of beef exports alone was worth 2 billion NZD (Ministry of Agriculture and Forestry, 2010).

In New Zealand, cattle destined for beef production come mainly from Angus, Hereford, Angus×Hereford and Friesian genetic lines. Beef cattle are predominantly raised in the North Island where, in 2009, 71 percent of the 4.1 million beef cattle in New Zealand were raised in the North Island (Statistics New Zealand, 2010). An estimated 42 percent of export beef are from steers (male castrates) and heifers (virgin females), collectively known as primes, with 37 percent and 21 percent from cows and bulls, respectively (Meat and Wool New Zealand, 2009).

Approximately 80 percent of beef produced in New Zealand is exported to international markets. The United States is by far New Zealand's largest beef market with typically a third of New Zealand's total beef export revenue coming from beef exported to the USA. North Asia (Japan, Taiwan and South Korea) are New Zealand's second largest beef export destinations followed by South Asia, the European Union and the Pacific nations (Meat and Wool New Zealand, 2009).

The large volume of New Zealand beef exported to the USA is due to the difference between the beef produced from both countries. In the USA, beef cattle are grain-finished resulting in beef with high intramuscular fat content which is seen as extensive marbling in the meat. These are sold as table cuts. In contrast, pasture-fed New Zealand beef are leaner and sold frozen to the USA. These are mixed with fatty trims for USA beef to get a standardised fat content and subsequently used for the manufacture of processed beef products such as patties and sausages. New Zealand's export trade with the USA has remained strong because New Zealand beef are destined for different food purposes from those locally produced beef in the USA.

In contrast, New Zealand beef exported to North Asia is marketed as chilled table cuts. Strong emphasis is placed on New Zealand beef as a leaner, healthier alternative to grain-finished beef. New Zealand beef is also marketed as a "natural" product with beef cattle reared in a free-range environment. The growth of New Zealand beef in North Asian market is also attributed to it being marketed as a safe, disease-free product. The detection of bovine spongiform encephalopathy (BSE) in North American beef in 2003 resulted in the ban of USA beef exported to North Asia. This left New Zealand and Australia with exclusive access to the Japanese and South Korean beef markets between 2003 and 2006 (Ministry of Agriculture and Forestry, 2007). Within these years, New Zealand's beef market share in Japan quadrupled from 2.1 to 8.5 percent (Beef and Lamb New Zealand, 2007).

Current market trends for New Zealand beef in the international market

With the onset of the global economic recession in 2009, the outlook for New Zealand beef is challenging. As the New Zealand dollar remains susceptible to large shifts in the global economy, the forecast for New Zealand beef prices was expected to be slightly lower for 2010 (Ministry of Agriculture and Forestry, 2010). The global recession has also caused a shift in consumer's choices as more consumers are now purchasing cheaper, alternative meat and processed beef products over higher value prime cuts.

Added to the expected reduction in New Zealand beef prices is the presence of strong competition from Australia and USA, particularly in North Asian markets. With the ban of North American beef to Japan and South Korea now lifted, New Zealand's beef market share in these countries is expected to decrease as a result of the strong New Zealand dollar and the Asian preference for soft, marbled beef produced by North American grain finishing systems (Ministry of Agriculture and Forestry, 2010).

With the challenges of the current economic climate and increasing competition combined with the growing numbers of discerning consumers in key markets, more emphasis is now placed in providing a product that is safe, of consistent quality, convenient and competitively priced. Hence, research-driven innovations and solutions to existing quality issues of New Zealand beef are critical if the international growth and success of New Zealand beef is to be fully realised.

This thesis addresses the inconsistent tenderness particularly associated with beef, a current issue for the New Zealand meat industry. The production of consistently

acceptable beef is dependent upon understanding of meat science. This thesis investigates the biochemical mechanisms for the inconsistent tenderness in beef.

The conversion of muscle to meat

The severing of critical blood vessels during slaughter (termed "sticking" in the meat industry) effectively terminates the supply of oxygen and nutrients to all tissues of the body including muscles. The cessation of the oxygen supply to muscles results in muscle metabolism rapidly becoming anaerobic. This has significant implications on the biochemistry and structure of muscle cells as muscle enters *rigor mortis* to become meat as we know it.

The structure of muscle

Meat is heterogeneous and composed of varying amounts of muscle, fat, connective tissue and blood vessels. Skeletal muscle can constitute up to 65 percent of the carcass weight (Hendrick, Aberle, Forrest, Judge & Merkel, 1994). The terms meat and muscle are often used interchangeably. For the purpose of this thesis, the term meat is used to describe muscle in rigor.

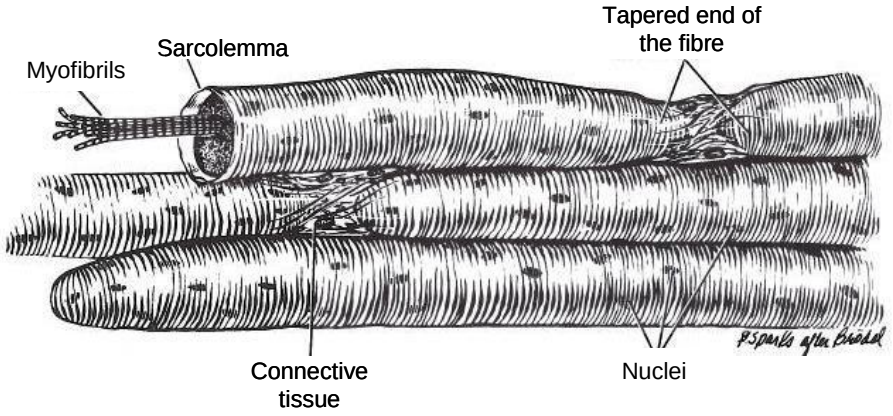

Figure 1: The structure of skeletal muscle fibres. Adapted from (Hendrick *et al.*, 1994)

Skeletal muscles vary in size and shape depending on their function in the live animal, and are directly or indirectly attached to bones, cartilage, ligaments or skin. Muscles are covered and permeated to the cellular level by connective tissue. Connective tissue also provides channels that allow nerve fibres and blood vessels to enter and exit the muscle structure.

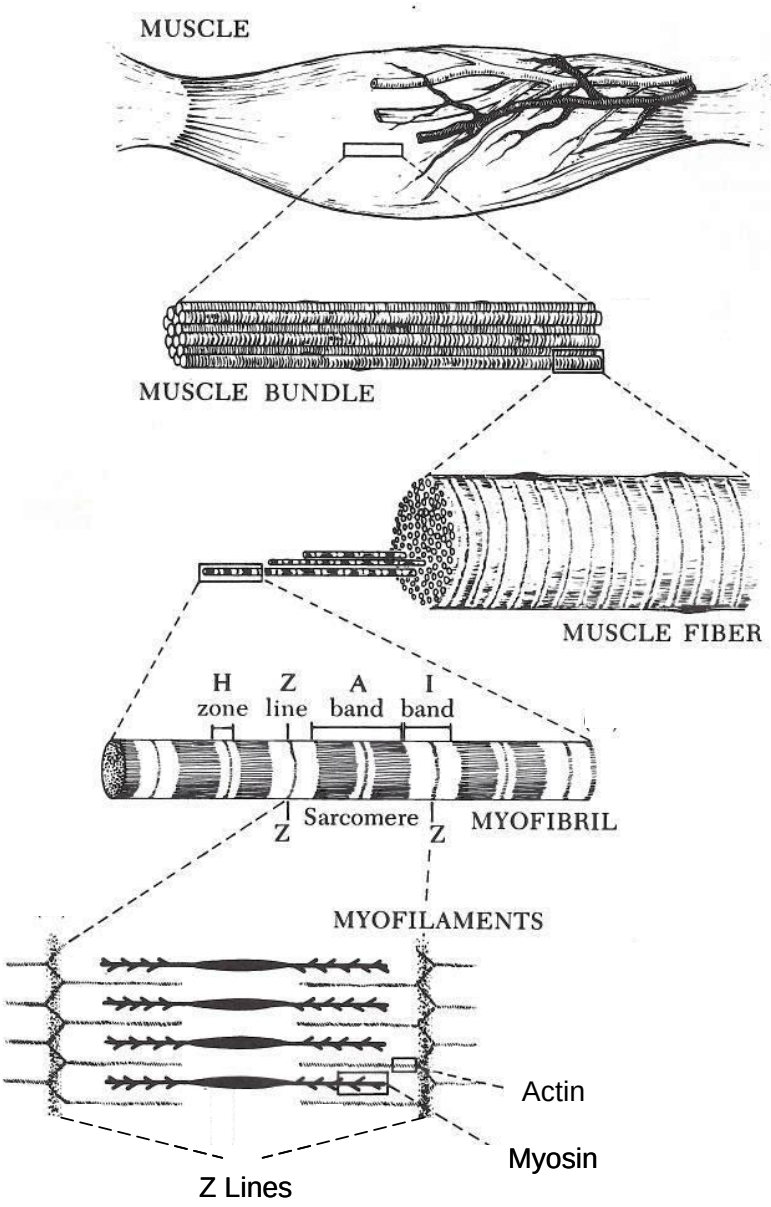

Figure 2: A schematic diagram showing the organisation of the skeletal muscle. Adapted from (Hendrick *et al.*, 1994).

Muscle comprises of structural units called muscle fibres that make up to 92 percent of the total muscle volume (Hendrick *et al.*, 1994). Muscle fibres are long, thread-like cells with tapered ends (Figure 1). They vary considerably in diameter and they can be up to several centimetres in length in large animals. Muscle fibres are covered by muscle membranes known as sarcolemmas. Groups of muscle fibres are then bound together into muscle bundles and a group of muscle bundles are in turn covered by a sheath of connective tissue to form individual muscles.

Muscle fibres are composed of subunits known as myofibrils that run longitudinally along the length of the muscle fibre (Figure 2). Myofibrils are ordered in such a way that they are divided into repeating subunits known as sarcomeres with one sarcomere unit between two consecutive Z-lines. Sarcomeres are predominantly

composed of myofilaments simply known as thick and think filaments. Thick filaments are arranged in parallel and aligned with myofibrils longitudinally. Thin filaments are attached to the Z-lines and are similarly arranged longitudinally across the myofibril and in alignment with the thick filaments. Under a transmission light microscope, the repeating arrangement of sarcomeres gives skeletal muscles a striated appearance of alternating dark and light areas along the myofibrils. It is for this reason that skeletal muscle is also referred to as striated muscle.

Muscle metabolism *post mortem*

The sticking and bleeding of animals during slaughter effectively terminates continuous supply of glucose and oxygen required for glycolysis and the aerobic metabolism of acetyl-CoA in the Krebs cycle. The cessation of the circulatory system also means that metabolic products from the subsequent anaerobic metabolism cannot be transported out of the muscle. Consequently, these metabolites accumulate in the muscle cells resulting in significant changes in the muscle.

In an effort to maintain cell homeostasis in an oxygen-depleted environment, muscle cells transit to anaerobic metabolism to break down alternative energy rich substrates to renew declining concentrations of ATP. Creatine phosphate (CP) is initially metabolised by the muscle cells to maintain ATP concentrations. Briefly, the enzyme creatine phosphokinase catalyses the breakdown CP to creatine and inorganic phosphate, which is subsequently transferred to ADP to produce ATP (Pösö & Puolanne, 2005). Glycolysis rates vary depending on the species and muscle, but CP concentrations in muscle are typically depleted within two hours *post mortem*. During this time, muscle ATP concentrations generally remain constant (Figure 3).

A significant biochemical change in muscle *post mortem* is the drop in muscle pH. This is due to the enzymatic breakdown of glycogen to glucose-6-phosphate units and the subsequent metabolism of glucose-6-phosphate via the glycolytic pathway (Hamm, 1977). In the absence of oxygen, pyruvate can no longer be converted to acetyl-CoA and undergo aerobic metabolism via the Krebs cycle. Instead, it is anaerobically converted to lactic acid. For one glucose moiety of glycogen, only three molecules of ATP are produced, a significantly lower yield compared with the aerobic pathway, resulting in the gradual disappearance of ATP from *post mortem* muscle as glycogen concentration declines (Figure). In addition, as a result of the failure of the blood circulation system, lactic acid cannot be transported away from the muscle. Consequently, it accumulates, increasing muscle acidity as seen in Figure . Glycolysis

usually ceases before all glycogen has been depleted. This may be due to glycolytic enzymes becoming deactivated by the lowering of the muscle pH. In well-fed, unstressed animals the pH fall is typically from ≤7.0 to about 5.5 (Hamm, 1977). The pH that the muscle finally attains is known as the ultimate pH (pH_u).

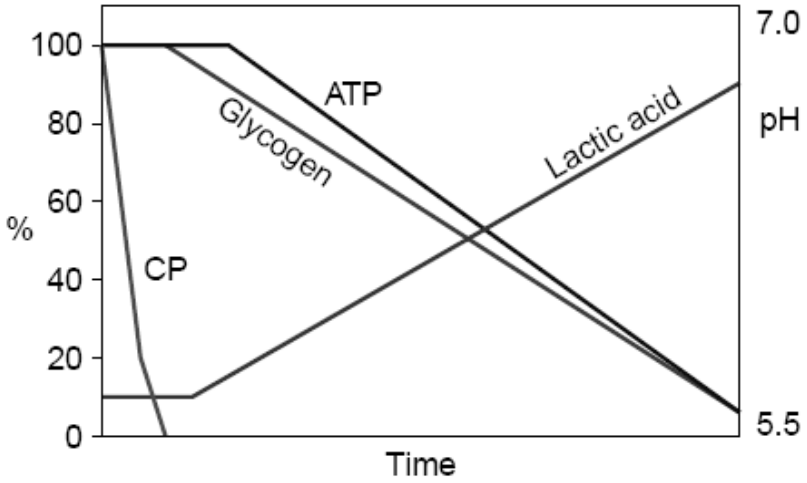

Figure 3: Kinetics of creatine phosphate (CP), glycogen, ATP and lactic acid in *post mortem* muscle. As lactic acid accumulates in the muscle, the pH drops to about 5.5 (Honikel & Werner Klinth, 2004).

Structural changes *post mortem*

Perhaps the most noticeable physical change in conversion of muscle to meat is the stiffening of muscles, a phenomenon known as *rigor mortis*. Muscles usually maintain their extensibility early *post mortem*. The loss of muscle extensibility observed during *rigor mortis* is due to permanent cross bridges forming between myosin heads (thick filaments) and actin (thin filaments). As these actomyosin cross-bridges form, muscles contract and the sarcomere length shortens. In muscle in the living animal, actomyosin bonds are dissociated by mechanisms requiring energy that is provided by the enzymatic hydrolysis of ATP. In *post mortem* muscle, the concentration of ATP is initially kept to pre-slaughter concentrations due to the hydrolysis of CP. However, as CP supply is exhausted and ATP synthesis via the anaerobic glycolysis of glycogen is minimal compared with the yield from aerobic metabolism, ATP required for the dissociation of actomyosin bonds is inevitably depleted. It is for this reason that loss of muscle extensibility and the progression of rigor is correlated with the depletion of glycogen and ATP in muscles *post mortem* (Jeacocke, 1984; Lawrie & Ledward, 2006). At the completion of rigor mortis, all

myosin binding sites have formed actomyosin bridges and muscle extensibility is at its minimum. It is at this point where the conversion of muscle to meat is complete.

With increasing time *post mortem*, further structural changes in the structure of muscle cells and proteins take place. Most notably, the extensibility of meat, as measured by shear force increases during prolonged chilled storage of carcasses (Pulford, Fraga Vazquez, Frost, Fraser-Smith, Dobbie & Rosenvold, 2008). The resolution of meat extensibility is not due to the dissociation of actomyosin bonds. Rather, it is caused by the breakdown of the muscle ultra structure due to the degradation of certain structural and myofibrillar proteins by proteolytic enzymes. The mechanisms and proteins involved during ageing are discussed in detail further in this chapter.

Meat tenderness

One of the most undesirable consumer traits in cooked beef is toughness. Although much research has been conducted on the development of meat tenderness, the biochemical mechanisms causing variation in meat tenderness remain an enigma. This research is reviewed below.

Meat tenderness and pH_u

Many studies have shown that meat quality attributes are influenced by the meat's pH_u. The influence that pH_u has over meat quality attributes is perhaps not surprising as pH has a dominant role in the chemical and physical changes taking place during the conversion of muscle to meat and during meat ageing (Bate-Smith, 1948; Devine, 1994b).

Several studies have shown that a significant curvilinear relationship exists between pH_u and cooked tenderness. These studies have characterised the relationship between pH and tenderness during ageing such that between pH 5.4 to 6.0, meat toughness peaks at approximately pH 6 then subsequently decreases as pH_u increases beyond pH 6.0 (Dransfield, Nute, Mottram, Rowan & Lawrence, 1985; Jeremiah, Tong & Gibson, 1991; Pulford *et al.*, 2008; Purchas & Aungsupakorn, 1993; Purchas, Yan & Hartley, 1999; Watanabe, Daly & Devine, 1996a). It is clear from these studies that intermediate pH_u meat take longer to age to acceptable tenderness, however, tenderness in this pH_u group is still variable even after extended ageing (Simmons, Auld, Thomson, Cairney & Daly, 2000). Normal and high pH_u meat are generally acceptably tender, but

high pH_u meat cannot be sold as chilled table cuts due to its undesirable dark colour and susceptibility to microbial degradation (Newton & Gill, 1981).

Due to these differences in meat quality associated with pH_u, meat can be conveniently classed into three pH_u ranges: normal ($5.4 \leq pH_u \leq 5.79$), intermediate ($5.8 \leq pH_u \leq 6.19$) and high ($pH_u \geq 6.2$) (Watanabe *et al.*, 1996a; Wiklund, Farouk, Stuart, Dobbie, Lomiwes & Frost, 2009)

Causes of pH_u variability in meat

As highlighted earlier, the pH_u muscles attain depend on their pre-slaughter glycogen content, or more accurately, glycogen concentration. Muscle with high (and normal) glycogen contents will yield normal pH_u meat, while carcasses with depleted glycogen will yield either intermediate or high pH_u meat. The extent to which muscle glycogen is depleted primarily depends on the physical condition and stress levels of the animals preceding slaughter. To produce normal pH_u meat, it is important to ensure that animals are handled in a low-stress manner because it takes at least 48 hours of rest with food for bulls (Warriss, Kestin, Brown & Wilkins, 1984) and up to 10 days for lambs (Devine *et al.*, 2006) to replenish depleted muscle glycogen stores.

Among other causes, stress experienced by mixing unfamiliar mobs of beef cattle and the duration of these mobs in lairage prior to slaughter is known to lead to a higher proportion of high pH_u meat (Ferguson, Shaw & Stark, 2006; Kreikemeier, Unruh & Eck, 1998; Lahucky, Palanska, Mojto, Zaujec & Huba, 1998; McVeigh & Tarrant, 1982; Mounier, Dubroeucq, Andanson & Veissier, 2006). This is exacerbated by mob size during lairage, where mean pH_u from larger mob sizes is generally higher (Kreikemeier *et al.*, 1998; Smith, Wright & Muir, 1996). Additionally, the pH_u of beef from animals stressed with electric prodders pre-slaughter was found to be similar to beef from animals that were not treated with electric prodders. However, meat from stressed animals were found to be tougher, less juicy, have less flavour and have a lower 'liking' score compared with meat from unstressed animals as determined by an untrained consumer panel (Warner, Ferguson, Cottrell & Knee, 2007).

Some studies have also reported the seasonal variation in the incidence of high pH_u beef (Brown, Bevis & Warriss, 1990; Kreikemeier *et al.*, 1998; Tarrant & Sherington, 1980; Young, Thomson, Merhtens & Loeffen, 2004). The cause of this variation has been attributed to seasonal temperatures. Heifers exposed to average temperatures below 0°C a few days before slaughter are more likely to yield high pH_u meat (Scanga, Belk, Tatum, Grandin & Smith, 1998). High transport temperatures

during the summer months result in animal discomfort and stress resulting in the higher prevalence of high pH_u meat (Immonen, Ruusunen, Hissa & Puolanne, 2000; Mounier *et al.*, 2006). The quality and availability of pasture in grass-fed beef cattle as the seasons progress throughout the year are also thought to contribute to the variation in body condition of the animals and therefore in the pH_u of beef. This is thought to lead to lower muscle glycogen reserves and consequently, higher pH_u meat (Brown *et al.*, 1990).

Animal age and gender have also been found to affect meat quality attributes. Meat from older animals tends to be less tender. This is due to the prevalence and stability of collagen cross-links within the muscle connective tissue resulting in higher background toughness (Purchas & Werner Klinth, 2004). The gender effects on meat quality is well reported, with higher incidences of high and intermediate pH_u meat observed in bulls (Brown *et al.*, 1990; Wiklund *et al.*, 2009; Young *et al.*, 2004)

The dietary composition of animals has a large influence on muscle characteristics at slaughter and can influence the eating quality of meat. The flavour of pasture fed cattle are generally more intense than their corn-finished counterparts (Warriss, 2000). Supplementing the diet of corn-fed cattle with antioxidants such as selenium (an antioxidant precursor) and vitamin E, increases colour stability in raw meat (Oddy, Harper, Greenwood & McDonagh, 2001; Wood *et al.*, 2004). Antioxidants also improve the shelf life of meat by combating lipid oxidation (Geay, Bauchart, Hocquette & Culioli, 2001). Finally, providing animals with a high energy diet prior to transportation to slaughter also reduces the incidence of high pH_u beef (Immonen *et al.*, 2000).

The pH_u profile of New Zealand beef

Past surveys to determine the pH_u profile of New Zealand beef have found a high incidence of higher pH_u beef produced in the New Zealand pastoral system. A survey published in 1994 reported approximately 45 percent of beef animals had a pH_u greater than 5.7 in an indicator muscle (*M. longissimus dorsi*). Additionally, bulls were more disposed to producing higher pH_u meat with about 80 percent of bulls recording a pH_u greater than 5.7 compared with only nine percent of steers (male castrates) (Graafhuis & Devine, 1994).

A more recent survey with 13,700 animals conducted by Young *et al.* (2004) found that 19 percent of prime cattle and 62 percent of bulls had a pH_u above 5.7. Although these surveys were conducted six years apart, it is clear that there is still an

unacceptably high incidence of higher pH_u beef, especially in bulls. The continuing high incidence of elevated pH_u meat in bulls and its association with variable toughness in intermediate pH_u meat remains a problem for the New Zealand meat industry. It is inevitable to relate the incidence of high pH_u values to stress levels in connection with slaughter, which can be regarded as a welfare issue. Empirically, this issue would be eliminated by the better handling of animals that would lead to the production of low pH_u meat. However, due to the high throughput of animals being slaughtered within one day, stresses associated with elevated pH_u meat and inconsistent meat quality is unavoidable. Therefore, understanding the biochemical mechanisms of the intermediate pH_u needs to be understood. The following section describes known mechanisms and factors that are believed to contribute to cooked meat tenderness.

Factors involved in the development of tenderness in beef

Scientists agree that meat tenderness is due to the proteolytic degradation of the organised myofibrillar structure. However, the enzyme systems involved in meat tenderisation are still a topic of contention. Beyond proteolytic degradation, carcass processing conditions have significant impact on meat tenderness. In addition, collagen and connective tissues within and between muscles also provide background toughness.

Meat tenderness and proteolytic enzyme activities

There is a general consensus that the tenderisation of muscle as it is converted to meat during *post mortem* storage (often called ageing) is an enzymatic process involving proteolytic enzymes also known as proteases. Briefly, proteases hydrolyse cytoskeletal and myofibrillar proteins by using them as substrates resulting in the degradation of the structure of muscle fibres, consequently resulting in meat tenderisation. There are several views as to which protease systems are important. Two proteases recognised as being potentially involved in *post mortem* proteolysis leading to meat tenderness are calpain, specifically μ-calpain (Huff-Lonergan & Lonergan, 2005; Koohmaraie & Geesink, 2006), and lysosomal cathepsins (Ouali, 1992; Ouali *et al.*, 2006).

μ-Calpain

μ-Calpains are Ca^{2+}-dependent proteases endogenous to skeletal muscle cells (and other cell types). In *post mortem* muscle, the development of *rigor mortis* is associated with the release of calcium ions from the sarcoplasmic reticulum and the

mitochondria to the micromolar concentrations required for μ-calpain activation (Jeacocke, 1993). *In vitro* experiments have demonstrated that the pattern of degradation of myofibrillar proteins by exogenous μ-calpain is similar to the degradation pattern in *post mortem* muscle (Huff-Lonergan, Mitsuhashi, Beekman, Parrish, Olson & Robson, 1996). μ-Calpain has also been reported to degrade the cytoskeletal proteins titin and nebulin at a faster rate than other myofibrillar proteins filamin desmin and troponin-T (Hopkins & Taylor, 2004). The degradation of these proteins leads to the disruption of the highly organised myofibrillar structure resulting in meat tenderisation.

μ-Calpain activity is regulated by calpastatin, which inhibits calpains, also in a calcium-dependent process (Goll, Thompson, Li, Wei & Cong, 2003). Upon activation, the conformation of μ-calpain is modified allowing calpastatin to associate with so-called inhibitory domains of μ-calpain, resulting in the enzyme inhibition. Quantitative assays to determine calpastatin activity have reported a positive correlation between calpastatin activity and toughness in beef (Shackelford, Koohmaraie, Cundiff, Gregory, Rohrer & Savell, 1994) and pork (Kemp, Sensky, Bardsley, Buttery & Parr, 2010). Furthermore, the slower tenderisation of rate of beef compared with pork, may be due to the higher ratio of calpastatin:calpain activity in beef (Koohmaraie, Whipple, Kretchmar, Crouse & Mersmann, 1991).

Other factors affecting μ-calpain activity in *post mortem* muscle include enzyme autolysis and muscle pH. The activation of μ-calpain is concomitant with the progressive autolysis of the enzyme and thus an increasing decline in net proteolytic activity (Baki, Tompa, Alexa, Molnar & Friedrich, 1996; Edmunds, Nagainis, Sathe, Thompson & Goll, 1991). μ-Calpain activity is also optimal at physiological pH (Dayton, Goll, Zeece, Robson & Reville, 1976) and calpain levels in *post mortem* muscle becomes increasingly limited as muscle acidifies. Thus, μ-calpain proteolysis of myofibrillar proteins is also regulated by μ-calpain autolysis and pH.

Cathepsins

The contribution of cathepsins to meat tenderisation is doubted by some meat scientists as cathepsins do not fulfil characteristics critical for a protease system to contribute to meat tenderisation (Hopkins *et al.*, 2004; Koohmaraie *et al.*, 2006; Koohmaraie *et al.*, 1991). These characteristics, as defined by Koohmaraie

(1992b), require candidate protease systems to be endogenous to skeletal muscle, have access to myofibrillar proteins and be capable of hydrolysing, *in-vitro,* the same proteins that are degraded during *post mortem* storage. As cathepsins are inherently confined within the lysosomes, they are inaccessible to muscle myofibrils and are thus unable to degrade them (Hopkins *et al.*, 2004). Additionally, muscle protein degradation early *post mortem* has been attributed μ-calpain activity (Koohmaraie, Babiker, Merkel & Dutson, 1988) and although actin and myosin are primary substrates of cathepsins (Goll, Otsuka, Nagainis, Shannon, Sathe & Muguruma, 1983), these were not significantly degraded in *post mortem* muscle (Koohmaraie *et al.*, 1991).

However, there is evidence for the involvement of cathepsins in meat tenderisation. Studies have revealed that lysosomal membranes become increasingly permeable at acidic pH, consequently resulting in the diffusion of cathepsins to the muscle sarcoplasm so allowing access to myofibrillar substrates (Sentandreu, Coulis & Ouali, 2002). Coincidentally, the translocation of cathepsins to the sarcoplasm favours myofibrillar protein degradation as most cathepsins are active between the pH range of 3.0 and 6.0 (Etherington, 1984). The more extensive degradation of titin and troponin-T, two proteins associated with meat tenderness, in normal pH_u meat is attributed to cathepsin proteolysis (Dutson, 1983; Zeece & Katoh, 1989). In addition, cathepsins have been demonstrated to destabilise Z-lines myofibrils near the A-band and I-Zone junction (Ouali, Garrel, Obled, Deval, Valin & Penny, 1987). Thus, it has been proposed that meat tenderisation, particularly in normal pH_u meat, is due to the combined proteolytic activities of μ-calpain and cathepsins early *post mortem* and during storage, respectively (Jiang, 1998; Ouali, 1992). Early tenderisation may be attributed to μ-calpain activity which is optimal at physiological pH levels. However, the decline in μ-calpain activity due to increasingly acidic pH and enzyme autolysis coincides with increasing cathepsin activity which are active at more acidic pH levels and are increasingly released from the lysosomes during *post mortem* storage.

Processing conditions affecting cooked meat tenderness

The chilling regime of carcasses and fresh meat cuts is important in ensuring the microbial safety of the product. However, the temperature at which a carcass is kept pre rigor is critical. Rapid chilling of carcass *pre rigor* induces cold shortening, consequently resulting in tough meat, even after ageing (Locker & Wild, 1984).

Cold shortening occurs when a muscle is chilled below 10°C before the onset of rigor mortis (Locker & Hagyard, 1963). In living muscle, intracellular calcium ion concentrations are moderated by the leakage of calcium ions from the sarcoplasmic reticulum and the reuptake of the calcium ions by the calcium pump. Immediately after slaughter, where the ATP concentrations are still high, the mechanisms moderating calcium activity are still active. The activity of the calcium pump is temperature-dependent, and at lower temperatures, calcium reuptake becomes significantly slower than calcium leakage resulting in the intracellular build up of calcium ions (Young, Lovatt, Devine & Simmons, 2000). This inevitably induces muscle contraction as seen in cold shortened beef. As the mechanisms surrounding calcium reuptake are enzymatic, they are inhibited by low pH such that muscle will not cold shorten once the muscle pH falls below 6.0 (Locker *et al.*, 1963).

Electrical stimulation is applied by passing an electric current through the carcass to ensure that cold shortening is avoided, especially under rapid chilling conditions. Applying a current through the carcass early post mortem induces muscle contraction and the anaerobic metabolism of muscle glycogen. This results in the pH fall and accelerates the onset of rigor mortis.

As muscle pH decline is accelerated by electrical stimulation, optimising the chilling regime of carcasses can ensure that cold shortening is avoided. Studies have recommended the use of electrical stimulation followed by slow carcass chilling (10°C for 24 hours, then 2°C for 48 hours) or delayed carcass chilling (16°C for 24 hours, then chill down to 2°C) to obtain optimum meat tenderness (Hwang, Devine & Hopkins, 2003; Ockerman & Basu, 2004; White, O'Sullivan, Troy & O'Neill, 2006). When discussing carcass chilling, it is important to consider hygiene conditions that may result in the contamination or proliferation of harmful pathogens. However, on the condition that good handling practices are followed, hot-boned meat will be microbially safe as long as it is chilled to 21°C within 9 hours after boning (Waylan, Kastner & Werner Klinth, 2004).

Connective tissue

Connective tissue is important in maintaining the muscle structure by holding myofibres to muscle bundles and also by providing support for muscles during contraction. Intramuscular connective tissue is mainly composed of collagen and elastin. Approximately 90% of intramuscular connective tissue is found in the perimysium, the layer of connective tissue that surrounds the muscle fibre bundles. It is

the perimysium that is often implicated in the contribution of connective tissue to meat toughness (Sifre *et al.*, 2005). Connective tissue is thought to explain about 12% of the variation in texture found in cooked beef measured objectively or by a sensory panel (Taylor & Werner Klinth, 2004) as it is resistant to proteolytic breakdown and remains intact during ageing. It is for this reason that connective tissue is thought to provide background toughness to cooked meat.

Studies have reported a significant relationship between animal age and toughness. Shorthose and Harris (1990) found that for most muscles included in their study, objective and subjective measurements for toughness increased with animal age. Additionally, the study found that the rate of increase with age varied between muscles. This was thought to be due to the variation in connective tissue strength found in each muscle, in turn related to a muscle's role in the living animal. Age related toughness is considered to be due to the increase in collagen cross-links and fibril size as the animal matures, which results in a more interlaced network that is more thermally resistant and thus less soluble during cooking. However, there has been no conclusive evidence to confirm this (Oddy *et al.*, 2001; Purslow, 2005).

Animal growth rate was found to contribute to toughness from connective tissue. Several studies have shown that toughness provided by connective tissue was lower in animals slaughtered after a period of rapid growth. Compensatory growth after a period of weight loss has also been shown to lower connective tissue toughness (Oddy *et al.*, 2001; Purslow, 2005). It is hypothesised that newly synthesised connective tissue during compensatory growth is less cross-linked and condensed compared with mature connective tissue in animals that have had relatively constant growth, resulting in meat with less background toughness.

Apoptosis, small heat shock proteins and meat tenderness: a new hypothesis

Although tenderness in normal and high pH_u beef may be explained by the optimal proteolytic activity in these pH_u groups, the mechanisms causing inconsistent tenderness in intermediate pH_u beef is not understood. Ouali *et al.* (2006) proposed a hypothesis of tenderness based on apoptosis, a process of programmed cell death seen in damaged or potentially dangerous cells in living organisms where cells effectively self-destruct without damaging surrounding cells. Marked changes in the biochemistry of muscle *post mortem* driven by the termination of nutrient and oxygen supply to the muscles purportedly trigger apoptosis of muscle cells (Ouali *et al.*, 2006). In response

to impending cell death, small heat shock proteins (sHSP) are synthesised to combat apoptosis (Beere, 2004) as an attempt to maintain muscle cell homeostasis.

sHSP belong to a large group of proteins known as chaperone proteins and are ubiquitous in all cellular organisms. Chaperone proteins are known for their role in assisting the correct conformation of nascent polypeptides by preventing proteins from entering incorrect alternative conformation pathways that would produce non-functional or harmful structures (Ellis, 1996; Ellis & van der Vies, 1991). Studies have found that sHSP may also have a protective role in cells, and their expression is particularly prominent where cells have been exposed to adverse conditions such as hyperthermia (hence the name heat shock), hypoxia and harmful oxidants (Escobedo, Pucci & Koh, 2004; Oesterreich, Benndorf & Bielka, 1990). In addition, sHSP are also known to repair damaged proteins and maintain cell survival by keeping proteins from fatally aggregating in stressed cells (Soti, Sreedhar & Csermely, 2003). Considering that meat tenderness is dependent upon the degradation of myofibrillar proteins *post mortem*, sHSP have therefore been implicated in the development of meat tenderness.

sHSP are abundant in bovine muscles and their expression is stimulated after slaughter (Pulford *et al.*, 2008). However, very little work has been conducted to investigate the contribution of small heat shock proteins with regard to meat tenderness. Their role in the conversion of muscle to meat is largely unknown. It has only been in the past few years that researchers have been able to shed some light in the role that sHSP may have in meat quality. These are discussed in a review presented in this thesis (Paper III).

Summary

The New Zealand beef industry is mainly based on frozen commodity exports, a bulk of which is sourced from bulls. Most of this meat is ground (minced) in the destination market and used for manufactured meat products. A challenge for the New Zealand meat industry is to transform its frozen commodity products into higher value chilled cuts. The financial advantage of this approach is obvious as chilled New Zealand beef is typically worth twice as much as frozen beef.

A key limiting factor in the transformation of frozen beef into higher value chilled cuts is the inconsistent tenderness of New Zealand beef, particularly bull beef. This is typically sourced from meat that is in the intermediate pH_u class. Surveys conducted in New Zealand abattoirs have reported a high prevalence of intermediate

pH_u meat derived from bulls. Currently some New Zealand abattoirs apply a patented method that enables the sorting of carcasses into lower pH_u and higher pH_u classes. This method gives the opportunity to select to freeze or to keep the meat chilled and at the same time guarantee optimal meat tenderness and shelf life based on predicted pH_u of the meat. However, this assay downgrades all intermediate pH_u beef as unsuitable for higher value table cuts, when a significant portion of this meat will attain acceptable tenderness that may be marketed at a higher price.

The mechanisms causing inconsistent tenderness in intermediate pH_u beef are largely unknown. To transform bull beef from a frozen commodity to higher value chilled cuts, it is useful to understand these mechanisms. An improvement in the inconsistent tenderness of New Zealand beef would provide a greater portion of acceptably tender product to fulfil the growing North Asian market. Another important consideration in the transformation of intermediate pH_u meat is the microbiological properties of this pH_u group. Due to the elevated pH of intermediate pH_u meat, the shelf life of this pH_u group will inevitably be shorter compared with low pH_u meat (Newton *et al.*, 1981). Thus, steps to ensure the safety of the intermediate pH_u meat marketed as chilled cuts should also be taken in to account. The success of providing meat with consistent tenderness from intermediate pH_u bulls would enable chilled beef exports to the higher value North Asian markets to rise by 50 percent to an estimated 40 percent of total New Zealand beef exports, generating increased revenue from existing and new markets. This would add an additional net NZ$56 million per year to the value of New Zealand beef exports (cost benefit analysis done on behalf of AgResearch Ltd.).

Objectives

The main objective of this thesis was to determine the role, if any, of sHSP endogenous to skeletal muscle in the development of meat tenderness. It is hoped that understanding the basis of the behaviour of these proteins in *post mortem* muscle will contribute to the better understanding of various mechanisms involved in meat tenderisation during the conversion of muscle to meat and subsequent ageing. Further knowledge in this area will enable meat processors to tailor their processing protocols by targeting existing technology or develop new technology to reduce the production of tough beef.

The specific aims of this thesis were:

- To obtain a current overview of the pH_u status of New Zealand bull beef routinely slaughtered in representative commercial abattoirs in the North Island.

- To explore the chaperone function of the sHSP (HSP20, HSP27 and αβ-crystallin) in protecting myofibrillar proteins from proteolytic degradation.

- To further characterise the expression of the sHSP and protease activity (μ-calpain and cathepsin B) and to determine how these parameters may be related in the apoptotic death of muscle cells, and the variable degradation of myofibrillar proteins in *post mortem* muscle resulting in inconsistent tenderness in intermediate pH_u beef.

Materials and methods

Animal material and sampling

Paper I

First study. For the pH_u survey of bull beef, *M. longissimus dorsi* (LD) from bull carcasses (n = 1,597) were collected from four commercial abattoirs. All animals were electrically stunned before slaughter and electrically stimulated. Upon reaching the boning floor, the LD was hot boned from the left side of the carcass and a 2.5 cm thick muscle sample was cut at the 12^{th} rib, the quartering site. Samples were then labelled, packed in plastic bags and then stored at -1.5°C for 48 h. The ultimate pH was subsequently measured after storage at 48 h.

Second study. LD from the left side of electrically stimulated bull carcasses slaughtered in a commercial abattoir were collected in spring (n = 63) and summer (n = 94). Muscle samples from summer were collected over two consecutive days with 49 and 45 LD samples collected on the first and second day, respectively. All muscles were hot-boned upon reaching the boning floor and 10 g was dissected from the anterior end of each muscle, frozen in liquid nitrogen and stored at -80°C. The remaining muscle was packed in plastic bags and stored at -1.5°C for 24 h then each LD were cut into six equally sized sub-samples then vacuum packed. The sub-samples were randomly allocated an ageing timepoint (1, 2, 7, 14 and 28 days *post mortem*) and stored at -1.5°C. A 10 g sample was dissected from all sub-samples after their allocated ageing timepoint, frozen in liquid nitrogen and then stored at -80°C.

Paper II

The animal samples used to obtain the data in this study were the bull LD samples collected in the second study of Paper I.

Paper IV

First Study: LD from Angus heifers (n = 14) were used in this study. All animals were stunned by captive bolt and were not electrically stimulated. After the LD muscles were hot-boned from the carcass, they were packed in vacuum bags and held in a 37°C water bath. Muscle pH was monitored during storage and approximately 10 g of

muscle samples were collected from each LD at pH 7.4, 7.2, 7.0, 6.8 and 6.5 and used for subsequent biochemical assays

Second Study. The animal samples used to obtain the data in this study were the same bull LD samples collected in the second study of Paper I.

Paper V

The muscle samples used in this study were the same bull LD samples collected in the summer from the second study of Paper I.

Methods

pH measurement

For Paper I (first study), muscle pH measurement were conducted using a portable pH meter (Mettler Toledo, MP 125, Switzerland) fitted with a combination electrode (InLab® 427) with automatic temperature compensation. For Paper IV (first study), muscle pH was measured with a combination puncture electrode pH meter (Mettler-Toledo GmbH, Switzerland). All other pH measurements of muscle samples in the presented papers were measured with a Testo® 230 pH meter (Lenzkirch, Germany). All pH meters were calibrated at pH 7.0 and 4.0 with buffers (Mallinckrodt Chemicals, Philipsburgh, NJ) at ambient temperature (20°C).

Shear force and cook loss

Shear force and cook loss was determined by measuring the weight of all sub-samples prior to cooking, the cooking the samples in weighted plastic bags in a 100°C water bath to an internal temperature of 75°C. After cooking in an ice water bath, the samples were patted dry with a paper towel, and then reweighed. Cook loss was calculated as the difference of the initial uncooked weight of the sample and the surface-dried cooked sample, and expressed as the percentage of the initial cooked weight. The shear force of samples was measured with a MIRINZ tenderometer as described by Pulford *et al.* (2008).

Sample preparations

For the preparation of total muscle extracts (Papers II, IV and V), approximately 0.5 g of frozen muscle fragments was dispersed in 5 mL of total muscle extraction

buffer (50 mM Tris-HCl; pH 5.8, 10% glycerol, 2% SDS and 2% 2-mercaptoethanol) for 20 seconds at 20,000 rpm with an Ika®-Labortechnik Ultra Turrax T25 disperser (Staufen, Germany) fitted with a 18 mm S25 tool. The homogenate was then centrifuged at 10,000 gravities for 5 min at 4°C. The total muscle extract supernatant was frozen at -80°C awaiting further analysis.

Whole muscle homogenates (Papers II and V) were prepared by dispersing 0.4 g frozen muscle fragments in whole muscle extraction buffer (10 mM sodium phosphate buffer, 0.2 % SDS; pH 7.0). The homogenate was then centrifuged at 1,500 gravities for 15 min at 25°C and the supernatant stored at -80°C.

For the preparation of sarcoplasmic fractions in Paper V (first study), 2 g of muscle was homogenised in soluble fraction extraction buffer (50 mM Tris-HCl, 10 mM EDTA, 1 mM DTT, 0.1 mM phenylmethanesulfonyl fluoride (PMSF) with COMPLETE™ protease inhibitors (Roche, 11 836 170 001); pH 7.4). The homogenate was then centrifuged at 3000 gravities for 10 min at 4°C and the supernatant stored at -80°C until analysed. Sarcoplasmic fractions in Papers IV (second study) and V were prepared as described by Pulford *et al.* (Pulford *et al.*, 2008).

Bovine myofibrillar fractions with associated proteins (Paper V (first study)) were prepared as described by Wang *et al.*(1988). Muscle fibres were teased apart in myofibrillar extraction buffer (50 mM Tris-HCl, 10 mM EGTA, 2mM $MgCl_2$, 0.1 mM PMSF, 0.1% Triton X-100; pH 7.0) and stored in the buffer at 4°C overnight. The muscle fibres were then gently homogenised in the same buffer and centrifuged at 3000 gravities for 5 min. The supernatant was discarded and the myofibrillar pellet was washed three times in myofibril extraction buffer without Triton X-100. The resulting myofibrillar fraction was stored in 50 percent glycerol in a standard salt solution (20 mM NaH_2PO_4:Na_2HPO_4, 100 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM NaN_3; pH 6.8) at -20°C until analysed.

For the preparation of stripped myofibrillar fractions (Paper V (first study)), 0.5 g of muscle sample was teased apart in stripped myofibrillar extraction buffer (20 mM NaH_2PO_4:Na_2HPO_4, 100 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 0.1 mM PMSF, 0.1 mg mL^{-1} soybean trypsin inhibitor, 0.5% Triton X-100; pH 7.0), finely chopped with scissors then agitated at 200 rpm in an oscillating shaker in extraction buffer at room temperature for 1 h. The resulting suspension was centrifuged at 2000 g at 4°C for 5 min. The supernatant was discarded and the pellet was washed four times with BASS buffer (0.1 M NaH_2PO_4:$NaHPO_4$, 25 mM KCl, 39 mM boric acid, 0.1 mM PMSF, 0.1 mg mL^{-1} soybean trypsin inhibitor; pH 7.0). The pellet was then resuspended in BASS

buffer containing 0.5 mg mL^{-1} collagenase type 1 (Roche, 04 834 606) and 0.1 mM $CaCl_2$ and digested at 25°C for 30 min before redispersing the digested pellet in the buffer followed centrifugation at 2000 g for 5 min. The resulting pellet was washed four times with a boric acid-based buffer, BASS buffer and resuspended in ice cold phosphate buffer (0.1 M NaH_2PO_4:Na_2HPO_4; pH 7.0) followed by centrifugation at 300 g for 5 min. Myofibrils remaining in the suspension were pelleted from the supernatant by centrifugation at 3000 g for 5 min. Some myofibrils were then 1: stripped further using phosphate buffer containing 0.5 M KCl for 10 min on ice, washed three times phosphate buffer, suspended in phosphate buffer containing 0.1 mM PMSF and 50% glycerol then stored at -20°C. This treatment removed all myofibril associated proteins, but maintained the myofibrillar structural protein integrity or 2: resuspended in phosphate buffer. These myofibrils had most associated proteins removed, including μ-calpain, but still had αβ-crystallin attached.

Protein measurement of sample preparations

Protein concentrations of sarcoplasmic and myfibrillar preparations in Paper V (first study) were measured using a Bradford Protein Assay kit (BioRad Laboratories, Hercules, CA). Protein concentrations of total muscle extracts (Papers II and IV) was determined using a RC-DC protein assay kit (BioRad Laboratories). Whole muscle homogenate (Papers II and V) and sarcoplasmic fraction (Papers IV (second study) and V) protein determination was measured with a DC protein assay kit (BioRad Laboratories).

Measurement of μ-calpain activity

The determination of relative μ-calpain activities in Papers I, IV (second study) and V was conducted by dispersing 5 g of frozen muscle in 25 mL ice cold extraction buffer (75 mM Tris-base, 10 mM EDTA, 10 mM 2-mercaptoethanol, 0.2% Triton X-100; pH 7.5) with an Ika®-Labortechnik Ultra-Turrax T25 (Staufen, Germany) fitted with a S25 dispersing tool. The homogenate was centrifuged at 30,000 g for 30 min at 4°C and the supernatant was then filtered through cotton gauze and glass wool. Separation of μ-calpain was conducted using a step-wise salt gradient on a DEAE Sephacel ion exchange chromatography as originally described by Wheeler & Koohmaraie (1991). The relative μ-calpain activity of extracted muscle calpain was conducted using a fluorescent substrate described by Wiklund *et al.* (2010).

In Paper V (first study), µ-calpain activity was determined according to Mittoo *et al.*(2003). Briefly, µ-calpain (Calbiochem, #208712) was diluted in 100 µL Calpain Assay Buffer (50 mM Tris-HCl; pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.05% ($^{v}/_{v}$) 2-mercaptoethanol) and dispensed into a black-walled 96 well plate (Nunc, #265301) together with 1 µM fluorogenic calpain-1 substrate (Calbiochem, #208748). The reaction was started with the addition of 5 mM $CaCl_2$. A change in fluorescence was measured over 60 min with a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany) using 490 nm excitation and 520 nm emission filters. When appropriate, varying amounts of αβ-crystallin (0.5 to 10 µg), 1 µg calpastatin (Calbiochem, #208900), 1 g casein or 1 g bovine serum albumin (BSA) were added to the reaction mixture 5 min prior to the addition of µ-calpain.

Measurement of cathepsin B activity

Extraction and measurement of cathepsin B activity (Papers I, II and V) was determined according to Caballero *et al.* (2007) with minor modifications. Two grams of frozen muscle sample was dispersed in 18 mL of extraction buffer (10 mM potassium phosphate, 1 mM EDTA, 50 mM NaCl, 250 mM sucrose; pH 7.4). Cathepsin B standard curves were prepared from purified cathepsin B (Sigma, C6286) and diluted in incubation buffer (100 mM sodium acetate, 1 mM EDTA, 5 mM DTT, 0.1% Brij 35; pH 5.5). Forty microlitre sample extracts and standards were diluted with 300 µL of incubation buffer, then 25 µL of each diluted mixture was dispensed into a 96 well black-walled fluorescence microtitre plate (Nunc, #265301). Seventy microlitres of incubation buffer was dispensed into each well to make up the total volume in each well to 95 µL. After incubation at 37°C for 10 min, 5 µL of 40 µM cathepsin B fluorogenic substrate III (Calbiochem, 219392) was dispensed into each well. The fluorescence emitted by samples and standards were measured every minute for 1 h at 37°C with a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany) fitted with 360 nm excitation and 460 emission filters.

Coomassie Blue SDS-PAGE

A 0.2 mL aliquot of each thawed total muscle extract (Papers II, IV and V) was mixed with the same volume of reduced sample buffer (62.5 mM Tris-HCl; pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.02% bromophenol blue). The solution mixtures were heated for 20 min at 50°C followed by centrifugation at 10,000 g for 10 min and the supernatant was loaded onto gels.

For the separation of larger protein (>100 kDa), 100 µg of protein was loaded into each well and separated at room temperature in a BioRad Criterion cell system at 10 mA for 17 h. Following electrophoresis, gels were stained in colloidal Coomassie Blue (17% ammonium sulphate, 2% phosphoric acid, 30% methanol, 0.04% Coomassie G-250) for 48 h. Stained gels were then washed with distilled water and images were captured with a GS700 calibrated densitometer scanner (BioRad Laboratories).

Gel electrophoresis and Western blots

Prior to the resolution of proteins in by SDS-PAGE, total muscle extracts (Papers II and IV (second study)), myofibrillar preparations (Paper V) and sarcoplasmic fractions (Papers IV and V) were diluted in sample loading buffer. Proteins were resolved on Tris-HCl gels (BioRad Laboratories) of varying acrylamide percentages, depending on the protein of interest. For the separation of titin fragments (Paper IV), proteins were resolved on 5% Tris-HCl gels. Desmin, myosin and µ-calpain were generally resolved on 7.5% Tris-HCl gels (BioRad Laboratories) (Papers II, IV and V). HSP20, HSP27 and αβ-crystallin were resolved on 12% Bis-Tris gels (BioRad Laboratories). All gels were resolved in a BioRad Criterion Cell system.

Proteins were subsequently blotted onto Immobilon-P PVDF membranes (Millipore, IPVH00010) and then blocked overnight at 4°C with 5% non-fat dry milk powder in PBS-Tween (0.08 M Na_2HPO_4, 0.02 M NaH_2PO_4, 0.1 M NaCl, 0.1% Tween). The blots were probed with anti-αβ-crystallin (Abcam, Ab13496), anti-HSP20 (Hytest, HSP20-11), anti-HSP27 (Hytest, #4HSP27), anti-µ-calpain (Thermo Scientific, MA3-940), anti-desmin (Sigma, D1033), anti-myosin (Sigma, M-4276) or anti-titin (Sigma, 9D10) monoclonal antibodies for 1 h at ambient temperature. All monoclonal antibodies were derived from mouse cells. αβ-Crystallin, HSP20 and HSP27 primary antibodies were diluted 1:10000 in PBS-Tween. Desmin, myosin, titin and µ-calpain antibodies were diluted 1:5000, 1:10000, 1:200 and 1:5000, respectively, in PBS-Tween. Membranes were washed four times for 10 min with PBS-Tween, then incubated with goat anti-mouse IgG (heavy and light chains) linked to horseradish peroxidase (BioRad, #172-1011), and diluted to 1:5000 in PBS-Tween. Following four washes with PBS-Tween, as previously described, membrane bound antibody was detected with ECL Western Blot substrate kit (Pierce, 32106). Western Blot images were captured with a G:Box Chemi HR16 image capture instrument (Syngene, Cambridge, UK).

Immunoprecipitation of myofibrillar and soluble proteins

Immunoprecipitation experiments in Paper IV was conducted by covalently bonding αβ-crystallin antibody (10 µg mL^{-1}) to 100 µL Dynabeads® Protein G magnetic beads (Invitrogen, 100-03) according to the manufacturer's protocol. Immunoprecipitation of soluble and myofibrillar proteins were performed in 1 mL immunoprecipitation buffer (50 mM Tris-HCl, Complete™ protease inhibitor (Roche, 11 836 170 001), 10 mM EDTA, 10 mM EGTA; pH 7.4) containing either 1: partially solubilised (with 0.5% Triton X-100) isolated bovine LD myofibrils or 2: soluble muscle proteins. Lysates were cleared by incubation with 100 µL magnetic beads for 1 h at 4°C in an oscillating shaker. Unbound proteins were incubated with αβ-crystallin/magnetic bead complex overnight at 4°C. The protein suspension was then placed in a magnet, the supernatant discarded, and the proteins bound to the magnetic bead complex were washed four times for 5 min with 1 mL PBS containing 0.1% Triton X-100. Proteins were eluted from the complex with 0.1 M citric acid, pH 5.2, and then immediately neutralised by addition of 1 M Tris-HCl to pH 7.4. Eluted proteins were diluted in Laemmli sample loading buffer then analysed by Western blotting.

A mixture of 2.5 µg mL^{-1} of purified µ-calpain (Calbiochem, #208712) and 10 µg of purified αβ-crystallin (Abcam, Cambridge, UK; ab74441) were incubated for 5 min at 37°C in 50 mM Tris-HCl (pH 7.4) containing either 10 mM EGTA or 10 µM $CaCl_2$. αβ-Crystallin was immunoprecipitated overnight 4°C as previously described. Eluted proteins were diluted in Laemmli sample loading buffer then analysed by Western blotting.

Purification of bovine αβ-crystallin

Purification of αβ-crystallin (Paper IV) conducted by homogenising bovine LD muscle in extraction buffer (10 mM Tris-HCl, 10 mM EDTA, 2 mM DDT; pH 7.5) then filtered through a fine wire mesh to remove large particles. The suspension was centrifuged at 15,000 gravities and the supernatant was stored at -80°C until purified.

Protein purification was conducted at 4°C using GE Pharmacia FPLC system, as described by Atomi *et al.*(1991) and Schoenmakers *et al.*(1969). Briefly, the soluble muscle suspension was initially passed through a Sephacryl S-200 size exclusion column (Amersham Biosciences, Fairfield, CT, USA; 17-0584-10), and the fractions containing αβ-crystallin, as determined by immunoblot were passed through a DEAE Sepharose fast flow FPLC column (Amersham Biosciences, 17-0907-01). Finally, αβ-crystallin was passed through a SP Sepharose fast flow column (Amersham

Biosciences, 17-0729-01) and αβ-crystallin was eluted using a linear salt gradient (0 to 0.5 M KCl), with αβ-crystallin eluting at approximately 0.3 M KCl. αβ-Crystallin fractions were pooled and dialysed against a buffer (10 mM NaH_2PO_4:$NaHPO_4$; pH 7.0), then concentrated to approximately 10 mg mL^{-1} using Centricon-10 filters (Millipore, 4206) and stored at -80°C. Purity and renaturation of the bovine αβ-crystallin was verified by sodium dodecyl gel electrophoresis (SDS-PAGE) (data not shown).

μ-Calpain induced proteolysis of stripped myofibrils

For Paper IV stripped myofibrils (10 μg) prepared as described earlier were resuspended in binding buffer (immunoprecipitation buffer excluding Complete™ protease inhibitor (pH 7.4)) and incubated at 37°C in the presence or absence of μ-calpain for 3 h. The myofibril preparation was centrifuged at 3000 g at 4°C for 5 min then the pellet was washed three times with the same buffer. The resulting pellet was denatured in Laemmli sample loading buffer and αβ-crystallin, desmin, and titin were detected by electrophoresis followed by Western blotting.

Quantitative determination of small heat shock proteins

Individual sHSP was measured by indirect enzyme-linked immunosorbent assay (ELISA) as described by Pulford *et al.* (2008), with some modifications. Sarcoplasmic fraction was adjusted to concentrations of 4 μg mL^{-1} protein with coating buffer (10 mM Na_2HPO_4, 15 mM NaCl; pH 7.4). Protein standards were prepared from purified (Hytest, 8HSP20), HSP27 (Hytest, 8HSP25) and αβ-crystallin (Sigma, C7858) diluted in coating buffer. An aliquot (100 μL) of samples and standards were dispensed into 96 well Costar® high binding polystyrene plates (Corning Inc., 3590) in duplicate. Plates were placed on an oscillating shaker overnight at 4°C. Following coating, the contents of all wells were discarded then all wells were blocked with 1% BSA in wash buffer (10 mM Na_2HPO_4, 15 mM NaCl, 0.1% Tween 20; pH 7.4) then washed four times with wash buffer. Following washing, 100 μL of mouse monoclonal antibody (HSP20, Hytest 4HSP20; HSP27, Hytest 4HSP27 and αβ-crystallin, Abcam ab74441) each diluted to 1:10,000 with 1% BSA in assay buffer then washed as previously described. Goat anti-mouse IgG (heavy and light chains) linked to horseradish peroxidase (BioRad Laboratories, #172-1011) was diluted to 1:5,000 in assay buffer (1% BSA) and 100 μL aliquots of this solution was dispensed into all wells and left to incubate for 1 h at room temperature. Following washing, 100 μL of 3,3',5,5'-tetramethybenzidine substrate

(Sigma, T0440) was dispensed into each well and left to incubate for 30 min. The reaction was stopped by dispensing 100 µL of 1 M H_2SO_4 into all wells. The absorbance of each well was measured at 450 nm with a Fluostar Optima plate reader (BMG Labtech, Ortenberg, Germany).

Statistical analysis

Paper I

Analysis of data was conducted using the REML directive of Genstat (Genstat, 2008). A REML analysis was conducted to determine significant differences in the means between seasons and slaughter plant. Analysis of deviance from binomial regression models was conducted to determine significant difference in the mean proportions of each pH_u category between slaughter plants for all seasons.

For the second trial, the data was analysed using a mixed model smoother (Upsdell, 1994). The model used time *post mortem* × Season + Day effect (a random term describing the day to day variability). The curves in the plots give the estimated means of the measured parameter at each time point for each of the seasons. The splines are 95% least significant intervals for testing differences between seasons. Thus, points within and between seasons are significantly different from each other if the bands do not overlap. Correlation analysis between pH_u and meteorological variables and variance analysis of cook loss, µ-calpain and cathepsin B activity were conducted using Minitab 15 (Minitab, 2006).

Papers II

Analysis of the shear force data was conducted using the REML directive of Genstat (Genstat, 2008) to determine variances and differences in mean values between low and high pH_u meat. Data analysis was also conducted using a mixed model smoother (Upsdell, 1994) with time *post mortem* × pH_u and time effect (a random term to describe variability between ageing timepoints) used as the model. The curves from the plots derived from the models are estimated curves of each of the measured parameters when grouped into timepoints *post mortem* and by pH_u. The splines are 95% least significant intervals for testing differences between groups. Thus, two groups are significantly different from each other if the splines do not overlap.

Paper IV

Statistical analysis of the data was conducted using a mixed model smoother (Upsdell, 1994). The model used was time *post mortem* × pH_u and time effect (a random term describing the variability between ageing timepoints). The curves in the plots are estimated means of the measured parameters plotted against pH_u and at increasing times *post mortem*. The splines are 95% least significant intervals for testing differences between and within groups. Points between and within groups that do not overlap are significantly different from each other.

Paper V

Statistical analysis of the data was conducted using the REML directive of Genstat (Genstat, 2008) to determine mean differences in shear force, relative μ-calpain activity, HSP20, HSP27, and αβ-crystallin between pH_u groups.

To test the significance between days and pH_u groups for each of the parameters measured, the data was analysed using a mixed model smoother (Upsdell, 1994). The model used was time *post mortem* × pH_u and time effect. Time effect was a random term describing the variability between ageing timepoints. The curves derived from the model show the estimated means of the parameters when grouped into time *post mortem* or ultimate pH_u. The splines are 95% least significant intervals for testing differences between groups. Thus, two groups are significantly different from each other if the splines do not overlap.

Summary of presented papers

Paper I

This study presents results from a survey of ultimate pH (pH_u) of slaughtered beef cattle in New Zealand with particular focus on bulls. Bulls (n = 1,597) slaughtered in four commercial abattoirs were categorised into low ($pH_u \leq 5.79$), intermediate ($5.8 \leq pH_u \leq 6.19$) or high ($pH_u \geq 6.2$) pH_u groups based on the pH of the *M. longissimus dorsi* at 48 hours *post mortem*. Overall, 51% of bull meat included in the survey were intermediate and high pH_u. There was a higher incidence of intermediate and high pH_u meat in autumn and spring compared with winter, and positive significant correlation between pH_u and mean maximum temperature and rainfall ($p < 0.01$ for both) on the day of slaughter. Variation in the pH_u distribution between the three abattoirs was also observed, with the incidence of intermediate and high pH_u meat being more prevalent in one abattoir than the other two in autumn and spring.

A follow-up study determined the effect of seasonal variation in pH_u, cook loss and shear force for bull meat from animals slaughtered in spring (n = 94) and summer (n = 63). The mean pH_u of meat from bulls slaughtered in summer was significantly higher than meat from bulls slaughtered in spring ($p < 0.05$), most likely owing to the higher incidence of intermediate and high pH_u in that season. Relative μ-calpain activity was also significantly higher at 0 day *post mortem* in summer than in spring. Although lower pH_u is usually believed to favour greater cook loss and cathepsin B activity, these parameters were curiously significantly higher in meat from bulls slaughtered in summer ($p < 0.05$), which had a higher mean pH_u compared with bulls slaughtered in spring.

Paper II

Bull *M. longissimus dorsi* (n = 63) were categorised into high ($pH \geq 6.2$), intermediate (pH 5.8 – 6.19) and low (≤ 5.79) ultimate pH (pH_u) groups and aged up to 28 days *post mortem* at -1.5°C. High pH_u samples were acceptably tender at 1 day *post mortem* and significantly more tender than low pH_u meat at all ageing timepoints ($p < 0.05$). Rapid autolysis of μ-calpain in high pH_u meat was associated with the more rapid degradation of titin, nebulin and filamin in this pH_u group. Desmin degraded faster in low pH_u meat and was concurrent with an increase of cathepsin B activity. The results from this study support the hypothesis that beef tenderisation is pH_u

compartmentalised. Tenderness in high and low pH_u meat is characterised by variable rate of degradation of high and low molecular weight myofibrillar proteins during ageing, which are in turn regulated by differential μ-calpain and cathepsin B activities.

Paper III

The development of meat quality is a complex interaction between the biological traits and biochemical processes during the conversion of muscle to meat. It was hypothesised that muscles inevitably engage towards apoptotic cell death due to the termination of oxygen and nutrient supply to the muscle following exsanguination. Thus, factors that regulate the process of apoptotic cell death of muscle cells are believed to ultimately influence meat quality. Proteomic studies have associated the regulation of small heat shock proteins (sHSPs) with various meat quality attributes including tenderness, colour, juiciness and flavour. Due to the anti-apoptotic and chaperone functions of sHSPs, they are proposed to be directly involved with development of meat quality. In this review, we discuss the possible chaperone and anti-apoptotic role of sHSPs during the conversion of muscle to meat and consider the repercussions of this on the development of meat tenderness.

Paper IV

This study aimed to determine how small heat shock proteins (sHSP) protect myofibrillar proteins from μ-calpain degradation during ageing. Immunoprecipitation experiments with *M. longissimus dorsi* (LD) from Angus heifers (n = 14) explored the interaction between αβ-crystallin, desmin, titin, HSP20, HSP27 and μ-calpain. Results showed that αβ-crystallin associated with desmin, titin, HSP20, HSP27 and μ-calpain. Exogenous αβ-crystallin reduced desmin and titin degradation in myofibrillar extracts and significantly reduced μ-calpain activity. In a second experiment, bull LD muscles (n = 94) were aged at -1.5°C for up to 28 days *post mortem*. μ-Calpain autolysed faster in high ultimate pH (pH_u) meat ($pH_u \geq 6.2$) and this was concomitant with the more rapid degradation of titin and filamin in this pH_u group. Desmin stability in intermediate pH_u meat (pH_u 5.8 to 6.19) may be due to the protection of myofibril-bound sHSP combined with the competitive inhibition of μ-calpain by sHSP.

Paper V

Bull *M. longissimus dorsi* (n = 94) categorised into high (pH ≥ 6.2), intermediate (pH 5.8 -6.19) and low (pH ≤ 5.79) ultimate pH (pH_u) groups and aged at -1.5°C for 28 days. Shear force was higher and more variable ($p < 0.05$) in intermediate pH_u samples during ageing. Titin, filamin and desmin degradation was also less extensive in intermediate pH_u samples compared to the other two pH categories. The extent of the decrease of HSP20, HSP27 and αβ-crystallin concentrations during *post mortem* ageing was pH_u related in that high pH_u meat maintained the highest concentration of small heat shock proteins followed by intermediate and low pH_u meat. μ-Calpain autolysis was slowest in intermediate pH_u and cathepsin B activities remained consistently low during ageing in this group ($p < 0.05$). Meat toughness in the intermediate pH_u group may be attributed to the combination of a larger pool of sHSP with a sub-optimal cathepsin B activity and intermediary μ-calpain activities.

Discussion

Meat tenderness is correlated with ultimate pH_u with a significant curvilinear relationship between meat pH_u and tenderness reported in beef, lamb and pork (Bouton, Harris & Shorthose, 1971; Devine, 1994a; Dransfield *et al.*, 1985; Jeremiah *et al.*, 1991). The rate at which meat tenderises is also pH_u related. Taking 11 kgF as the upper limit for acceptable tenderness in meat (Bickerstaffe, Bekhit, Robertson, Roberts & Geesink, 2001), shear force values reported in this thesis are consistent with previous studies with high and low pH_u meat attaining acceptable tenderness within 1 and 7 days *post mortem*, respectively (Paper II)

In contrast to low and high pH_u meat, intermediate pH_u tenderised at a slower rate and was significantly tougher compared with high and low pH_u meat even after ageing. Additionally, the shear force of intermediate pH meat was more variable compared with low and high pH_u meat suggesting that some intermediate pH_u will be tough even after extended ageing (Paper V). These findings agree with previous research reporting the delayed tenderisation (Bouton *et al.*, 1971; Pulford *et al.*, 2008; Watanabe *et al.*, 1996a) and higher shear force (Jeremiah *et al.*, 1991; Purchas *et al.*, 1993) in intermediate pH_u meat.

The current pH_u status of New Zealand bull beef

Previous pH_u surveys of New Zealand beef cattle have consistently reported the significant production of intermediate and high pH_u meat. In a survey of beef slaughtered in four commercial abattoirs Graafhuis & Devine (1994) reported a high incidence of intermediate and high pH_u meat from bulls (71%) compared with steers and cows (9% and 30%, respectively). With 30% of bull beef surveyed attaining intermediate pH_u, the incidence of intermediate pH_u meat in bulls was significantly higher than for steers and cows. A more recent study of 13,700 beef cattle (Young *et al.*, 2004) found that 62% of beef from bulls attained a pH_u greater than 5.7 compared with only 19% of beef from primes (steers and heifers). In this thesis, a survey of four commercial abattoirs was conducted to obtain a current outlook of the pH_u status of New Zealand bull beef (Paper I), realising however that the three studies are far from being ideally comparable.

Compared with previous surveys (Graafhuis *et al.*, 1994; Young *et al.*, 2004), 19% of all bull beef surveyed for this thesis attained intermediate pH_u, and the mean pH_u of bull beef was also lower than in the previous studies. This trend is supported by previous studies showing a declining trend of bull beef pH from 6.35 to 6.21 between 1990 and 1993 (Purchas, 1990; Purchas *et al.*, 1993). The decline in the mean pH of bull beef further declined to pH 6.16 in 1994 (Graafhuis *et al.*, 1994) and 5.97 as reported in this thesis.

The overall pH_u distribution of bulls may be related to the season of slaughter as indicated by the higher incidence of intermediate and high pH_u meat in autumn and spring compared with winter. Analysis of data from the different abattoirs revealed further variation in pH_u distribution between abattoirs within and between seasons. Overall, a lower incidence of intermediate and high pH_u meat was observed in winter for all abattoirs. These observations parallel results from other studies that similarly report a higher incidence of high pH_u meat from beef cattle slaughtered in autumn (Brown *et al.*, 1990; Tarrant *et al.*, 1980). However, one abattoir had higher combined proportions of intermediate and high pH_u meat in all seasons compared with the other abattoirs included in the survey.

Higher incidences of intermediate and high pH_u meat during autumn may be attributed to the sub-optimal quality of pastures due to the maturation of plants during autumn leading to pastures with high dead matter (Litherland *et al.*, 2002; Machado, Morris, Hodgson & Fathalla, 2005). This leads to plants in these pastures with decreased digestibility, low carbohydrate content and an overall low nutritive content. This results in depleted muscle glycogen reserves in beef cattle and consequently, animals that are more susceptible to higher pH_u.

The seasonal variation in the pH_u distribution of bull beef may be partly attributed to extreme environmental conditions increasing the susceptibility of animals to stress, resulting in the depletion of muscle glycogen and leading to higher incidences of intermediate and high pH_u meat (Scanga *et al.*, 1998). Elevated temperatures as observed during summer and autumn induces stress in cattle during transport and lairage consequently resulting in increased levels of meat with elevated pH_u (Fabiansson *et al.*, 1984; Mounier *et al.*, 2006). Young *et al.* (2004) reported that rainfall and mean maximum daily temperature on the day and days preceding slaughter is related with *post mortem* glycogen levels. These findings are consistent with results in Paper I that showed lower daily maximum temperatures on the day of slaughter favoured the production of low pH_u meat.

However, although Abattoirs 1 and 3 had similar mean maximum daily temperatures on the day of slaughter, the combined proportion of intermediate and high pH_u meat was consistently higher in Abattoirs 1. Furthermore, the combined proportion of intermediate and high pH_u meat for Abattoirs 2 and 3 were similar for autumn and spring, even though conditions were cooler in Plant 2 for both seasons. These results suggest that factors other than temperature may contribute to the variation in pH_u distribution observed between abattoirs.

The inconsistency in the pH_u profiles of bull beef between abattoirs may be due to lairage conditions, pre-slaughter facilities and handling of animals leading to slaughter. Mixing of unfamiliar mobs in open lairage pens was shown to induce stress brought about from aggressive behaviours as the animals attempt to establish a social hierarchy within the new group (McVeigh *et al.*, 1982; Warriss *et al.*, 1984). The detrimental results of mixing unfamiliar mobs is clear, with the incidence of high pH_u in bulls kept in open lairage pens three time higher compared with bulls kept in individual pens (Fabiansson *et al.*, 1984). A recent study of on-farm mixing of bulls prior to slaughter transport in New Zealand and the effects of this on muscle glycogen content, pH_u, overall meat quality and blood metabolites indicated that there is an increased risk of producing higher pH_u beef if bulls are mixed within 24 hours pre-slaughter (McDade, 2010). Although slaughter plants in New Zealand attempt to prevent the mixing of unfamiliar animals prior to slaughter, the high throughput of animals slaughtered within one day makes this unavoidable.

When comparing shear force values of beef from bulls slaughtered in spring and summer, beef from summer slaughtered bulls attained acceptable tenderness earlier and were significantly more tender compared with spring slaughtered bull beef, even when the mean pH_u of beef from both seasons were similar. However, variation in the pH_u distribution between the two seasons was observed, with a higher and lower incidence of low and intermediate pH_u meat, respectively, in spring slaughtered bull beef. It can be argued that these differences are simply due to day-to-day variation of shear force as all muscle samples from spring were collected on the same day. However, meat from the two slaughter days in summer were similar in shear force, with meat from both these days being significantly more tender than beef from spring slaughtered bulls (data not shown). This indicates a seasonal variation in shear force and that that factors other pH_u are involved in the variation of tenderness in beef.

Regardless of season and abattoir, there is still a significant prevalence of intermediate pH_u meat derived from New Zealand bulls. Due to the inconsistent

tenderness of intermediate pH_u meat, they are sold as frozen manufacturing grade commodities, even though a bulk these will be acceptably tender and could be marketed as higher value chilled table cuts. To fully realise the economic potential of bull beef, the challenge for the meat industry is to reduce the incidence of intermediate and high pH_u meat and to segregate intermediate pH_u meat that will attain acceptable tenderness from those that will be unacceptably tough. Understanding the mechanisms causing inconsistent tenderness in beef may be critical in achieving this aim.

The development of tenderness in low and high pH_u meat

Meat tenderness is attributed to the weakening of the organised myofibrillar structure due to the proteolytic degradation of key proteins *post mortem*. Researchers have attempted to relate the degradation of these proteins with the ultimate tenderness that meat will eventually attain. This part of the thesis aimed to further investigate the mechanisms surrounding the different rate of tenderisation as observed in low and high pH_u meat (Paper II).

The variation in the tenderisation of low and high pH_u meat may be due to the differential degradation rates of myofibrillar proteins in these pH_u groups (Papers II, IV and V). Analysis of whole muscle extracts by SDS-PAGE Coomassie blue revealed the more rapid rate of degradation of high molecular weight proteins titin, nebulin and filamin in high pH_u meat than in low pH_u meat. Conversely, Western blot analysis of lower molecular weight proteins myosin and desmin were found to be more extensively degraded in low pH_u meat. These findings are consistent with previous studies (Watanabe & Devine, 1996b; Yu & Lee, 1986) that report the faster degradation rates of titin and nebulin in high pH_u meat. Additionally, the preferential degradation of myosin in low pH_u meat is consistent with previous research that relate the degradation of myosin to the fragmentation of the M-line of the myofibrils, contributing to the tenderness of low pH_u meat (Yates, Dutson, Caldwell & Carpenter, 1983; Yu *et al.*, 1986).

It is widely accepted that *post mortem* myofibrillar protein degradation is due to the proteolytic action of endogenous proteases in muscle. However, there is still some disagreement as to which proteases are variably involved in muscle tenderisation. Two proteases implicated in *post mortem* myofibrillar protein degradation are μ-calpain and cathepsin B. This thesis investigated the activities of these enzymes in relation to myofibrillar protein degradation and meat tenderness at different pH_u values.

μ-Calpains are optimally active at physiological pH and are activated by the accumulation of Ca^{2+} in muscle sarcoplasm *post mortem* (Jeacocke, 1993; Koohmaraie, 1992a). Quantitative determination of relative μ-calpain activity found that μ-calpain activity across the pH_u spectrum declined during ageing such that at 7 days *post mortem*, minimal μ-calpain activity was detected (Papers IV and V). In addition, a negative relationship was observed between μ-calpain and pH_u such that enzyme activity in high pH_u was generally lower than in low pH_u meat at 0 and 1 day *post mortem*.

Activation of μ-calpain occurs concomitantly with the autolysis of the enzyme (Baki *et al.*, 1996). Initial autolysis of the large μ-calpain sub-unit from 80 kDa to 78 kDa has been found to enhance its proteolytic activity, but the enzyme eventually becomes inactivated following further autolysis of the intermediary 78 kDa sub-unit to 76 kDa (Edmunds *et al.*, 1991; Li, Thompson & Goll, 2004). Results reported in this thesis found the earlier and more rapid autolysis rates of μ-calpain in high pH_u meat compared with low pH_u meat (Papers II, IV and V). Taking autolysis as an indicator of enzyme activation, μ-calpain was already active at 0 day *post mortem* in high pH_u meat. In comparison, enzyme activation in low pH_u meat was delayed, with enzyme autolysis detected at 1 day *post mortem*.

Upon activation, μ-calpain increasingly associates with myofibrils (Boehm, Kendall, Thompson & Goll, 1998) and is proteolytically active until autolysed extensively. However, these activities are often not represented in quantitative assays as myofibril-bound μ-calpains are difficult to extract and to not react with exogenous substrates used to measure μ-calpain activity (Delgado, Geesink, Marchello, Goll & Koohmaraie, 2001). Thus, lower quantitative activities and autolysis of μ-calpain early *post mortem* as observed in high pH_u meat may be interpreted as an indicator of μ-calpain activation with greater levels of myfibrillar-bound μ-calpain in this group early *post mortem*.

Several studies have demonstrated the ability of μ-calpain to degrade myofibrillar proteins associated with meat tenderness. The fragmentation of linkages in the I-band and weakening of N_2 lines in the myofibrils was attributed to the degradation of titin and nebulin by μ-calpain (Boyer-Berri & Greaser, 1998; Taylor, Geesink, Thompson, Koohmaraie & Goll, 1995). Furthermore, μ-calpain was found to degrade several myofibrillar proteins *in vitro* that are similarly degraded in meat during *post mortem* ageing (Huff-Lonergan *et al.*, 1996).

Although the contribution of cathepsin B to meat tenderisation has been disputed (Hopkins *et al.*, 2004), the decline in muscle pH observed in *post mortem* muscle results in the release of cathepsins from the lysosome making them available to hydrolyse myofibrillar proteins (Sancho, Jaime, Beltran & Roncales, 1997). Cathepsins are active at pH values between 3.0 and 6.0 (Etherington, 1984) and have been shown to destabilise myofibrillar A-band and Z-lines *in vitro* (Ouali *et al.*, 1987; Zeece *et al.*, 1989) and degrade desmin, titin and troponin T (Dutson, 1983; Zeece *et al.*, 1989).

Measurements of cathepsin B activity in this thesis found the activity of this enzyme progressively increased in low pH_u meat *post mortem* with ageing. In contrast, cathepsin B activity in high pH_u was significantly lower than low pH_u meat and remained low throughout the ageing period (Papers II and V). The low activity of cathepsin B activity in high pH_u meat is due to the minimal release of the enzyme in this group owing to the higher stability of lysosomal membranes at higher pH values (Ertbjerg, Henckel, Karlsson, Larsen & Moller, 1999). Supposing that cathepsins were released from the lysosome in high pH_u meat, they would still be limited by the near neutral pH level of meat in this group, which does not favour cathepsin activity.

Based on the degradation of myofibrillar proteins and proteolytic activity reported in this thesis, it is hypothesised that the aetiology of meat tenderness is likely to be compartmentalised by pH_u. The activation of µ-calpain, as indicated by autolysis, in high pH_u meat immediately *post mortem* was concomitant with the faster degradation rates of higher molecular weight proteins titin, nebulin and filamin during ageing consequently resulting in the immediate tenderisation observed in this pH_u group. As cathepsin B activity is limited by the inherent pH of high pH_u meat, cathepsin B does not contribute to the tenderisation of this pH_u group.

In contrast with high pH_u meat, early tenderisation of low pH_u meat is due to the degradation of higher molecular weight proteins in this group, which corresponded with the slower autolysis of µ-calpain. However, autolysis and pH decline during the conversion of muscle to meat limits µ-calpain activity in low pH_u meat. At latter ageing timepoints the increased activity of cathepsin B coincided with the further degradation of desmin and filamin and decline in shear force. Thus tenderness in low pH_u meat is attributed to the degradation of higher molecular weight proteins by µ-calpain *pre rigor* and combined with the fragmentation of smaller molecular weight proteins by cathepsin B during ageing.

Inconsistent tenderness in intermediate pH_u meat

Higher shear force values in intermediate pH_u meat corresponded with the delayed and less extensive degradation of myofibrillar proteins in this group even after ageing for 28 days *post mortem* (Papers IV and V). Although relative µ-calpain activity in this group was intermediary in relation to µ-calpain activity in high and low pH_u meat, the rate of µ-calpain autolysis in intermediate pH_u meat was much slower compared to low and high pH_u meat, suggesting the slower activation of this enzyme (Papers IV and V). In addition, cathepsin B activity remained consistently low in intermediate pH_u throughout ageing due to the stability of lysosomal membranes in the intermediate pH_u range (Paper V). Thus, less extensive degradation of myofibrillar proteins in intermediate pH_u meat is due at least to the sub-optimal activity of µ-calpain and cathepsin B consequently resulting in tougher meat in this group.

However, the high shear force variance in this group suggests that while some meat will be tough, a bulk of intermediate pH_u meat will attain acceptable tenderness. This indicates that existing proteolytic activity in this pH_u group is sufficient to induce adequate myofibrillar protein degradation resulting in acceptably tender meat. Thus, mechanisms beyond enzyme proteases may be involved in regulating the proteolytic degradation of myofibrillar proteins leading to inconsistent tenderness observed in intermediate pH_u beef.

Ouali *et al.* (2006) and Herrera-Mendez (2006) proposed that the *post mortem* termination of oxygen and nutrient to supply to the muscle combined with the decline in pH results in the inevitable apoptotic death of muscle cells. Skeletal muscle cells have been demonstrated to undergo apoptotic cell death *post mortem* (Becila *et al.,* 2010). In response to impending cell death, the up-regulation of sHSP is triggered to combat or impede the onset of apoptosis and maintain cellular homeostasis.

A review of the constitutive role of sHSP in muscle cells during normal development and stress is presented in Paper III. Briefly, sHSP are chaperone proteins that passively bind and stabilise denaturing proteins, preventing their irreversible aggregation during periods of heat and oxidative stress (Fu & Liang, 2003; Haslbeck *et al.,* 1999; Jakob, Gaestel, Engel & Buchner, 1993; Rao, Horwitz & Zigler, 1993). In addition, sHSP interfere with cellular signal transduction pathways that regulate apoptotic cell death, thereby negatively regulating normal apoptosis in living organisms (Arrigo, 2005; Beere, 2004; Samali & Orrenius, 1998). In this section, results supporting the chaperoning and anti-apoptotic function of sHSP in bovine muscle *post mortem* is presented and the implications of these on meat tenderness is discussed.

The protection of skeletal myofibrils from proteolytic damage by small heat shock proteins

In an attempt to find new biomarkers for meat quality, meat scientists have attempted to relate the proteomic profile of muscle with several meat quality attributes. These studies revealed a significant negative correlation between the expression of HSP27 and shear force in beef (Kim *et al.*, 2008) and pork (Hwang, Park, Kim, Cho & Lee, 2005). Due to the chaperone ability of sHSP to maintain the integrity of myofibrillar proteins, the down-regulation of HSP27 and αβ-crystallin was proposed to favour proteolytic degradation consequently resulting in tender meat (Bernard, Cassar-Malek, Le Cunff, Dubroeucq, Renand & Hocquette, 2007).

To determine the role of sHSP in meat tenderness, this thesis sought to investigate whether sHSP offered any protection to the proteolytic degradation of myofibrillar proteins and aimed to characterise the mechanisms driving this (Paper IV). Immunoprecipitation experiments to determine the localisation of sHSP in *post mortem* muscle cells revealed the association of αβ-crystallin with HSP20, desmin and μ-calpain in both sarcoplasmic and myofibrillar muscle fractions. αβ-Crystallin also increasingly associated with the muscle myofibrils as intramuscular pH declined from pH 7.4 to 6.5. The pH-related association of sHSP with muscle myofibrils agrees with results by Bennardini *et al.* (1992) who similarly reported the increased binding of αβ-crystallin to cardiac myofibrils in acidic buffer. In addition the association of αβ-crystallin with the Z-disc and I-band regions of the myofibril reported by Golenhofen *et al.* (2004) is also consistent with results in this thesis. Because desmin is a constituent of the Z-disc region of myofibrils, the association of αβ-crystallin with desmin confirms their localisation in the Z-disc region of skeletal myofibrillar proteins in *post mortem* muscle.

To determine whether sHSP protected myfibrillar proteins from proteolytic degradation *in vitro*, stripped myofibrils with or without added exogenous αβ-crystallin were incubated with activated μ-calpain. The degradation of titin and desmin was less extensive where αβ-crystallin was added. These results suggest that αβ-crystallin is able to attenuate the μ-calpain induced degradation of desmin and titin.

Further analysis of αβ-crystallin in myofibrils with exogenous αβ-crystallin by Western blotting revealed the progressive degradation of this sHSP with increasing incubation time. With the association of αβ-crystallin with μ-calpain already demonstrated in previous experiments, these results suggest that αβ-crystallin is also a substrate for μ-calpain. This finding was confirmed by the reduced catalysis of

fluorogenic μ-calpain-specific substrate in the presence of αβ-crystallin. These results suggest that αβ-crystallin acts as a competitive inhibitor of μ-calpain activity against myofibrillar proteins.

Overall, evidence for the protection of myofibrillar proteins from μ-calpain induced degradation by sHSP early *post mortem* are presented in this thesis. sHSP have been demonstrated to reduce the degradation of myofibrillar proteins *post mortem* muscle. The chaperoning function of sHSP on muscle myofibrils is facilitated by the increasing association of sHSP with muscle myofibrils as the intramuscular pH of muscle declines early *post mortem*. Aside from preventing the irreversible degradation of damaged proteins, sHSP may also inhibit μ-calpain activity by functioning as a competitive substrate for the enzyme. It is hypothesised that the association of sHSP to the myofibrils is optimal in the intermediate pH_u range leading to the greater protection from myfibrillar protein degradation by μ-calpain in this group and consequently, tougher meat.

The role of small heat shock proteins in the development of meat tenderness

To determine whether the *in vitro* protection of myofibrillar proteins by sHSP from μ-calpain proteolysis actually occurs in *post mortem* muscle during ageing, the sHSP levels in *post mortem* muscle at various ageing timepoints was determined and related to the degradation of myofibrillar proteins, proteolytic activity and meat tenderness. Quantitative determination of HSP20, HSP27 and αβ-crystallin in muscle sarcoplasm revealed the decline of all sHSP to minimum concentrations within the first two days *post mortem* (Papers IV and V). However, the extent of sHSP decline in muscle sarcoplasm was pH_u-related in that high pH_u meat maintained higher concentrations of sHSP throughout ageing. The loss of sHSP from the muscle sarcoplasm was also confirmed by Western Blot analysis which showed the more extensive disappearance of sHSP band densities in low pH_u meat followed by intermediate then high pH_u meat.

Previous experiments in this thesis (Paper IV) showed the increasing association of αβ-crystallin with muscle myofibrils at decreasing pH. Considering the kinetics of sHSP in the sarcoplasmic and myofibrillar fraction, the increasing association of sHSP with muscle myofibrils at declining pH_u suggest that sHSP translocate from the sarcoplasm to the myofibrillar fraction. However, the association of sHSP with muscle myofibrils was suggested to be limited by their respective isoelectric points, such that sHSP form non-functional aggregates once pH declines to levels below the isoelectric

point of each sHSP (pH 6.49, 5.98 and 6.76 for HSP20, HSP27 and αβ-crystallin, respectively) (Pulford *et al.*, 2008).

Interestingly, Paper V shows that sarcoplasmic sHSP were found to be degraded in high pH_u meat, but not in intermediate and low pH_u meat. This is attributed to the proteolytic activity of μ-calpain, which was shown to degrade αβ-crystallin *in vitro*. The absence of sHSP degradation in intermediate and low pH_u meat is due to the minimal activity of μ-calpain, which is limited by the inherent pH in these groups.

Although overall sHSP concentrations are greater in high pH_u meat, the amount of myofibril-bound sHSP to protect myofibrils from proteolytic degradation is minimal in this group as sHSP are predominantly located in muscle sarcoplasm. Additionally, sHSP in high pH_u degraded in high pH_u meat during ageing is speculated to be due to the μ-calpain which is optimally active in this pH_u group. In contrast, the chaperone activity of sHSP in low pH_u meat is nullified by the aggregation of sHSP as the inherent pH declines below the isoelectric points of each sHSP. Thus, the chaperoning activity in high and low pH_u meat is limited by sHSP degradation and aggregation, respectively.

On the whole, results from the analysis of sHSP in muscle fractions indicate the potentially greater affinity for sHSP to bind to muscle myofibrils in intermediate pH_u meat. Two pathways by which greater levels of myofibrillar-bound sHSP in intermediate pH_u meat consequently results in toughness in this group is proposed in this thesis. First, greater concentrations of sHSP in intermediate pH_u confer greater protection of myofibrillar proteins from proteolytic degradation during ageing leading to less extensive myofibrillar protein degradation and consequently, tougher meat.

Finally, sHSP impede the onset of apoptosis in *post mortem* muscle by interfering with the activation of caspases that mediate apoptotic cell death (Arrigo, 2005; Beere, 2004; Beere, 2005). Pulford *et al.* (2009) reported the delayed activation of effector caspase 3/7 in intermediate pH_u meat compared with low and high pH_u meat. In addition, the pH decline of muscle during the conversion of muscle to meat is associated with meat tenderness (Hwang & Thompson, 2001). A distinguishing feature of apoptosis is the simultaneous inversion of acidic phosphotidylserine groups from the internal to the external leaflet of the cellular membrane and the basic phosphatidylcholine and phosphatidylethanolamine groups from the external to the internal leaflet (Becila *et al.*, 2010; Martin *et al.*, 1995). The inversion of these phospholipid groups modifies the buffering capacity and charge distribution within muscle cells. It was proposed that sHSP interfere with the phospholipid inversion

during apoptosis resulting in the delayed muscle pH decline and leading to tougher meat (Ouali *et al.*, 2007).

The reasons for the large variation in meat tenderness in intermediate pH_u meat is still an enigma to meat science. With the correlation between sHSP expression and meat tenderness, it is hypothesised that the meat tenderness in this group is related with the expression of sHSP. Specifically, intermediate pH_u meat with elevated sHSP levels will inevitably yield unacceptably tough meat and the inverse will be true for tender intermediate pH_u meat. However, the mechanisms by which sHSP are recruited to chaperone denaturing proteins and regulate apoptosis requires further characterisation to verify their proposed role in the development of meat tenderness.

Implications

Based on the results reported in this thesis, contribution of specific myofibrillar proteins to meat tenderisation requires reassessment. Research by Takahashi (1996) and Yu *et al.* (1986) reported the weakening of the M-line, indicated by myosin degradation, as contributing to meat tenderness. However, results in this thesis show that significant degradation of myosin in low pH_u meat at 1 day *post mortem* did not coincide with any significant decline in shear force in this group. Minimal myosin degradation was observed in high pH_u meat, which was already acceptably tender at 1 day *post mortem*. Furthermore, although desmin was found to degrade in both low and high pH_u, any contribution of desmin to meat tenderness may be more significant in low pH_u meat where desmin degradation was concomitant with shear force decline. The contribution of desmin degradation to tenderness in high pH_u meat seems to be negligible as this group was already acceptably tender long before desmin degradation was initially detected.

Continuous contractile activity, induced by low voltage electrical stimulation, was found to up-regulate the expression of αβ-crystallin and HSP27 in muscle (Koh & Escobedo, 2004; Neufer & Benjamin, 1996). Furthermore, electrical stimulation favoured the translocation of sHSP from the sarcoplasm to the Z-disc contributing to the stabilisation of this region (Koh *et al.*, 2004). These studies implicate the widespread practice of electrical inputs on beef carcasses in New Zealand. Electrical stimulation of carcasses is conducted to avoid the phenomenon of shortening which occurs when muscles are rapidly cooled *pre rigor* (Locker *et al.*, 1963), particularly in hot-boned muscles. However, electrical stimulation and immobilisation of carcasses may favour

the translocation of sHSP from the sarcoplasm to the myofibrils leading to greater protection of myofibrils from proteolytic degradation, particularly in intermediate pH_u meat. With the emerging role of sHSP in the development of meat tenderness, processing protocols such as the use of electrical inputs on carcasses and their effects on the sHSP expression and localisation on muscle require evaluation.

Conclusions

- Data from a pH_u survey of New Zealand bull beef revealed that 19 percent of bulls included in the survey generated intermediate pH_u beef in an indicator muscle (*M. longissimus dorsi*). Seasonal variation and between plant variations in the pH_u distribution was also observed. Higher incidences of intermediate and high pH_u meat were observed from autumn and spring bull beef compared with meat from bulls slaughtered in winter. The combined frequency of intermediate and high pH_u meat was noticeably higher in one of the abattoirs surveyed compared with the others.

- Shear force, cook loss and proteolytic activity were found to be significantly different between beef from spring and summer slaughtered bulls. Although the mean pH_u of beef from both seasons were similar, significantly lower shear force and higher early *post mortem* μ-calpain activity were observed in summer slaughtered bull beef, suggesting that factors other than pH_u contribute to the seasonal variation in bull beef quality.

- Results presented in this thesis support the hypothesis of the pH_u compartmentalisation of tenderness in beef. The rapid tenderisation of high pH_u meat is owing to the rapid activation of μ-calpain resulting in faster degradation rates of higher molecular weight proteins such as titin, nebulin and filamin. Tenderness in low pH_u meat is proposed to be due to the early *post mortem* degradation of higher molecular weight proteins by μ-calpain combined with the degradation of lower molecular weight proteins such as desmin by cathepsin B at latter ageing timepoints.

- αβ-Crystallin was found increasingly associated with muscle myofibrils early *post mortem* as muscle pH declined. Exogenous αβ-crystallin reduced the μ-calpain-induced degradation of titin and desmin in myofibrils. Furthermore, αβ-crystallin reduced μ-calpain activity by functioning as a competitive inhibitor of the enzyme.

- The less extensive degradation of myofibrillar proteins in intermediate pH_u meat is also due to the sub-optimal activity of μ-calpain and cathepsin B proteases in this

group. The decline of sHSP levels in muscle sarcoplasm corresponded with increasing association of αβ-crystallin myofibrils suggesting that sHSP translocate to the myofibrils in *post mortem* muscle. However, this association is pH_u-dependent in that myofibril-bound sHSP is highest in intermediate pH_u meat. Therefore, toughness in intermediate pH_u meat is attributed to the sub-optimal μ-calpain and cathepsin B activities combined with the protection of myofibrillar protein from proteolytic degradation.

Future investigations

The results in this thesis provide support to the potential role of sHSP in the development of tenderness in meat. With regards to the contribution of sHSP to the inconsistent tenderness observed in intermediate pH_u bull beef, the following future investigations are recommended:

- Further investigations to confirm that muscle cells indeed undergo apoptotic cell death of *post mortem* in beef carcasses during the conversion of muscle to meat, and to confirm the ability of sHSP to inhibit or delay the onset of apoptosis is required.

- Further characterise the kinetics of sHSP in the myofibrillar fraction of muscle during ageing in low, intermediate and high pH_u meat to confirm the hypothesis that myofibril-bond sHSP concentrations are greater in intermediate pH_u leading to the greater protection of myofibrils from proteolytic degradation in this group

- Investigate the effects of electrical stimulation on the expression of sHSP in muscle and the translocation of these sHSP from the muscle sarcoplasm to the myofibrils and the effects of these on myofibrillar protein degradation, proteolytic enzyme activity and meat tenderness during ageing.

- Determine the role of sHSP in the inconsistent tenderness in intermediate pH_u meat by characterising the expression and localisation of sHSP in tough and tender intermediate pH_u meat and related these with shear force, proteolytic enzyme activity, pH_u and myofibrillar protein degradation.

- Elucidate the structural and post-transcriptional changes taking place in *post mortem* muscle during ageing and how these relate to the chaperoning function of sHSP and meat tenderness.

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I

The pH status and seasonal variation of meat quality attributes in New Zealand beef

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Abstract

This study presents results from a survey of ultimate pH (pH_u) of beef cattle in New Zealand with particular focus on bull beef. Bulls (n = 1,597) slaughtered in three commercial abattoirs were categorised into low ($pH_u \leq 5.79$), intermediate ($5.8 \leq pH_u \leq 6.19$) or high ($pH_u \geq 6.2$) pH_u groups based on the pH of the *M. longissimus dorsi* at 48 hours *post mortem*. Overall, 51% of bull meat included in the survey were intermediate and high pH_u. There was a higher incidence of intermediate and high pH_u meat in autumn and spring compared with winter, and significant correlation ($p < 0.05$) between pH_u and mean maximum temperature and rainfall on the day of slaughter was observed. Variation in the pH_u distribution between the three abattoirs was also observed, with the incidence of intermediate and high pH_u meat being more prevalent in one abattoir compared to the other two in autumn and spring.

A follow-up study determined the effect of seasonal variation in pH_u, cook loss and shear force for bull meat from animals slaughtered in spring (n = 94) and summer (n = 63). The mean pH_u of meat from bulls slaughtered in summer was significantly higher compared with meat from bulls slaughtered in spring ($p < 0.05$), most likely owing to the higher incidence of intermediate and high pH_u in this season. Relative μ-calpain activity was also significantly higher at 0 day *post mortem* in summer. Although lower pH_u favours greater cook loss and cathepsin B activity, these were significantly higher in bull slaughtered in summer ($p < 0.05$), which had a higher mean pH_u compared with bulls slaughtered in spring.

Introduction

Due to the extensive pastoral production system of cattle in New Zealand, psychological and physiological factors combine to generate a range of meat ultimate pH (pH_u) values in beef. Meat pH_u is widely used as an indicator of meat quality, and carcasses are often categorised according to its pH_u. Briefly, low pH_u meat ($pH_u < 5.8$) is most ideal with regards to consumer acceptability and palatability. High pH_u meat ($pH_u \geq 6.2$) is darker in colour and more susceptible to microbial spoilage (Newton & Gill, 1981). However, high pH_u meat has excellent manufacturing properties (Young, Zhang, Farouk & Podmore, 2005) and is commonly used for the production of processed meat products.

Meat with a pH_u between 5.8 and 6.19 is categorised as intermediate pH_u. Intermediate pH_u meat is notorious for being inconsistently tender. Although a portion of intermediate pH_u meat can age normally and reach acceptable tenderness (Silva, Patarata & Martins, 1999), a significant portion will be initially tough and tenderise at a slower rate compared with low and high pH_u meat (Bouton, Harris & Shorthose, 1971; Purchas, Yan & Hartley, 1999). The mechanisms driving the delayed tenderisation and toughness in intermediate pH_u is still largely unknown.

To determine the pH_u status of New Zealand beef, a comprehensive survey of four abattoirs in representative regions of the country was conducted in the 1990s (Graafhuis & Devine, 1994). In this survey, pH_u measurements from *M. longissimus dorsi* were determined 24 hours *post mortem*. The findings from the survey reported significantly lower incidence of low pH_u meat in bulls (29 percent) compared with steers and cows (91 and 70 percent, respectively). With 30 percent of bull beef categorised as intermediate pH_u, the prevalence of intermediate pH_u in bulls was considerably higher compared with steers and cows. In addition, seasonal variation in the distribution of pH_u was observed for bulls, with a lower incidence of low pH_u beef in autumn (66 percent) compared with spring (83 percent).

Although some pH data of New Zealand beef have been reported subsequent to the survey conducted by Graafhuis & Devine (1994), these have been restricted to one abattoir. Thus, there was a need for representative survey of the current pH_u status of beef cattle in New Zealand, and is the main objective of this study. This research particularly focuses on bull beef due to the high prevalence of intermediate and high pH_u in this animal category and the financial potential for the meat industry in adding value to this product.

Materials and methods

Animals and sample collection

Four abattoirs were chosen to represent typical beef abattoirs in New Zealand. The survey was conducted at three different times of the year (summer, winter and spring) over a period of two years. All abattoirs in the survey practiced hot boning for all beef carcasses. Upon reaching the boning floor, the *M. longissimus dorsi* (LD) from bull carcasses (n = 1,597) was hot boned from the left side of the carcass and a 2.5 cm thick muscle sample was cut at the 12^{th} rib, the quartering cut site. Samples were labelled, packed in plastic bags and then stored at -1.5°C for 48 hours.

For a second part of this study, LD from the left side of bull carcasses that were slaughtered in a commercial abattoir were collected in spring (n = 63) and summer. Muscle samples in the summer were collected over two consecutive days with 49 and 45 LD samples collected on the first and second day, respectively. The LD muscles were hot-boned upon reaching the boning floor and 10 grams was dissected from the anterior end of each muscle, frozen in liquid nitrogen and stored at -80°C until analysed. The remaining muscle was packed in plastic bags and stored at -1.5°C for 24 hours then each LD were cut into six equally sized sub-samples then vacuum packed. The sub-samples were randomly allocated an ageing timepoint (1, 2, 7, 14 and 28 days *post mortem*) and stored at -1.5°C. A 10 g sample was dissected from all sub-samples after their allocated ageing timepoint, frozen in liquid nitrogen, then stored at -80°C until analysed.

Meteorological data

Data from meteorological stations that supply the National Institute of Water and Atmospheric Research national climate database in New Zealand was used in this study. Data were gathered from stations closest or located in the same towns as the abattoirs. Mean maximum temperatures and rainfall at each of the slaughter days were averaged for each season for both trials (Table 1). For Trial 2, maximum temperatures and rainfall in the summer were similar to the daily mean for that slaughter month.

Measurement of pH, cook loss and shear force

Ultimate pH was measured in all samples at 2 days *post mortem* with a portable pH meter (Mettler Toledo, MP 125, Switzerland) fitted with a Mettler Toledo combination

electrode (InLab® 427) with automatic temperature compensation. The pH meter was calibrated at pH 7.0 and 4.0 with buffers (Mallinckrodt Chemicals, Phillipsburgh, NJ, USA) stored at room temperature (20°C).

The shear force and cook loss of all sub-samples in the second study was determined by recording the weight of all uncooked sub-samples prior to cooking, then cooking the samples in weighted plastic bags in a 100°C water bath to an internal temperature of 75°C. After cooling in an ice water bath, the samples were patted dry with a paper towel, and then reweighed. Cook loss was calculated from the difference of the initial uncooked weight of the sample and the cooked sample, after drying the surface with a paper towel and expressed as the percentage of the initial uncooked weight. The cooked shear force of each sub-sample was measured with a MIRINZ tenderometer as described by Pulford *et al.* (2008).

Measurement of µ-calpain activity

Five grams of frozen muscle was crushed with a pestle and then dispersed in 25 mL ice cold extraction buffer (75 mM Tris-Base, 10 mM EDTA, 10 mM 2-mercaptoethanol, 0.2% Triton X-100; pH 7.5) with an Ika®-Labortechnik Ultra-Turrax T25 (Staufen, Germany) fitted with a S25 dispersing tool. The homogenate was centrifuged at 30,000 g for 30 minutes at 4°C and the supernatant was then filtered through cotton gauze and glass wool. Separation of µ-calpain was conducted using a step-wise salt gradient on a DEAE Sephacel ion exchange chromatography as originally described by Wheeler & Koohmaraie (1991). The relative µ-calpain activity of extracted muscle calpain was conducted using a fluorescent substrate described by Wiklund *et al.* (2010).

Measurement of cathepsin B activity

Extraction and measurement of cathepsin B activity was determined according to Caballero *et al.* (2007) with minor modifications. Two grams of frozen muscle sample was dispersed in 18 mL of extraction buffer (10 mM potassium phosphate, 1 mM EDTA, 50 mM NaCl, 250 mM sucrose; pH 7.4). Cathepsin B standard curves were prepared from purified cathepsin B (Sigma, C6286) and diluted in incubation buffer (100 mM sodium acetate, 1 mM EDTA, 5 mM DTT, 0.1% Brij 35; pH 5.5). Forty microlitre sample extracts and standards were diluted with 300 µL of incubation buffer, then 25 µL of each diluted mixture was dispensed into a 96 well black-walled fluorescence microtiter plate (Nunc, #265301). 70 µL of incubation buffer was

dispensed into each well to make up the total volume in each well to 95 µL. After incubation at 37°C for 10 minutes, 5 µL of 40 µM Cathepsin B fluorogenic substrate III (Calbiochem, 219392) was dispensed into each well. The fluorescence emitted by samples and standards were measured every minute for 1 hour at 37°C with a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany) fitted with 360 nm excitation and 460 emission filters.

Statistical analysis

Data analysis from the first trial was conducted Genstat (Genstat, 2008). To determine significant differences in the means between seasons and abattoir, REML analysis was conducted. Analysis of deviance from binomial regression models was conducted to determine significant difference in the mean proportions of each pH_u category between abattoirs for all seasons.

For the second trial, the data was analysed using a mixed model smoother (Upsdell, 1994). The model used time *post mortem* × Season + Day effect (a random term describing the day to day variability). The curves in the plots give the estimated means of the measured parameter at each time point for each of the seasons. The bands are 95% least significant intervals for testing differences between seasons. Thus, points within and between seasons are significantly different from each other if the bands do not overlap. Correlation analysis between pH_u and meteorological variables and variance analysis of cook loss, µ-calpain and cathepsin B activity were conducted using Minitab 15 (Minitab, 2006).

Results

Ultimate pH survey of New Zealand beef

The distribution of pH_u combined for all animal categories, abattoirs and seasons are presented in Figure 1. Bulls produced significantly higher proportions of intermediate and high pH_u meat, leading to a higher mean pH of 5.97 ± 0.46. For steers and cows, the majority of animals attained low pH_u meat. This explains the lower overall pH_u means for steers and cows, 5.61 ± 0.29 and 5.53 ± 0.21, respectively.

A comparison of the present results with those from a similar New Zealand survey published in 1994 (Graafhuis *et al.*, 1994) is shown in Table 2. It is clear that the proportion of low pH_u meat markedly increased in bulls in this survey compared

with that conducted by Graafhuis *et al.* (1994). Although there has been an improvement in the proportion of low pH_u meat produced from bulls, there was still a high incidence of intermediate and high pH_u bulls, with 51% in these two categories.

The seasonal variation in the pH_u distribution of bulls is shown in Figure 2. There was a higher proportion of low pH_u meat in the winter compared with autumn and spring. The distribution of low and high pH_u meat in autumn and spring were similar, but there was a higher incidence of intermediate pH_u meat in bulls slaughtered in the autumn.

It is evident that there is variation in the pH_u distribution of bull meat between abattoirs for all three seasons (Figure 3). Due to logistic reasons, a single pH_u survey could only be conducted in Abattoir 4 during the spring of the first year. Thus, data from Abattoir 4 was included in analysis of the overall and seasonal comparisons of pH_u distributions but not in the between-abattoir comparisons. In autumn, Abattoir 1 had a higher frequency of intermediate and high pH_u meat compared with the other abattoirs. Abattoir 3 clearly had the greatest proportion of low pH_u bull beef. The incidence of intermediate and high pH_u bull meat in Abattoirs 1 and 3 was reduced in winter, coincident with an increased proportion of low pH_u bull meat. In spring, Abattoir 1had a higher proportion of high pH_u bull meat and a lower frequency of low pH_u bull meat compared with Abattoirs 2 and 3.

Seasonal variation in the pH_u distribution of bull meat was also observed within each abattoir, particularly for Abattoirs 1 and 3. The prevalence of high pH_u meat was highest in spring for Abattoirs 1 and 3, but there was a higher incidence of intermediate pH_u meat in autumn for Abattoir 1. For Abattoir 2, although a higher proportion of intermediate pH_u bull meat occurred in spring, the pH_u distribution of bull beef was more consistent between seasons in this abattoir.

To determine if there was a correlation between meteorological conditions and meat pH_u, correlation coefficients were determined for pH_u and meteorological variables (Table 3). The positive relationship between pH_u and mean maximum temperatures and rainfall on the day of slaughter were significant ($p < 0.01$) such that higher temperatures and rainfall on the day of slaughter is significantly related with higher pH_u values. However, these relationships were significant only for Abattoir 1 ($p < 0.01$).

Seasonal variation in bull meat quality

For the second part of this study, a total of 157 bulls were sampled from one abattoir in spring (n = 94) and summer (n = 63). The proportion of low pH_u meat was higher in bulls slaughtered in the spring and the prevalence of intermediate pH_u meat was higher in summer compared with spring (Table 4). The proportion of high pH_u meat was similar for both seasons, with 30 and 32 percent in spring and summer, respectively. Although there was some variation in the proportion of each pH_u category between seasons, the mean pH_u of bull meat from both seasons were not significantly different ($p > 0.05$).

The mean percentage cook loss from bulls collected from both seasons remained unchanged during the first 21 days *post mortem* (Figure 4A). At 28 days *post mortem*, the cook loss of bulls from both seasons dramatically increased. Percentage cook loss was significantly higher in bulls slaughtered in summer compared with those slaughtered in spring ($p < 0.05$) throughout the ageing period.

Seasonal variation was also observed in shear force measurements determined for all animals throughout the ageing period (Figure 4B). With 11 kgF as the upper limit for acceptable tenderness in New Zealand beef, the shear force of bull meat slaughtered in spring and summer had declined to acceptable levels after 7 days *post mortem* and continued to decline thereafter. However, meat from bulls slaughtered in spring had significantly higher shear force values compared with meat from bulls slaughtered in summer throughout the ageing period ($p < 0.05$).

To determine if there was any seasonal variation in the proteolytic activity of bull beef during ageing, quantitative analysis of μ-calpain and cathepsin B were conducted at all ageing time points (Table 5). Relative μ-calpain activity was significantly higher in meat from bulls slaughtered in summer compared with bulls slaughtered in spring at 0 day *post mortem*. No significant seasonal differences were observed in the relative activity of μ-calpain at subsequent ageing time points. Unlike meat from bulls slaughtered in summer, relative μ-calpain activity increased from 0 to 1 day *post mortem* in spring, suggesting that the activation of μ-calpain in this group may have delayed.

Cathepsin B activity generally increased during ageing for bull meat in both spring and summer. Although no significant seasonal differences was observed during the first 2 days *post mortem*, cathepsin B activity was significantly higher in summer bull meat at 7 and 28 days *post mortem* compared with spring bull meat. Higher

relative μ-calpain activity early *post mortem* and cathepsin B activity *post rigor* corresponded with the lower shear force values of summer bull meat.

Discussion

Several studies published subsequent to the pH survey of New Zealand beef by Graafhuis & Devine (1994) have reported varying distributions of pH_u across the different animal classes. In a survey including 967 animals sourced from several farmers (Webby, Fisher, Lambert, Daly, Knight & Turner, 1999), 68 percent of beef derived from steers (n = 967) were reported to be low pH_u. Data from a more recent study with 14,000 cattle reported 62 percent of bull meat with a pH_u above 5.7 (Young, Thomson, Merhtens & Loeffen, 2004). Although these studies provided some insight into the pH distribution of New Zealand beef, all animals in these studies were collected from the same abattoir. The present survey aimed to obtain a picture of the current pH_u status of New Zealand bulls across representative regions in New Zealand.

When comparing the pH_u distribution of the past survey (Graafhuis *et al.*, 1994) with the present survey, it is clear that there has been significant reduction in the incidence of intermediate and high pH_u meat derived from bulls. In addition, a declining trend in the pH_u of New Zealand bulls has also been observed. Early studies have indicated the decline in the mean pH_u of New Zealand bull meat from 6.35 to 6.21 between 1990 and 1993 (Purchas, 1990; Purchas & Aungsupakorn, 1993). Further, mean pH_u decline reported in subsequent survey by Graafhuis & Devine (1994) and this study confirm the progressive decrease in the mean pH_u of bull meat over the years. However, with approximately 50 percent of bulls surveyed having a pH_u greater than 5.8, there is still a significant production of intermediate and high pH_u bull beef. Although bulls undergo similar handling and slaughter processes as cows and steers, the elevated meat pH_u in this animal category confirms that they are more susceptible to pre-slaughter stress, consequently resulting in the higher frequency of intermediate and high pH_u meat (Brown, Bevis & Warriss, 1990; Lowe, Devine, Wells & Lynch, 2004).

The high incidence of elevated pH_u in bulls is not confined to New Zealand. Significant research efforts were invested worldwide in the 1980's and 1990's to solve the meat quality problems related to high pH_u meat originating from bulls, particularly to assist the beef industries. Surveys of beef cattle in the United Kingdom, Finland and Sweden have found greater proportions of bull beef with pH_u above 5.8 compared with steers and cows (Brown *et al.*, 1990; Fabiansson, Erichsen, Laser Reuterswärd &

Malmfors, 1984; Puolanne & Aalto, 1981). This consequently results in the mean pH_u of bull beef being significantly higher compared with other animal groups as observed by Page *et al.* (2001).

Several studies have shown seasonal variation in the incidences of intermediate and high pH_u meat for all animal classes. Brown *et al.* (1990) and Tarrant *et al.* (1980) observed that there was a higher proportion of high pH_u beef from cattle slaughtered in autumn. These results are consistent with the present survey that found an increase in the proportion of intermediate and high pH_u meat from bulls and steers slaughtered in that season. However, this pattern was not observed in cows, which had higher proportions of intermediate and high pH_u beef in spring.

Environmental factors in which the animals have been reared is linked with the depletion of muscle glycogen, resulting in higher incidences of high pH_u meat during periods of extreme weather conditions (Scanga, Belk, Tatum, Grandin & Smith, 1998) Young *et al.* (2004) reported that muscle glycogen levels, as measured by a patented enzymatic method, was significantly related with rainfall and the mean maximum temperature on the day and days preceding slaughter for both bulls and steers. Transport and lairage of cattle on warm days, as observed in autumn and summer, induces stress in cattle consequently resulting in elevated pH_u meat (Fabiansson *et al.*, 1984; Mounier, Dubroeucq, Andanson & Veissier, 2006).

The sub-optimal quality of pastures during summer and autumn may also be a contributing factor in the higher incidences of intermediate and high pH_u during these seasons. Carbohydrate levels and metabolisable energy of New Zealand pastures are lowest during summer and autumn due to the maturation of plants within these months leading to a high dead matter and low nutritive value of stems (Litherland *et al.*, 2002; Machado, Morris, Hodgson & Fathalla, 2005). The decreased digestibility and carbohydrate content in pastures during summer and autumn can result in lower muscle glycogen reserves leading to animals being more susceptible to higher pH_u (Brown *et al.*, 1990; Tarrant *et al.*, 1980)

Further analysis of the bull data revealed the variation in the pH_u profile of bulls at the different abattoirs surveyed and that the incidence of intermediate and high pH_u bull meat varied between abattoirs in all seasons. Overall, there was a higher frequency of low pH_u meat during the winter in all abattoirs. Regression analysis also revealed that lower daily maximum temperatures and rainfall during the day of slaughter favoured low pH_u meat production. This is consistent with the findings reported by Young *et al.* (2004) who found that the maximum temperature on the day and days

preceding slaughter was significantly related with pH_u. Although Abattoirs 1 and 3 had similar mean maximum temperatures on the day of slaughter, the proportion of intermediate and high pH_u meat in Abattoir 1 was higher for all seasons. Regression analysis revealed that mean maximum temperature on the day of slaughter was only significantly related with pH_u for Abattoir 1. In addition, the combined proportion of intermediate and high pH_u meat for Abattoirs 2 and 3 were similar in autumn and spring, even though temperature conditions were cooler in Abattoir 2 for both seasons (Table 1). This suggests that although temperature may play a role in the seasonal variation of pH_u, the variation in the pH_u distribution between abattoirs may be attributed to factors other than temperature, such as lairage and pre slaughter facilities and handling of animals leading to slaughter.

In a survey conducted by Fabiansson *et al.* (1984), the incidence of high pH_u meat in bulls slaughtered in an abattoir with open lairage pens was three times higher than in abattoirs with individual pens. Keeping bulls in open pens while in lairage results in the mixing of unfamiliar mobs of bulls from different farms that result in stress brought about by aggressive behaviours as the animals try to establish a social hierarchy within the new group (McVeigh, Tarrant & Harrington, 1982; Warriss, Kestin, Brown & Wilkins, 1984). Results from all previous research around handling of bulls and prevention of high ultimate meat pH values led to recommendations for abattoirs on how to handle the bulls in connection with slaughter and keeping optimal meat quality (Fabiansson, Shorthose & Warner, 1989). However, the present study together with previous surveys indicates that New Zealand can still improve on handling bulls in order to reduce the incidence of high pH_u meat. Although abattoirs attempt to prevent the mixing of unfamiliar animals once they have reached the abattoir, the high throughput of animals during the day of slaughter makes this unavoidable. Thus the pH_u variation of bull meat between abattoirs in the present study may be due to the different handling methods of animals during transport and while in lairage.

To demonstrate the seasonal variation in bull meat quality, measurements of quality attributes from bulls slaughtered during spring and summer were determined. Mean daily maximum temperatures on the day of slaughter were considerably higher during summer (Table 1), which may contribute to the higher incidence of intermediate and high pH_u bulls in summer compared with spring. However, there was no significant difference in the mean pH_u of meat from bulls slaughtered in summer compared with those slaughtered in spring.

Considering the strong negative correlation between cook loss and pH_u (Bouton *et al.*, 1971; Silva *et al.*, 1999), it is interesting to note that the mean percentage cook loss in summer was significantly higher throughout ageing compared with spring despite the pH_u between the two seasons being similar. This suggests that factors other than pH_u are involved in the seasonal variation of cook loss. A possible explanation for this is that muscle from bulls slaughtered in summer are in *rigor mortis* at a faster rate as they attain a higher pH_u than those slaughtered in the spring. Consequently, the temperature at which *rigor mortis* is attained in summer is higher, resulting in increased cook loss in beef from animals slaughtered in that season (Farouk & Swan, 1998).

Seasonal variation in shear force was also observed, with the mean shear force of spring bull meat being significantly higher than summer bull meat. With 11 kgF as the upper limit for acceptable meat tenderness (Bickerstaffe, Bekhit, Robertson, Roberts & Geesink, 2001), bull meat from both seasons attained acceptable tenderness within 14 days *post mortem*. However, summer bull meat attained acceptable tenderness earlier compared with spring.

Calpains, particularly µ-calpain, and cathepsins are two enzyme systems that have been most implicated in the degradation of key myofibrillar proteins *post mortem* leading to meat tenderisation (Huff-Lonergan, Mitsuhashi, Beekman, Parrish, Olson & Robson, 1996; Zeece & Katoh, 1989). Briefly, calpains and cathepsins are optimally active at near neutral and acidic pH levels, respectively (Koohmaraie, Schollmeyer & Dutson, 1986; Ouali, 1992). Thus, any calpain activity is believed to be early *post mortem* where the muscle pH is still high. In contrast, proteolysis of myofibrillar proteins by cathepsins, particularly in low pH_u meat, occur at latter stages *post mortem* once meat had attained lower pH levels and cathepsins had been released from the lysosomes to the sarcoplasm (Dutson, 1983; Sentandreu, Coulis & Ouali, 2002). This is consistent with the findings in this paper that report the general decline in µ-calpain activity and increase in cathepsin B activity with ageing. Furthermore, the higher shear force of bull meat in spring was consistent with the significantly lower relative activity of µ-calpain at 0 day *post mortem*, and cathepsin B activity at 7 and 28 days *post mortem*.

The reasons for the apparent seasonal variation in activities of both proteolytic enzymes are unknown. Although the pH_u of muscle is an important factor in the proteolytic activity of enzymes, it does not completely explain the elevated levels of both µ-calpain and cathepsin B activity in summer. This suggests that like cook loss, factors other than pH_u are involved in the seasonal variation of enzyme activities during

ageing, and consequently meat tenderness. Further work to identify factors outside of temperature and pH_u and their contribution to the seasonal variation in meat quality attributes is required.

Conclusion

Compared with the last New Zealand survey published in 1994, there was an overall decline in intermediate and high pH_u bull beef suggesting improved animal husbandry practices since the last survey. However, with approximately 50 percent of bull beef categorised as intermediate or high pH_u, there is still a significant production of bull beef with unacceptably high pH_u. Seasonal variation in pH_u distribution was also observed for all animal categories, with higher incidences of intermediate and high pH_u observed for bulls and steers in autumn. It was also evident there was seasonal variation in both cook loss and shear force and proteolytic activity in bull meat from spring and summer slaughter. Although the mean pH_u of bull meat from spring and summer slaughters were similar, significantly higher mean cook loss and cathepsin B activity were observed in summer bull meat. This suggests that factors other than pH_u are also critical in contributing to the seasonal variation in meat quality as observed in bull beef.

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Table 1: Mean maximum daily temperatures and rainfall on the slaughter days for each of the abattoir surveyed in autumn, winter, spring and summer for both trials.

	Mean maximum daily temperature on slaughter days (°C)				Mean rainfall on slaughter days (mm)			
	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer
Trial 1								
Abattoir 1	21.1	15.2	19.2		0.1	0.4	0.1	
Abattoir 2	17.2	15.3	12.4		8.2	6.5	2.3	
Abattoir 3	22.4	12.4	20.5		1.4	0.2	0.1	
Trial 2			18.2	25.0			0.0	2.9

Table 2: The mean pH_u ± standard deviation of bulls from the pH_u survey in comparison with the last comprehensive pH_u survey of New Zealand beef.

	1994[a]			2009		
	Mean pH_u	n	Proportion with pH_u below 5.8 (%)	Mean pH_u	n	Proportion with pH_u below 5.8 (%)
Bulls	6.16	766	29.0	5.97±0.46	1597	49.2

[a]Graafhuis & Devine (1994)

Table 3: Linear correlation coefficients between pH_u and meteorological variables during the slaughter days in spring and summer.

	Mean maximum temperature on slaughter days (°C)	Mean rainfall on slaughter days (mm)	Significance
Overall data	0.26	0.12	$p < 0.01$
Abattoir 1	0.6	0.59	$p < 0.01$
Abattoir 2	0.24	0.18	n.s.
Abattoir 3	0.15	0.03	n.s.

Table 4: Mean pH_u and percentage frequencies of low, intermediate and high pH_u bulls slaughtered in spring and summer.

	Spring	Summer
n	94	63
Mean pH_u	5.9	6.0
Percentage frequency		
Low pH_u	56	49
Intermediate pH_u	14	19
High pH_u	30	32

Table 5: Mean cathepsin B and relative μ-calpain activities in bull beef slaughtered in spring and summer.

Days *post mortem*	Cathepsin B (± SEM) (mU/mg protein)			Relative μ-calpain activity (± SEM)		
	Spring (n = 94)	Summer (n = 63)	Significance	Spring (n = 94)	Summer (n = 63)	Significance
0	1.33 ± 0.06	1.25 ± 0.07	ns	144 ± 18	251 ± 15	$p < 0.001$
1	1.37 ± 0.08	1.32 ± 0.09	ns	222 ± 22	234 ± 14	ns
2	1.35 ± 0.09	1.49 ± 0.10	ns	203 ± 16	233 ± 10	ns
7	1.12 ± 0.10	1.68 ± 0.13	$p < 0.001$	194 ± 14	181 ± 9	ns
14	1.60 ± 0.13	1.88 ± 0.15	ns			
28	1.63 ± 0.17	2.04 ± 0.16	$p < 0.01$			

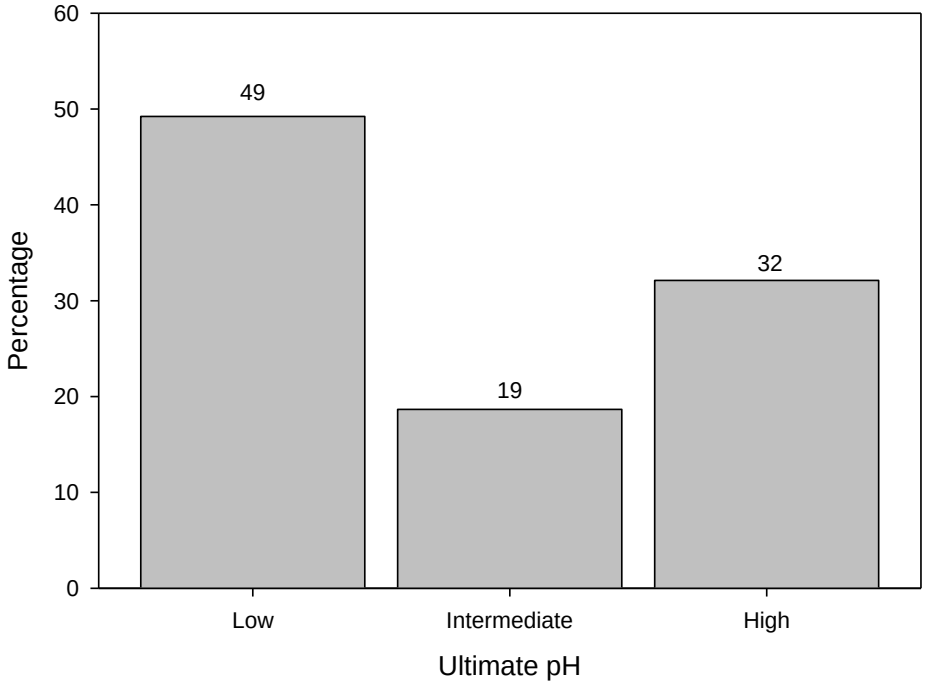

Figure 1: The overall frequency of low, intermediate and high pH_u beef in bulls. *M. longissimus dorsi* were collected from three commercial abattoirs in autumn, winter and spring.

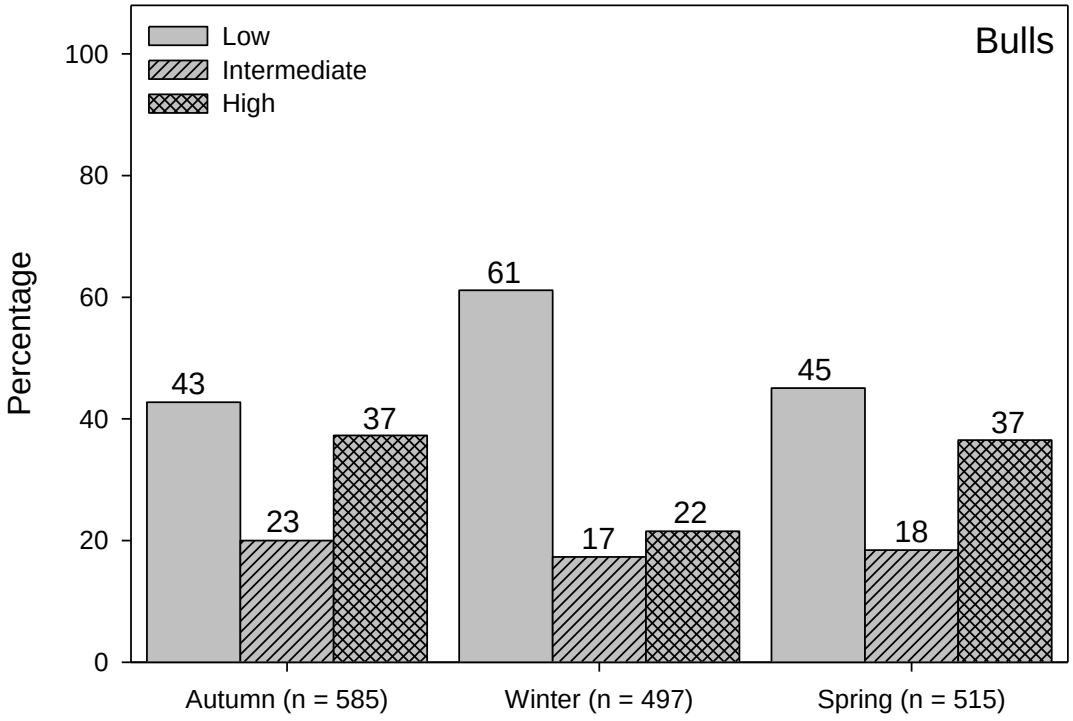

Figure 2: The variation in the pH_u profile of beef derived from bulls slaughtered in autumn, winter and spring.

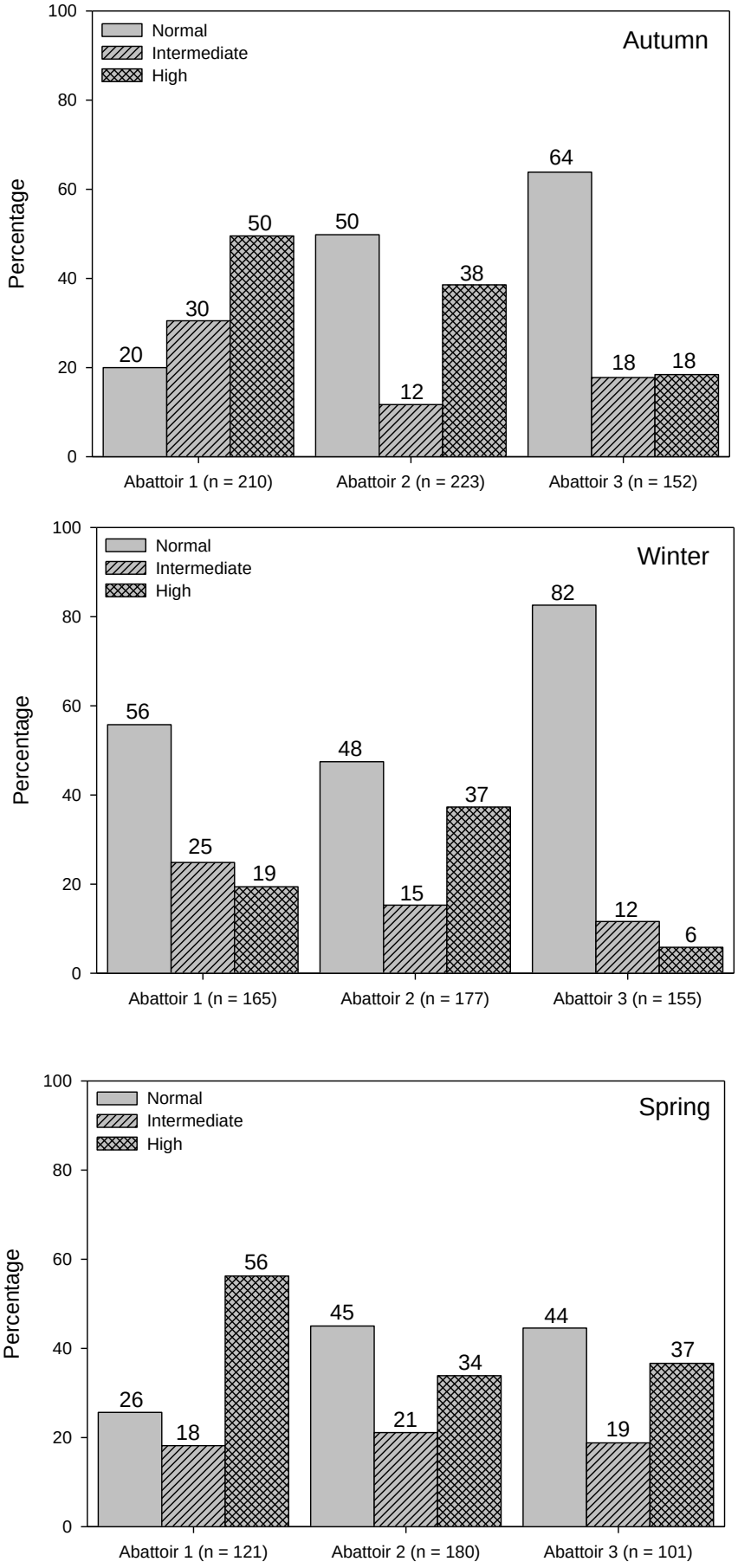

Figure 3: Between-abattoir variation in the frequency of low, intermediate and high pH_u bull beef over autumn, winter and spring.

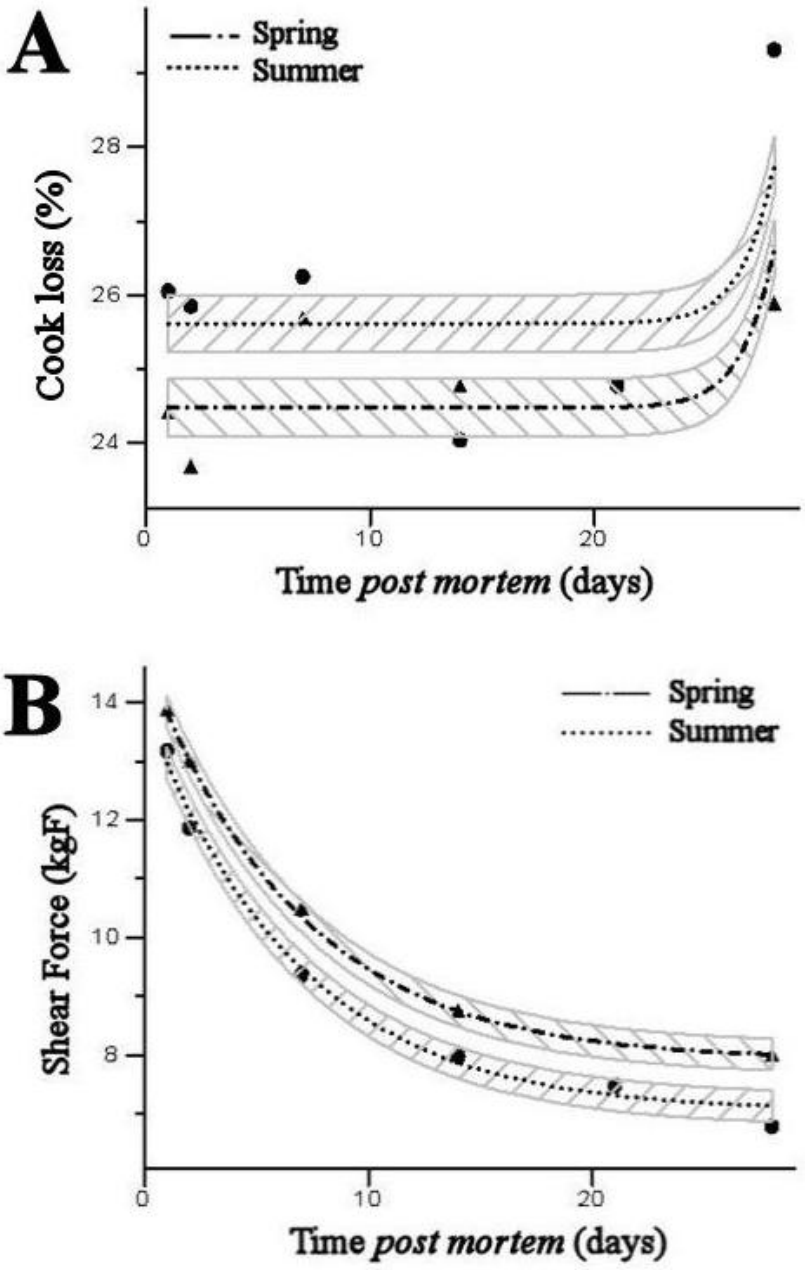

Figure 4: Cook loss (A) and shear force (B) of *M. longissimus dorsi* from bulls slaughtered in spring and summer, and aged up to 28 days *post mortem* at -1.5°C. Splines are 95% least significant intervals. If shaded areas do not overlap, the splines are significantly different.

II

The aetiology of meat tenderness is likely to be compartmentalised by ultimate pH[1]

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Abstract

Bull *M. longissimus dorsi* (n = 63). were categorised into high (pH ≥ 6.2), intermediate (pH 5.8 – 6.19) and low (≤ 5.79) ultimate pH (pH_u) and aged up to 28 days *post mortem* at -1.5°C. High pH_u samples were acceptably tender at 1 day *post mortem* and significantly more tender than low pH_u meat at all ageing timepoints ($p < 0.05$). Rapid autolysis of μ-calpain in high pH_u meat was linked with the more rapid degradation of titin, nebulin and filamin in this pH_u group. Desmin degraded faster in low pH_u meat and was concurrent with an increase of cathepsin B levels. The results from this study support the hypothesis that beef tenderisation is pH_u compartmentalised with tenderness in high and low pH_u meat characterised by variable rate of degradation of high and low molecular weight myofibrillar proteins during ageing, which are in turn regulated by μ-calpain and cathepsin B activities.

Keywords

Tenderness, compartmentalisation, μ-calpain, cathepsin B, ultimate pH, beef

Introduction

Meat tenderness has long been associated with ultimate pH (pH_u), with meat that attain high ($pH \geq 6.2$) and low ($pH \leq 5.79$) pH_u being acceptably tender after ageing (Bouton, Harris & Shorthose, 1971; Devine, Graafhuis, Muir & Chrystall, 1993; Jeremiah, Tong & Gibson, 1991; Purchas, Yan & Hartley, 1999). Additionally, the rate at which meat tenderises is also pH_u related, with high pH_u meat tenderising more rapidly than low pH_u meat (Watanabe, Daly & Devine, 1996). Biochemical mechanisms driving changes in *post mortem* muscle are pH dependent. It is therefore speculated that the plethora of changes that take place in low and high pH_u muscle *post mortem*, which result in meat tenderisation for both, are potentially different due to the pH difference between the two groups.

Meat tenderness is attributed to the weakening of the highly organised myofibrillar structure due to the proteolytic degradation of key proteins *post mortem*. Calpains and cathepsins are two enzyme systems that have been implicated in *post mortem* proteolysis. Briefly, the failure of ionic pumps *post mortem* as ATP is depleted results in the accumulation of sarcoplasmic Ca^{2+} to concentrations required to activate μ- and m- isoforms of calpain that in turn hydrolyse myofibrillar proteins (Goll, Thompson, Li, Wei & Cong, 2003; Koohmaraie, 1994). In addition, the weakening of the lysosomal membrane as the muscle pH declines *post mortem* releases cathepsins from the lysosomes making them available to hydrolyse myofibrillar proteins (Sancho, Jaime, Beltran & Roncales, 1997; Sentandreu, Coulis & Ouali, 2002).

Significant research has been invested on determining the contribution of several myofibrillar proteins to meat tenderness. The faster and more extensive and degradation rates of desmin, troponin T, myosin, filamin, nebulin and titin in tender meat compared with tough meat has been consistently reported (Huff-Lonergan, Mitsuhashi, Beekman, Parrish, Olson & Robson, 1996; Huff-Lonergan, Parrish & Robson, 1995; O'Halloran, Troy & Buckley, 1997; Whipple, Koohmaraie, Dikeman, Crouse, Hunt & Klemm, 1990). By following the degradation of these proteins during the conversion of muscle to meat and subsequent ageing, researchers have attempted to reconcile their findings with the ultimate tenderness that meat eventually attains. However, mechanisms surrounding the different rate of tenderisation as observed in high and low pH_u meat are still poorly understood.

This study evaluates the differential breakdown of myofibrillar proteins and the proteolytic activity in low and high pH_u meat during ageing in relation to the rate of tenderisation of the respective pH_u groups. It is hypothesised that the tenderisation of

low and high pH_u groups is pH_u compartmentalised due to the differential *post mortem* breakdown of myofibrillar proteins and proteolytic activity in these groups.

Materials and Methods

Animals

Sixty three bulls were head-only electrically stunned and slaughtered in a commercial abattoir on the same day. All carcasses were electrically stimulated following exsanguination. Upon reaching the boning floor, the *M. longissimus dorsi* (LD) were hot boned from the left side of the carcasses and about 10 g was excised from the anterior end of each muscle, snap frozen in liquid nitrogen, crushed and stored at -80°C. The rest of the muscle was packaged in a vacuum pack bag and stored at -1.5°C. After 24 h, each LD was divided equally into six pieces each randomly allocated to an ageing period of 1, 2, 7, 14 and 28 days *post mortem*, vacuum packed then stored at -1.5°C. At each ageing timepoint, 10 g was excised from each loin, frozen in liquid nitrogen, crushed and stored at -80°C. The rest of the loin was used to measure pH and shear force.

pH and shear force measurement

The pH measurements of all loins at all ageing timepoints were determined with a Testo® 230 meter (Lenzkirch, Germany). The pH meter was calibrated at pH 7.0 and 4.0 with buffers (Mallinckrodt Chemicals, Phillipsburgh, NJ, USA) stored at room temperature (20°C).

The shear force of loins aged at 1, 2, 7, 14 and 28 days *post mortem* were measured using a MIRINZ tenderometer as described by Pulford, Fraga Vazquez, Frost, Fraser-Smith, Dobbie & Rosenvold (2008).

Sample preparations

For the preparation of total muscle extracts, approximately 0.5 g of the frozen muscle fragments collected at each timepoint was dispersed in 5 mL of total muscle extraction buffer (50 mM Tris-HCl; pH 5.8, 10% glycerol, 2% SDS and 2% 2-mercaptoethanol) for 20 s at 20,000 rpm with an Ika®-Labortechnik Ultra Turrax T25 disperser (Staufen, Germany) fitted with a 18 mm S25 tool. The homogenate was then

centrifuged at 10,000 g for 5 min at 4°C. The total muscle extract supernatant was frozen at -80°C awaiting further analysis.

Whole muscle homogenates were prepared by dispersing 0.4 g frozen muscle fragments in whole muscle extraction buffer (10 mM sodium phosphate buffer, 0.2 % SDS; pH 7.0). The homogenate was then centrifuged at 1,500 g for 15 min at 25°C and the supernatant stored at -80°C.

Cathepsin B lysates were prepared by homogenising 2 g of FMF in cathepsin extraction buffer (10 KH_2PO_4, 1 mM EDTA, 50 mM NaCl, 250 mM sucrose; pH 7.4) and the homogenate centrifuged at 1,000 g for 10 min then 4,000 for 15 min at 2°C. The supernatant was filtered through a swab gauze pad followed by centrifugation at 20,000 g for 20 min at 2°C. The supernatant was recovered for protein and cathepsin B determination.

Protein determination

Protein measurement of total muscle extracts was determined using a RC-DC protein assay kit (BioRad Laboratories, Hercules, CA, USA). Similarly, protein concentrations of WMH and cathepsin B lysates were determined with a DC Protein Assay kit (BioRad Laboratories), a commercial kit based on methods outlined by Lowry, Rosenbrough, Farr & Randall (1951). For both assays, protein concentrations were determined from standard curves prepared with bovine serum albumin.

Coomassie blue SDS-PAGE

A 0.2 mL aliquot of each thawed total muscle extract supernatant was mixed with the same volume of reduced sample buffer (62.5 mM Tris-HCl; pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.02% bromophenol blue). The solution mixtures were heated for 20 min at 50°C followed by centrifugation at 10,000 g for 10 min and the supernatant was loaded onto gels.

For the separation of larger protein (>100 kDa), 100 µg of protein was loaded into each well and separated at room temperature in a BioRad Criterion cell system at 10 mA for 17 h. Following electrophoresis, gels were stained in colloidal Coomassie blue (17% ammonium sulphate, 2% phosphoric acid, 30% methanol, 0.04% Coomassie G-250) for 48 h. Stained gels were then washed with distilled water and images were captured with a GS700 calibrated densitometer scanner (BioRad Laboratories).

Immunoblots

Thawed supernatants of whole muscle extracts were initially adjusted to a protein concentration of 8 mg mL^{-1} with whole muscle extraction buffer then made up to a final concentration of 4 mg mL^{-1} with sample loading buffer (30 mM Tris, 30 mM EDTA, 3% SDS, 30% glycerol, 0.003% pyronin Y; pH 8.0) and 0.1 µL 2-mercaptoethanol. The samples were then heated to 50°C for 20 min. For desmin analysis, 40 µg of protein were loaded onto wells of 12% Bis Tris Criterion gels (BioRad Laboratories) and separated with XT MOPS separating buffer (BioRad Laboratories). For µ-calpain gels, 40 µg of protein were loaded onto 7.5% Tris-HCl gels (BioRad Laboratories). Electrophoresis of all gels was conducted in a BioRad Criterion cell system set at 120 V at room temperature.

Proteins from the gels were blotted onto Immobilon-P PVDF membranes (Millipore, IPVH00010) followed by overnight blocking with 5% non-fat dry milk powder in PBS-Tween (0.08 M Na_2PO_4, 0.02 M NaH_2PO_4, 0.1 M NaCl, 0.1% Tween). After washing with PBS-Tween, the membranes were incubated with monoclonal antibodies for 1 h at room temperature. Monoclonals used included desmin (Sigma, D1033), myosin (Sigma, M-4276) and µ-calpain (Thermo Scientific, MA3-940) diluted to 1:5000, 1:10000 and 1:5000, respectively in PBS-Tween. After washing with PBS-Tween, membranes were then incubated with goat anti-mouse IgG (H+L) HRP conjugate (BioRad Laboratories, #172-1011) diluted to 1:5,000 in PBS-Tween for 1 h at room temperature. Secondary antibody bound to the membrane was detected with an ECL Western Blot substrate kit (Pierce, 3216) and the resulting luminescent bands captured with a G:Box Chemi HR16 (Syngene, Cambridge, United Kingdom).

Cathepsin B activity

Cathepsin B activity of cathepsin B lysates were measured according to the procedures of Caballero *et al.* (2007) with some modifications. Lysates were mixed with incubation buffer (100 mM sodium acetate, 1 mM EDTA, 5 mM DTT, 0.1% Brij; pH 5.5) in 96 well fluorescent plates (Nunc, #265301) then heated to 37°C for 10 min. An aliquot of (5 µL) 40 µM Cathepsin B Fluorogenic Substrate III (Calbiochem, 219392) was subsequently dispensed into each well. The resulting fluorescence driven by the enzyme-substrate reaction was measured every minute for 1 h at 37°C with a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany) fitted with 360 nm excitation and 460 nm emission filters. Standard curves prepared from purified

cathepsin B (Sigma, C6286) were used to determine the activity of the enzyme in the samples.

Data analysis

Analysis of the data was conducted using the REML directive of Genstat (Genstat, 2008). Data analysis was also conducted using a mixed model smoother (Upsdell, 1994) with time *post mortem* × pH_u and time effect (a random term to describe variability between ageing timepoints) used as the model. The curves from the plots derived from the models are estimated curves of each of the measured parameters when grouped into timepoints *post mortem* and pH_u. The splines are 95% least significant intervals for testing differences between groups such that two groups without overlapping splines are significantly different ($p < 0.05$).

Results and Discussion

Shear Force

The *M. longissimus dorsi* from 63 bulls in this study were categorised as low ($\leq$ 5.79), intermediate (pH 5.8 – 6.19) and high (pH $\geq$ 6.2) pH_u based on the pH that muscles had attained 2 days *post mortem*. It is clear that pH_u is related to shear force, with shear force increasing as pH_u increased from 5.4 to 6.0, and then decreasing as pH_u increased to near neutral pH levels (Figure 1). The shear force of all pH_u groups generally decreased as the muscles were aged. Taking 11 kgF as the upper limit for acceptable cooked meat tenderness (Bickerstaffe, Bekhit, Robertson, Roberts & Geesink, 2001), high pH_u meat was already acceptably tender at 1 day *post mortem* (Table 1). In contrast, low pH_u meat was significantly tougher than high pH_u at all ageing times ($p < 0.05$). The data also suggest that low pH_u meat did not attain acceptable tenderness until about 7 days *post mortem*.

These findings are consistent with previous research that has demonstrated that although both pH_u groups eventually attain acceptable tenderness, high pH_u meat is consistently more tender than low pH_u meat even after extended ageing (Bouton *et al.*, 1971; Jeremiah *et al.*, 1991; Purchas *et al.*, 1999; Yu & Lee, 1986). Additionally, the early tenderisation in high pH_u (Purchas *et al.*, 1999; Yu *et al.*, 1986) was confirmed in the present study. Intermediate pH_u meat tenderness is not discussed in this study due to the delayed tenderisation in this group. A hypothesis providing an explanation to the

delayed tenderisation in intermediate pH_u beef is discussed in a separate study (Lomiwes, Farouk, Frost, Dobbie & Young, submitted).

Protein degradation and meat tenderness

To follow the degradation of higher molecular weight proteins during ageing, total muscle extracts from representative low and high pH_u samples were analysed by SDS-PAGE (Figure 2). Titin degradation occurred earlier in the high pH_u sample with the detection of the titin degradation product T2 at 0 day *post mortem*. It was not until 1 day *post mortem* that titin degradation was detected in low pH_u samples, with intact titin still faintly present at 2 days *post mortem*. Only T2 bands were present in both pH_u groups after 28 days ageing.

No nebulin degradation was observed at 0 day *post mortem* in either pH_u groups. Nebulin degraded at a faster rate in high pH_u meat, with detection of nebulin breakdown in the high pH_u sample at 1 day *post mortem* and the complete disappearance of intact nebulin at 2 days *post mortem*. In contrast, intact nebulin was still present in the low pH_u sample after 2 days *post mortem*. Evaluating nebulin at latter ageing timepoints was not possible due to the diffusion of protein bands in the area of the gel where nebulin is resolved. These are believed to be breakdown products of other larger molecular weight proteins.

Like titin and nebulin, filamin degraded earlier and more extensively in high pH_u samples. Doublet filamin bands – consisting of intact filamin and degraded filamin (F2) – were detected at 1 day *post mortem*. Filamin progressively degraded thereafter such that at 14 days *post mortem*, no intact filamin was detected in any of the high pH_u samples. In contrast, the appearance of the F2 band was detected at 7 days *post mortem* in the representative low pH_u sample with intact filamin still faintly detectable at 28 days *post mortem*.

Western blots of representative low and high pH_u samples showed a faster and more extensive degradation of myosin in the low pH_u sample at 1 day *post mortem*, (Figure 3). The degradation of desmin was initially detected at 7 days *post mortem* in both categories of meat. However, desmin was more extensively fragmented in high pH_u meat at the latter stages of ageing compared with low pH_u meat (Figure 4).

Titin and nebulin degradation results were consistent with previous research (Watanabe & Devine, 1996; Yu *et al.*, 1986). The preferential degradation of myosin in low pH_u meat was also observed by Yates *et al.* (1983) and Yu *et al.* (1986) and was related to the fragmentation found in the M-line of the myofibril. The weakening of the

actin/myosin bond *post rigor*, as indicated by myosin degradation, is thought to contribute to meat tenderisation.

Proteolytic enzyme activities and meat tenderness

The tenderisation of meat is believed to be predominantly due to the proteolysis of myofibrillar and cytoskeletal proteins. μ-Calpain and cathepsins are two enzyme systems most implicated in meat tenderisation. However, there is still a lack of knowledge on the specific functions of these enzyme systems in *post mortem* muscle, thus this is still a topic of contention among meat scientists.

μ-Calpain

The ability μ-calpain to degrade several myofibrillar and cytoskeletal proteins *in vitro* has been demonstrated to be similar to the degradation of these proteins in *post mortem* muscle (Huff-Lonergan *et al.*, 1996). In this study, immunoblots of whole muscle extracts from representative low (n = 4) and high (n = 7) pH_u samples were probed with μ-calpain antibody to track the autolysis of this enzyme during ageing. The immunoblots revealed that μ-calpain autolysis had already occurred at 0 day *post mortem* in the high pH_u samples with the detection of the active 78 and inactive 76 kDa subunits (Baki, Tompa, Alexa, Molnar & Friedrich, 1996; Maddock, Huff-Lonergan, Rowe & Lonergan, 2005) (Figure 5). In comparison, autolysis of the intact 80 kDa subunit was delayed in the low pH_u group with the appearance of the 78 kDa fragment at 1 day *post mortem*.

The faster autolysis rates of μ-calpain in the high pH_u samples suggest the early activation and higher activity of these enzymes early *post mortem* in high pH_u meat. In comparison, the delayed autolysis of this enzyme in the low pH_u samples indicate the suspended activation of these enzymes, possibly due to the slower onset of *rigor mortis* in low pH_u muscle or from the carryover of some other physiological factors not examined in the present study. In a previous study, μ-calpain was reported to be optimally active at pH 7.5 and retains up to 28% of its activity between pH 5.4 and 5.8 (Koohmaraie, Schollmeyer & Dutson, 1986). However, μ-calpain activity declines with time *post mortem* with lower than 4% of its initial activity at 7 days *post mortem* (Dutson, 1983). This is supported by the findings in the present study which demonstrated that μ-calpain activation and activity – as measured by autolysis – occurred much earlier and was sustained for longer in high pH_u meat. Due to the relatively rapid autolysis of μ-calpain to the inactive 76 kDa isoform in *post mortem*

cells, it is proposed that their role in meat tenderness is restricted to the first 48 h after slaughter. However, our studies showed the presence of the 78 kDa subunit even after 28 days *post mortem* in some low and high pH_u samples. Whether the 78 kDa subunit is still active beyond 7 days *post mortem* at the sub-optimal acidic environment of low pH_u meat is still controversial.

The degradation of titin at 0 day *post mortem* corresponded with the presence of activated μ-calpain at the same timepoint. Accelerated glycolysis by electrical stimulation, hastens the onset of *rigor mortis* resulting in the influx of calcium ions within the muscle, which in turn activates μ-calpain (Rhee, Ryu, Imm & Kim, 2000). This is particularly important in the rapid tenderisation of high pH_u meat, where the early onset of *rigor mortis* at high temperatures consequently results in the faster degradation of myofibrillar proteins leading to very tender meat early *post mortem*. The detection of intact filamin in low pH_u meat at 28 days *post mortem* may be due to the absence of μ-calpain activity, which may have been inactivated by self-autolysis and the acidic pH.

Cathepsin B

Cathepsin B activity was assayed for all animals at all timepoints. Our data showed significant differences in cathepsin B activity trends during ageing. Cathepsin B activity in high pH_u meat was consistently low throughout the ageing period (Figure 6). In comparison, cathepsin B activity in low pH_u meat progressively increased with ageing and was significantly higher than high pH_u at all ageing timepoints.

In *post mortem* muscle cathepsin B is released from the lysosome into the sarcoplasm of low pH_u meat, making them available to hydrolyse susceptible proteins (Dutson, 1983). Cathepsins have been shown to hydrolyse several myofibrillar proteins and also alter the A-band and Z-disks by degrading proteins associated with these regions (Zeece & Katoh, 1989). Cathepsin B activity is optimal between pH 5.0 and 6.0 (Ouali, 1992), and the contribution of this enzyme to myofibrillar protein degradation is minimal in intermediate and high pH_u meat due to the greater stability of lysosomal membranes at higher pH_u, consequently resulting in minimal cathepsins being released into muscle cytoplasm (Ertbjerg, Henckel, Karlsson, Larsen & Moller, 1999). Furthermore, assuming that lysosomal membranes were weakened and cathepsins were released into the cytoplasm, the inherent pH of intermediate and high pH_u meat would be suboptimal for cathepsin B activity, thus limiting its proteolytic action.

The contribution of cathepsin B to the tenderisation of low pH_u meat is most likely to be at the latter stages of ageing on the basis that cathepsin B needs to be released from the lysosomes. Western Blots and SDS-PAGE showed the progressive degradation of desmin, a known substrate of cathepsin B (Dutson, 1983), and filamin in low pH_u meat was concurrent with the rise in cathepsin B levels and the decrease in shear force *post rigor*. Interestingly, significant desmin fragmentation was not detected until 7 days *post mortem* in both low and high pH_u meat. Previous studies have reported desmin degradation in meat to occur as early as 2 to 3 days *post mortem* (Huff-Lonergan *et al.*, 1996; Pulford, Dobbie, Fraga Vazquez, Fraser-Smith, Frost & Morris, 2009) in tender, low pH_u meat. With μ-calpain activity being limited by the inherently acidic pH in low pH_u meat, it is likely that desmin and filamin degradation is due to a combination of μ-calpain activity early *post mortem* and cathepsin B activity at the latter ageing periods.

The pH_u compartmentalisation of the aetiology of tenderness

Meat tenderness has long been associated with the fragmentation of localised areas in the myofibril. The fragmentation of the I-bands (Dutson, 1983; Taylor, Geesink, Thompson, Koohmaraie & Goll, 1995), the weakening of the actin/myosin interaction *post rigor* (Takahashi, 1996; Taylor *et al.*, 1995), the weakening of inter-myofibril linkages in the N_2 lines (Taylor *et al.*, 1995) and the degradation of costameres and intra myofibril linkages in the I-band (Takahashi, 1996; Taylor *et al.*, 1995) have all been implicated in meat tenderisation.

Based on the results of the present study, it is apparent that the aetiology of tenderness development in meat is pH_u compartmentalised. That is, the mechanisms driving the tenderisation of high and low pH_u meat are not the same. In high pH_u meat, the more rapid degradation of predominantly larger molecular weight proteins such as titin, nebulin and filamin early *post* mortem correspond with the early tenderisation of beef. Conversely, tenderness in low pH_u meat is attributed to the fragmentation smaller molecular weight proteins such as desmin combined with the delayed degradation of large molecular weight proteins.

The effect of titin, nebulin and filamin degradation on meat tenderness is associated with the structural roles of these proteins. These proteins are located in the I-bands of the sarcomere. Titin and nebulin are also important components of N_2 lines which are areas where thick and thin filaments, respectively, coalesce with the Z-disks. The fragmentation of these regions results in the collapse of the integrity of the muscle

structure consequently leading to meat tenderisation (Dutson, 1983; Taylor *et al.*, 1995). Yu *et al.* (1986) reconciled the immediate tenderness found in high pH_u meat with the extensive degradation of Z-disks due to the rapid rate of titin and nebulin breakdown. It is therefore proposed that tenderness in high pH_u meat is predominantly due to the weakening of inter-myofibril links within myofibrils early *post mortem* due to the degradation of larger molecular myofibrillar proteins.

In contrast, the tenderisation rate of low pH_u meat was slower compared with high pH_u meat owing to the slower degradation of myofibrillar proteins in this pH_u group. The initial decrease in shear force in low pH_u meat may be explained by the faster degradation of myosin and partial fragmentation of titin and nebulin early *post mortem*. This is consistent with other studies that observed the more extensive degradation of myosin in low pH_u meat compared with intermediate or high pH_u meat (Yates *et al.*, 1983; Yu *et al.*, 1986).

In the present study, desmin was degraded extensively in both low and high pH_u meat. Desmin is important to the ultrastructure of muscle as it is a constituent of costameres and intermediate filaments that anchor myofibrils sarcolemma and link adjacent myofibrils to each other at the Z-disk level, respectively. The *post mortem* degradation of desmin has been found to be concomitant with meat tenderisation (Taylor *et al.*, 1995; Whipple *et al.*, 1990; Young, Graafhuis & Davey, 1980).

Implications

Based on the results presented in this paper, how the degradation of each myofibril protein contributes to meat tenderness requires reassessment. Myosin comprises 43% of myofibrillar proteins in muscle and is found in the M-line where it is a major constituent of the thick filaments. The weakening of the M-line, as indicated by myosin degradation, is thought to contribute to meat tenderness (Takahashi, 1996; Yu *et al.*, 1986). However, the extensive degradation of myosin at 1 day *post mortem* did not correspond with any significant change in the shear force of low pH_u meat. Moreover, minimal myosin degradation was observed in high pH_u meat, which was already acceptably tender at 1 day *post mortem*. This implies that although large molecular weight proteins represents only a fraction of the total myofibril proteins in muscle, their contribution to the tenderness of high pH_u meat is significant compared to the more abundant muscle proteins such as myosin and actin.

Although desmin degradation was observed in both low and high pH_u meat, any significant role of desmin in meat tenderisation is most likely to be in low pH_u meat,

where desmin degradation was concomitant with the drastic reduction in shear force. The role of desmin in the tenderisation of high pH_u seems to be negligible as this group was already acceptably tender long before desmin degradation had been initially detected.

Conclusions

This study provides evidence for the ultimate pH compartmentalisation of the aetiology of tenderness development in beef. The rapid tenderisation of high pH_u beef was found to be attributed to the early degradation of larger myofibrillar proteins such as titin and filamin due to the immediate activation of µ-calpain. Tenderisation of low pH_u meat is suggested to be due to the degradation of titin and nebulin by µ-calpain early *post mortem* combined with the and extensive degradation of desmin by the residual µ-calpain and cathepsin activity at the latter stages of ageing.

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Table 1: Mean and variance in shear force values for low and high pH_u bull beef aged at -1.5°C for up to 28 days *post mortem*. Mean values without the same superscript letter within day are significantly different ($p < 0.05$). Variance test was conducted using Bartlett's test for variance.

Time *post mortem* (days)	Low pH_u (n = 31)		High pH_u (n = 20)	
	Mean	Variance (kgF)	Mean	Variance (kgF)
1	13.7^a	14.6	10.1^b	21.2
2	12.1^a	13.1	9.0^b	14.9
7	10.0^a	9.8	6.8^b	9.9
14	8.6^a	4.2	5.9^b	7.6
21	8.3^a	4.0	5.7^b	4.4
28	7.8^a	6.6	4.8^b	1.4

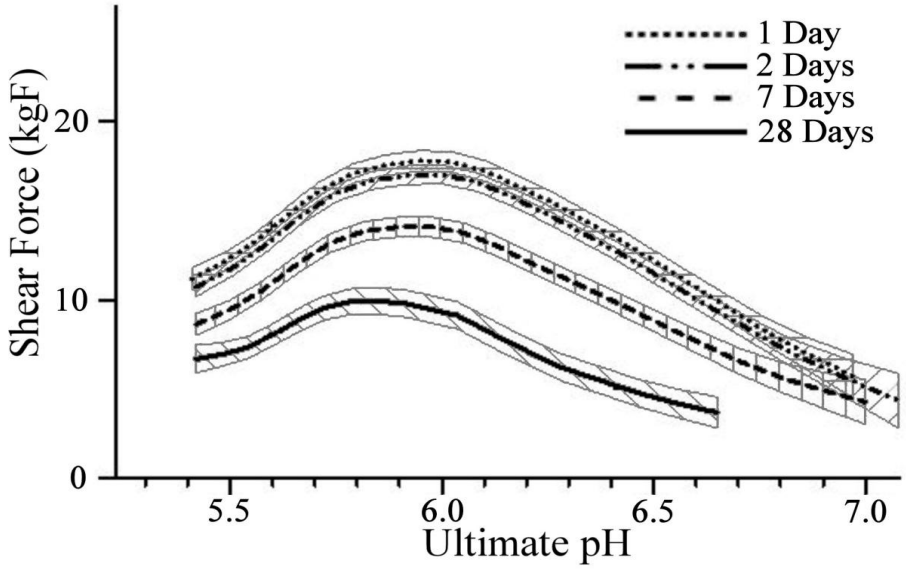

Figure 1: Shear force of bull beef (n = 63) across the pH_u spectrum during ageing. The adjusted means from each timepoint are representative by the corresponding dashed and solid lines. Splines are 95% least significant intervals.

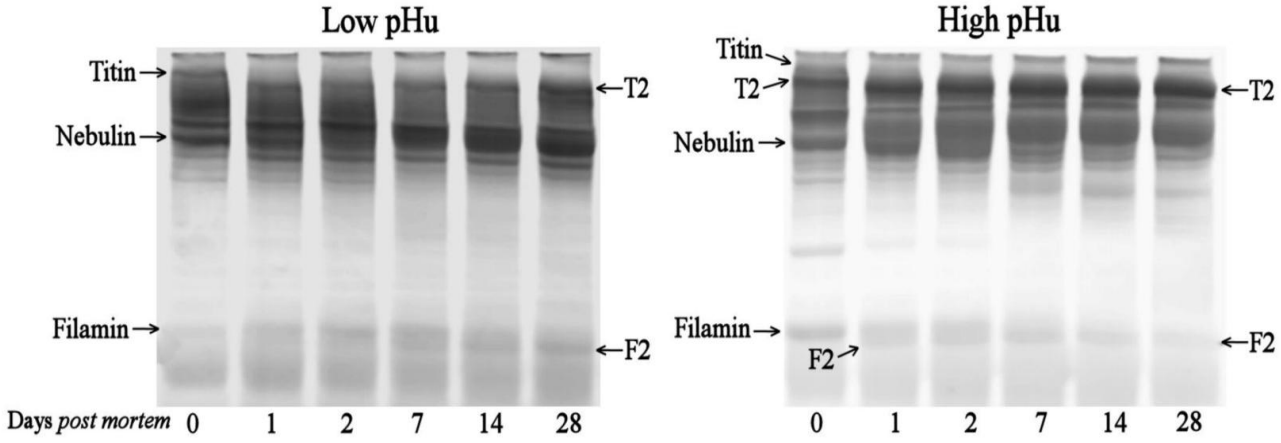

Figure 2: Coomassie blue stained SDS-PAGE gels showing the progressive degradation of larger molecular weight proteins in representative low and high pH_u bull beef samples during ageing. The breakdown product of intact titin is labelled T2. Filamin degradation products are labelled F2.

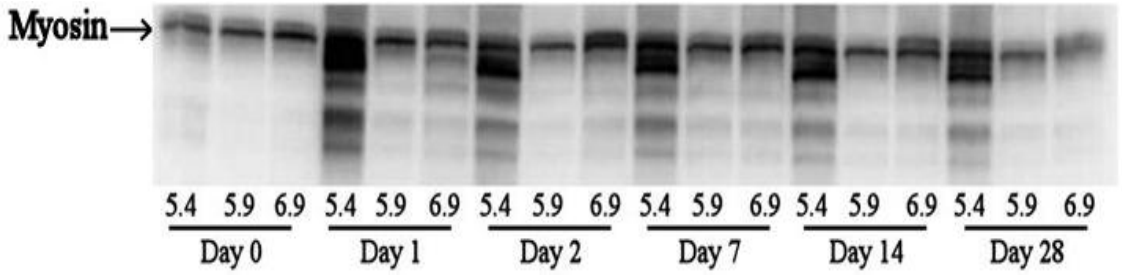

Figure 3: Western Blot analysis of myosin in representative low, intermediate and high pH_u bull beef samples at all ageing timepoints. Myosin degradation was detected in the low pH_u sample at 1 day *post mortem*.

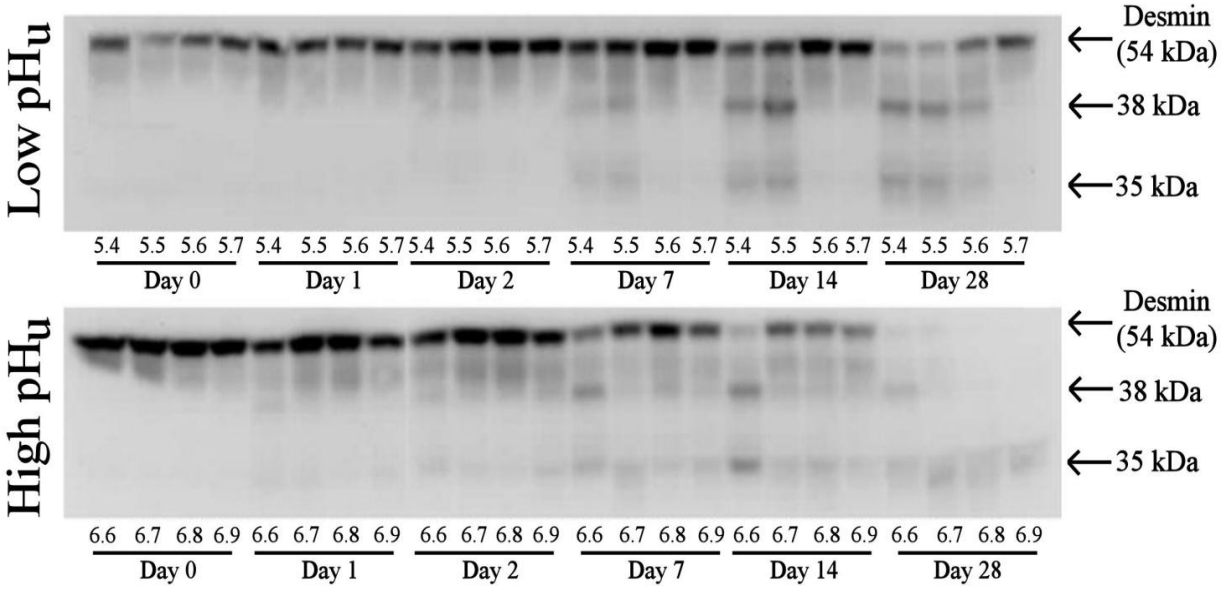

Figure 4: Degradation of desmin in representative low and high pH_u bull beef samples at all ageing timepoints. Desmin degradation products are labelled 38 kDa and 35 kDa.

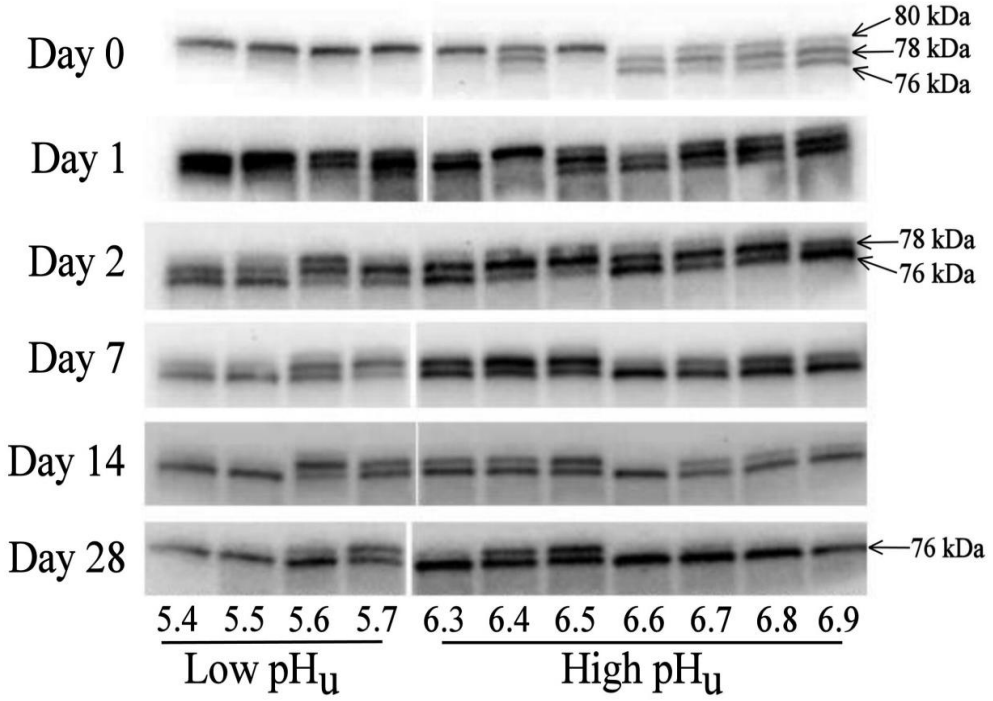

Figure 5: The autolysis of μ-calpain in representative low and high pH_u bull beef samples at all ageing timepoints. The intact μ-calpain subunit is labelled 80 kDa. Active and inactive autolysed subunits are labelled 78 kDa and 76 kDa, respectively.

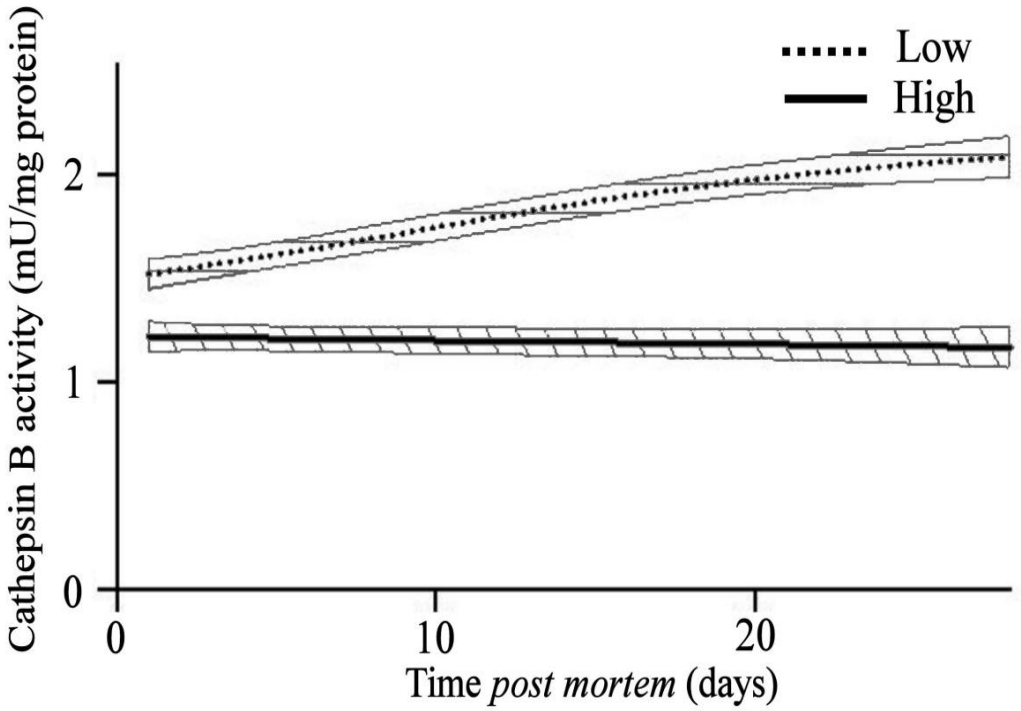

Figure 6: Cathepsin B activity in low and high pH_u bull beef samples aged at -1°C for up to 28 days *post mortem*. The adjusted means represented by the dotted and solid lines for low and high pH_u meat, respectively. Splines are 95% least significant intervals.

III

Small heat shock proteins and their role in meat tenderness: A review

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Abstract

The development of meat quality is a complex interaction between the biological traits and biochemical processes during the conversion of muscle to meat. It was hypothesised that muscles inevitably engage towards apoptotic cell death due to the termination of oxygen and nutrient supply to the muscle following exsanguination. Thus, factors that regulate the process of apoptotic cell death of muscle cells are believed to ultimately influence meat quality. Proteomic studies have associated the regulation of small heat shock proteins (sHSPs) with various meat quality attributes including tenderness, colour, juiciness and flavour. Due to the anti-apoptotic and chaperone functions of sHSPs, they are proposed to be directly involved with development of meat quality. In this review, we discuss the possible chaperone and anti-apoptotic role of sHSPs during the conversion of muscle to meat and consider the repercussions of this on the development of meat tenderness.

Keywords: Small heat shock proteins, meat quality, tenderness, intermediate pH, apoptosis, chaperone

1. Introduction

An ongoing challenge for the red meat industry is to produce higher value table cuts of consistent good quality. Three dominant meat attributes by which consumers judge meat quality are appearance, tenderness and flavour. The conformance of these attributes to consumers' expectations is critical and deviating from this will adversely affect the meat's marketability. Meat quality is increasingly understood to be an interaction between the biological traits of the live animal and biochemical processes that occur *post mortem* as muscle is converted to meat and during ageing. Although much work has been undertaken to optimise meat quality and minimise variability, the causes of large variation in meat quality is still not fully understood.

Recognising the fundamental importance of muscle proteins to meat quality traits, there has been a growing interest on how muscle proteins and the genes regulating their expression relate to meat quality. Proteomics is a tool increasingly used by meat scientists to determine the protein profile of muscle early *post mortem* and during ageing. Briefly, proteomics is the characterisation of the entire complements of proteins (proteome) that are expressed in a cell or tissue type (Bendixen, 2005). Determining the proteome of a muscle will provide clues on the function of genes and reveal a better understanding on how biochemical processes taking place in muscle affect meat quality.

The majority of proteomic studies in meat science have aimed to determine the proteome of muscle to identify molecular markers, or biomarkers, for meat quality traits. Several of these studies have consistently reported the differential expression of chaperone proteins, specifically small heat shock proteins, in muscle with variable tenderness, colour, water holding capacity and flavour (Bernard, Cassar-Malek, Le Cunff, Dubroeucq, Renand & Hocquette, 2007; Hwang, Park, Kim, Cho & Lee, 2005; Kim *et al.*, 2008; Sayd *et al.*, 2006). The aim of this review is to present the current findings on small heat shock proteins and meat quality and to discuss the biochemical mechanisms by which small heat shock proteins may affect tenderness.

2. Small heat shock proteins

Small heat shock proteins (sHSPs) belong to larger heat shock protein family. Heat shock proteins (HSP) can be categorised into five classes according to their monomeric molecular size. Four of these classes have molecular masses of about 60 kDa, 70 kDa, 90 kDa and 100 kDa, and are termed HSP60, HSP70, HSP90 and HSP100, respectively (Fink, 1999). Although they are the smallest of the HSP family,

sHSPs are the most variable in size having molecular masses ranging from 12-43 kDa (Haslbeck, Franzmann, Weinfurtner & Buchner, 2005). They are ubiquitous in all organisms, having been isolated from archaea, bacteria and eukaryotes (Gusev *et al.*, 2002). Interestingly, the number of sHSPs in any given organism varies considerably such that only two sHSPs is expressed in the yeast *Saccharomyces cerevisiae* compared with 19 sHSPs expressed in the plant *Arabidopsis thaliana* (Haslbeck *et al.*, 2005).

To date, 10 sHSPs have been identified in mammals but not all are universally expressed in mammalian cells (Taylor & Benjamin, 2005). Taylor *et al.* (2005) classified mammalian sHSPs according to the localisation of their expression within the organism: Class I sHSP are those that are ubiquitously expressed and Class II sHSPs refer to sHSPs which are only expressed in specific tissues. Currently, HSP20, HSP27 and αβ-crystallin have been implicated in meat quality. Thus, this review will predominantly discuss the structure and functional role of these sHSPs in muscle tissue. These proteins are Class I sHSPs and are reported to be genetically closely related (de Jong, Caspers & Leunissen, 1998).

3. The structure of small heat shock proteins

The monomeric structure of sHSPs have been described in detail by previous reviews (Gusev *et al.*, 2002; Haslbeck, 2002; Mymrikov *et al.*, 2011; Parcellier, Schmitt, Brunet, Hammann, Solary & Garrido, 2005) and illustrated in Figure 1. sHSP monomers are composed of several distinguishable domains. Perhaps the most obvious of these is the so-called α-crystallin domain located at the C-terminal end of the sHSP monomer. The α-crystallin domain is highly conserved and consists of an approximately 90 amino acid sequence that characterises membership of a protein to this group. The α-crystallin domain is proceeded by a flexible tail that is of variable length and amino acid sequence. At the N-terminal end of some sHSP monomers is the somewhat conserved WDPF domain. This is followed by a short and variable sequence of amino acid residues which has a slightly conserved site on the C-terminal end of the sequence. The variability in the molecular weights of sHSPs can be explained by the differences in length of the sites between the WDPF and α-crystallin domains and the variable length of the flexible C-terminal extension (Sun & MacRae, 2005b; Van Montfort, Slingsby & Vierling, 2001).

Certain sites in the α-crystallin domain are involved in the formation of β-pleated sheets that interact to form dimers and oligomers (Gusev *et al.*, 2002). These oligomers can either be homo- or hetero-oligomers composed of a single or multiple

sHSPs, respectively. The formation and structure of sHSP oligomers are in part dependent on the α-crystallin domain and the variable length and structure of the N- and C-terminal sequences of each sHSP monomer (Gusev *et al.*, 2002). sHSP oligomers are structurally flexible and variable in size (Haley, Horwitz & Stewart, 1998), with sub-units within an oligomer capable of rapidly exchanging with other sHSP oligomers (Bova, McHaourab, Han & Fung, 2000). Due to the high concentration of HSP20, HSP27 and αβ-crystallin in skeletal muscle (Kato, Goto, Inaguma, Hasegawa, Morishita & Asano, 1994), it is likely that these sHSPs variably interact with each other and exist as hetero-oligomers in muscle cells (Gusev, Bukach & Marston, 2005). This is supported by Kato *et al.* (1994) and Sugiyama *et al.* (2000) who reported the formation of HSP27, HSP20 and αβ-crystallin hetero-oligomers in skeletal muscle.

During periods of stress, sHSPs can undergo post-translational modifications resulting in protein phosphorylation at defined serine residues by protein kinases. Stress induces the activity of cAMP-dependent protein kinase which is known to phosphorylate Ser^{16} of HSP20 (Beall, Kato, Goldenring, Rasmussen & Brophy, 1997; Brophy, Dickinson & Woodrum, 1999a). Mitogen-activated protein (MAP) kinase activated protein kinase-2 phosphorylates HSP27 at Ser^{15}, Ser^{78} and Ser^{82} (Rogalla *et al.*, 1999; Stokoe, Engel, Campbell, Cohen & Gaestel, 1992) and Ser^{59} in αβ-crystallin (Kato, Ito, Kamei, Inaguma, Iwamototo & Sagal, 1998). Similarly, phosphorylation of αβ-crystallin Ser^{45} is catalysed by p44/p42 MAP kinase (Kato *et al.*, 1998).

The phosphorylation of sHSPs promotes the dissociation of oligomeric complexes into smaller oligomers, dimers and monomers. The degree of dissociation and resulting size of each oligomer is dependent upon the extent of sHSP phosphorylation (Ito, Kamei, Iwamoto, Inaguma, Nohara & Kato, 2001; Rogalla *et al.*, 1999). This in turn affects the properties of sHSPs such that the isoelectric point (p*I*) of singly phosphorylated HSP20 is higher than HSP20 with two phosphorylated residues at p*I* 6.0 and 5.7, respectively (Gusev *et al.*, 2005). Additionally, sHSP phosphorylation is also associated with the redistribution of sHSPs within cells as demonstrated by the translocation of phosphorylated αβ-crystallin from the cytosol to the Z-line of the myofibrils (Golenhofen, Ness, Koob, Htun, Schaper & Drenckhahn, 1998) and HSP27 from the cytosol to the nucleus (Arrigo, Suhan & Welch, 1988).

4. The function of small heat shock proteins in unstressed cells

4.1 sHSPs are molecular chaperones

Molecular chaperones are defined as proteins that bind to and stabilise unstable proteins and facilitates their correct assembly, but are not themselves components of the final functional structure (Ellis & van der Vies, 1991; Hendrick & Hartl, 1993). HSPs are widely regarded as molecular chaperones, with HSP60 and HSP70 recognised for their role in facilitating the correct assembly and folding of newly synthesised proteins. They are also involved in preventing the irreversible aggregation of proteins by assisting in proper reassembly of aberrantly folded and denatured proteins in an ATP-dependent process, and assist in the translocation of proteins to their correct cellular compartment (see reviews (Ellis *et al.*, 1991; Georgopoulos & Welch, 1993; Hendrick *et al.*, 1993)). Unlike larger members of the heat shock protein family, sHSPs function in an ATP-independent manner (Jakob, Gaestel, Engel & Buchner, 1993). Although there is a limited understanding regarding the chaperoning function of sHSPs in mammalian cells under normal conditions, the importance of sHSPs to the function of cells is indisputable. For example, the increased expression of HSP27 has been correlated with the growth status of mammalian cells (Welch, 1992) and mutations in αβ-crystallin lead to cataract development in the eye and desmin-related myopathies (Perng, Wen, van den, Prescott & Quinlan, 2004). In addition, mutations of HSP27 cause distal hereditary motor neuropathies and Charot-Marie-Tooth disease (Sun *et al.*, 2005b).

4.2 Actin polymerisation is modulated by sHSPs

Actin is a major component in the microfilaments of the cytoskeleton in muscle cells and is essential in the maintenance and structural integrity of muscle. Actin can exist in a monomeric globular form (G-actin) that is localised in muscle cytoplasm. G-actin subunits polymerise to form F-actin filaments which are arranged in a double helical conformation (Holmes, Popp, Gebhard & Kabsch, 1990). Upon initiation of actin polymerisation, actin subunits assemble at the so-called plus end of the microfilament, which can grow up to ten times faster than the opposite end, the minus end. Conversely, F-actin can also depolymerise into G-actin sub-units. Under stable conditions, actin polymerisation and depolymerisation rates are equivalent at the plus and minus ends, respectively, ensuring that the actin microfilament maintains a constant length (Mounier & Arrigo, 2002).

sHSPs have been implicated in the regulation of microfilament dynamics by their ability to inhibit actin polymerisation in muscle tissue thereby regulating microfilament formation (Figure 2). This was originally reported in smooth muscle cells, where actin polymerisation was found to be inhibited by HSP27 (Miron, Vancompernolle, Vandekerckhove, Wilchek & Geiger, 1991; Miron, Wilchek & Geiger, 1988). Conserved sequences in the C-terminal end of the α-crystallin domain and the Pro-Glu-Gly sequence upstream from the α-crystallin domain of sHSPs share some homology to known sites where actin monomers bind as they polymerise (Holmes *et al.*, 1990). Thus, sHSPs could mimic an actin monomer and bind to these actin-actin binding sites effectively acting as a cap to inhibit actin polymerisation (Mounier *et al.*, 2002).

Inhibition of actin polymerisation by sHSPs is regulated by their phosphorylation status. HSP27 was only capable of inhibiting actin polymerisation as a non-phosphorylated monomer and phosphorylated HSP27 monomers and non-phosphorylated oligomers did not affect actin polymerisation (Benndorf, Hayeß, Ryazantsev, Wieske, Behlke & Lutsch, 1994; Schneider, Hamano & Cooper, 1998). In addition, overexpression of non-phosphorylated HSP27 was associated with reduced F-actin concentrations (Lavoie, Hickey, Weber & Landry, 1993). As the existence of monomeric non-phosphorylated sHSPs is favoured in stable, unstressed cells, it is proposed that sHSPs regulate actin filament assembly and maintenance of the cytoskeletal network.

4.3 sHSPs regulate intermediate filament interactions

Vicart *et al.* (1998) reported that a missense mutation of αβ-crystallin (R120G) caused desmin-related myopathy, highlighting the importance of αβ-crystallin to the intermediate filament network. Desmin is an intermediate filament involved in the structural integrity and function of muscle cells (Paulin & Li, 2004). It was proposed that mutated αβ-crystallin is no longer capable of participating in the assembly of desmin filaments, resulting in the formation of desmin and αβ-crystallin aggregates characteristic of desmin-related myopathies (Chavez Zobel, Loranger, Marceau, Theriault, Lambert & Landry, 2003).

Earlier studies demonstrated that αβ-crystallin is capable of inhibiting the assembly of intermediate filaments in cytoskeletal fractions (Nicholl & Quinlan, 1994). In the same study, αβ-crystallin was also reported to increase the soluble pool of the intermediate filament protein, glial fibrillary acidic protein, in the presence of

intermediate filaments. These findings suggest a role of αβ-crystallin in intermediate filament regulation and remodelling during cell development. In unstressed cells, αβ-crystallin and HSP27 associate to intermediate filaments, thus regulating intermediate filament interactions with other cellular proteins and preventing protein aggregation that may be disadvantageous to the cell (Perng, Cairns, van den, Prescott, Hutcheson & Quinlan, 1999). In addition, intermediate filament interactions of αβ-crystallin and HSP27 in striated muscle were implicated in the maintenance and remodelling of myofibrillar structures following eccentric exercise (Feasson *et al.*, 2002). It was hypothesised that the inability of mutated αβ-crystallin to control these interactions resulted in the defective assembly of intermediate filament networks leading to a desmin-related myopathy (Ghosh, Houck & Clark, 2007; Perng *et al.*, 1999). Overall, these studies confirm the requirement of sHSPs for the normal management and assembly of intermediate filaments.

4.4 sHSPs regulate smooth muscle contraction

An increasing phosphorylation of HSP20 in smooth and cardiac muscles by cAMP- and cGMP-dependent kinases have been associated with the relaxation of smooth muscle (Beall *et al.*, 1999; Beall *et al.*, 1997; Bitar, Kaminski, Hailat, Cease & Strahler, 1991; Pipkin, Johnson, Creazzo, Burch, Komalavilas & Brophy, 2003). However, the mechanism by which sHSPs regulate muscle contraction/relaxation is contentious. At present, there are three prevailing views on how sHSPs regulate smooth muscle contraction (refer to reviews (Gusev *et al.*, 2005; Mymrikov *et al.*, 2011)). A brief overview of these is given below.

It has been proposed that sHSPs directly interact with actin filaments thereby interrupting the formation of the actomyosin complex, inducing the relaxation of smooth muscle and that this is mediated by sHSP phosphorylation (Brophy, Lamb & Graham, 1999b; Rembold, Foster, Strauss, Wingard & Van Eyk, 2000). Unphosphorylated HSP20 binds to α-actinin and F-actin and stabilises their associations with other contractile proteins, thus favouring muscle contraction. However, Brophy *et al* (1999a; 1999b) established that HSP20 phosphorylation results in the preferential binding of HSP20 from filamentous F-actin to G-actin. This results in the detachment of F-actin from myosin and other contractile proteins leading to muscle relaxation (Figure 3A). In a contrasting hypothesis, Rembold *et al.* (2000) proposed that HSP20 phosphorylation induced muscle relaxation by binding to F-actin and inhibiting F-actin from associating with myosin (Figure 3B). Mymrikov *et al.* (2011) have questioned the

validity of these models as both assume the direct interaction of HSP20 with actin filaments. However, HSP20 are predominantly localised in the cell cytoplasm during unstressed conditions, with only a minority bound to actin filaments in smooth muscle.

To further clarify the role of HSP20 phosphorylation on smooth muscle relaxation, Dreiza *et al.* (2004) proposed that phosphorylated HSP20 displaces phosphorylated cofilin from its association with the protein 14-3-3, a protein involved in maintaining the structural integrity of cells. The liberated phosphorylated cofilin becomes susceptible to desphosphorylation by slingshot phosphatases, which in turn catalyses the depolymerisation of F-actin, leading to muscle relaxation (Figure 3C).

5. The function of small heat shock proteins in stressed cells

5.1 Stress leads to cell damage

Cells exposed to various forms of stress undergo physiological changes that alter the transcription, translation and folding of proteins within the cell and also disrupt the integrity of the three dimensional structure of proteins leading to their loss of function (Parsell & Lindquist, 1993). Heat stress promotes the decline in intracellular pH, cytosolic calcium and ATP concentrations (see review by Welch (1992)). Collectively, these lead to the damage of cellular components ultimately leading to cell death.

Hyperthermic conditions were demonstrated to deactivate and promote the aggregation of the enzymes β-galactosidase and luciferase in mouse cells (Nguyen, Morange & Bensaude, 1989). In skeletal muscle cells, eccentric exercise, lengthening contractions and oxidative stress lead to myofibrillar disorganisation and muscle degradation (Koh & Escobedo, 2004; Lieber & Fridén, 1999). Oxidative stress also induces biochemical and functional modifications in cardiac muscle that lead to muscle degeneration under ischemic conditions (Chi & Karliner, 2004; Fan *et al.*, 2005b). The increase in reactive oxygen species that accompany heat and oxidative stresses have been attributed to the detrimental changes following stress insult in these studies.

5.2 Stress induces apoptotic cell death

Apoptosis is a constitutive process of programmed cell death that plays an opposite but complementary role to mitosis and is fundamental in maintaining tissue homeostasis (Kerr, Wyllie & Currie, 1972). In addition, apoptosis is indispensible in the survival of organisms as it is critical in the elimination of non-functional, damaged cells that are potentially harmful in multicellular organisms (Thompson, 1995).

The morphology of apoptosis was originally described by Kerr *et al.* (1972) (Figure 4). In the first phase of apoptosis, the dying cell undergoes cytoplasmic condensation with the characteristic blebbing of the plasma membrane that separates into several membrane-bound apoptotic bodies containing intact organelles. These apoptotic bodies are swiftly recognised and ingested by phagocytes or neighbouring cells (Kerr *et al.*, 1972; Wyllie, Kerr & Currie, 1980). Unlike necrosis, apoptosis is a non-inflammatory process making it well suited to normal cell turnover. Various stimuli elicit the activation of apoptosis in muscle tissue. Aging (Dirks & Leeuwenburgh, 2002), ischemia and reperfusion (Gill, Mestril & Samali, 2002), physical stress leading to cell damage (Sandri *et al.*, 1995), heat shock and nutrient withdrawal (Mailhos, Howard & Latchman, 1993) have all been shown to induce apoptosis of cells in affected tissues.

5.3 Stress up-regulates the expression of small heat shock protein in muscle tissues

In response to the diverse range of stresses that cells are continually challenged with, cells have evolved responses that are activated in response to injury or insult (Figure 5). Hyperthermia, ischemia and oxidative stress in mammalian cells are known to activate the constitutively expressed transcriptional factors known as heat shock factors. Upon activation, heat shock factors are translocated to the cell nucleus where they form trimers and undergo phosphorylation. This enables them to bind to so-called heat shock elements of the heat shock protein gene resulting in the synthesis of heat shock proteins. These processes are comprehensively described in the following cited reviews (Chi *et al.*, 2004; Santoro, 2000; Shamovsky & Nudler, 2008)

sHSPs are constitutively expressed at low levels in muscle tissue, but the activation of heat shock factors leads to increased sHSP synthesis (Lindquist & Craig, 1988) as demonstrated for HSP27 and αβ-crystallin in cells during hyperthermia (Oesterreich, Benndorf & Bielka, 1990; Sugiyama *et al.*, 2000). Oxidative stress by chemical induction or eccentric exercises also resulted in the increased expression of HSP27 and αβ-crystallin in skeletal muscle cells (Escobedo, Pucci & Koh, 2004; Neufer & Benjamin, 1996; Paulsen *et al.*, 2007).

5.4 sHSPs function as molecular chaperones in stressed cells

sHSPs prevent the irreversible aggregation of partially denatured proteins *in vitro*. Using model target proteins, HSP27 and αβ-crystallin stabilised carbonic

anhydrase, citrate synthase and α-glucosidase exposed to heat shock and promoted their subsequent refolding to their true functional form (Jakob *et al.*, 1993; Rao, Horwitz & Zigler, 1993). There appear to be variations in the chaperone properties of individual sHSPs. Van de Klundert *et al.* (1998) reported that the capacity of HSP20 to reduce the aggregation of the model target protein insulin B during heat shock was inferior to that of αβ-crystallin. However, sHSPs tend to be complementary in chaperoning denatured proteins. HSP27 enhanced the thermotolerance of αβ-crystallin, suggesting a combined role in conferring increased resistance of cells from heat and oxidative stress (Fu & Liang, 2003).

The chaperone mechanisms of sHSPs under normal conditions have been extensively reviewed (Haslbeck, 2002; McHaourab, Godar & Stewart, 2009; Narberhaus, 2002; Sun & MacRae, 2005a; Van Montfort *et al.*, 2001), thus a general overview of the chaperone mechanisms of sHSPs during periods of stress will be discussed in this paper. The onset of stress results in the unfolding of native proteins and the simultaneous dissociation of large sHSPs oligomers to smaller complexes. This structural change in the assembly of sHSPs exposes hydrophobic regions in sHSPs that passively bind to denaturing protein substrates to form a stable, soluble sHSP-protein substrate complex (Haslbeck *et al.*, 1999; Raman & Rao, 1994). Thus, oligomer disassembly is a critical process in the protective chaperone ability of sHSPs.

The mechanisms driving the dissociation of sHSP oligomers is unclear, but has been attributed to the phosphorylation of defined serine residues of sHSPs (Ito *et al.*, 2005; Ito, Okamoto, Nakayama, Isobe & Kato, 1997). The phosphorylation of sHSPs has been credited for the conferred resistance of cells to heat shock and oxidative stresses (Koteiche & McHaourab, 2003; Lavoie, Lambert, Hickey, Weber & Landry, 1995; Mounier *et al.*, 2002). However this was disputed by other studies that report the decrease in sHSP chaperone ability following sHSP phosphorylation (Ito *et al.*, 2001; Rogalla *et al.*, 1999). Benesch *et al.* (2008) suggested that chaperone activity cannot be defined simply by whether sHSPs are phosphorylated or not. sHSP oligomers can be categorised as low affinity or high affinity oligomers according to their ability to bind denaturing proteins. Benesch *et al.* (2008) proposed that the substructure of these oligomers are dimeric and monomeric, respectively (Figure 6). sHSP phosphorylation favours the formation of monomeric oligomers substructure which has a high binding affinity to substrate proteins due to high levels of exposed hydrophobic regions in their structure. As a result of their high binding affinity, sHSP oligomers consisting of predominantly monomeric substructures lose their ability to regulate the number of

denaturing protein substrates they bind to. As a consequence, they co-precipitate with denaturing proteins and lose their chaperone activity. In contrast, oligomers with predominantly dimeric substructures have negligible levels of hydrophobic regions exposed and resulting minimal binding to denaturing proteins and little chaperone activity. Therefore, the balance of monomeric and dimeric sHSP substructures regulates the chaperone activity of sHSP oligomers (Aquilina, Benesch, Ding, Yaron, Horwitz & Robinson, 2004; Benesch *et al.*, 2008).

A characteristic feature of the protective chaperoning activity of sHSPs is their translocation from the sarcoplasm to the myofibrils of muscle cells after the onset of stress (Golenhofen *et al.*, 1998; Paulsen *et al.*, 2007). The reduction of HSP27 concentrations in muscle sarcoplasm corresponded to an increase in the myofibrillar fraction following eccentric exercise (Paulsen *et al.*, 2007). In addition, there appears to be some specificity in the regions of the myofibrils that sHSPs bind to. For instance, HSP27 and αβ-crystallin have been reported to translocate to the I-band and Z-disc of muscle cells following various stress insults (Bullard *et al.*, 2004; Djabali, De Néchaud, Landen & Portier, 1997; Golenhofen *et al.*, 1998; Koh *et al.*, 2004). In these studies, sHSPs were proposed to stabilise and assist in the refolding of key proteins in these regions that are vulnerable to degradation following stress insults.

Although sHSP-denaturing protein complexes are relatively stable (Rao *et al.*, 1993), sHSPs are unable to renature damaged protein substrates. Rather, sHSPs cooperatively assist the ATP-dependent heat shock proteins HSP70 and HSP60 in refolding denatured proteins back to their original functional forms (Narberhaus, 2002; Sun *et al.*, 2005b; Van Montfort *et al.*, 2001). During periods of stress, cellular ATP is depleted (Wang & Spector, 2001) and HSP70 and HSP60 become preoccupied in chaperoning damaged proteins or are limited by the low ATP levels. However, the return of cells to normal physiological conditions upon the dissipation of the stress insult is concomitant with the restoration of cellular ATP levels. ATP have been shown to alter the conformation of sHSPs, thus releasing their denatured client proteins and allowing other molecular chaperones, such as HSP70, to renature the proteins back to their functional state (Wang *et al.*, 2001). As sHSP chaperone activity is independent of ATP, their function as an emergency chaperone in stressed cells is critical for cell survival.

5.5 sHSPs negatively regulates stress-induced apoptosis

The protective function of sHSPs extends beyond their chaperoning ability. As previously discussed, apoptosis is critical in normal cell development and maintenance of cell homeostasis. However, various stress stimuli also induces apoptosis that can lead to loss of cell function. sHSPs can enhance cell survival by interfering with cellular signal transduction pathways regulating apoptotic cell death (Figure 7).

Apoptosis is mediated by caspases, a group of cysteine peptidases endogenous to mammalian cells (Fadeel & Orrenius, 2005). Simply, caspases 8, 9 and 10 are collectively termed as initiating caspases and caspases 3, 6 and 7 are effector caspases according to their respective roles in complicated sequence of apoptotic cell death (Fuentes-Prior & Salvesen, 2004). Stress insults, such as heat shock, induce mitochondria-dependent apoptosis in mammalian cells by increasing the permeability of the mitochondrial membrane through mechanisms that are not yet fully defined (Samali & Orrenius, 1998). Increased mitochondrial membrane permeability leads to the release of pro-apoptotic factors from the mitochondria to the cytosol. These pro-apoptotic factors include the proteins cytochrome *c* (Yang *et al.*, 1997), Smac (Du, Fang, Li, Li & Wang, 2000) and DIABLO (Verhagen *et al.*, 2000). Once released in the cytosol, cytochrome *c* associates with apoptotic protease-activating factor-1 (Apaf-1), triggering their oligomerisation to form apoptosomes. Apoptosomes interact with and activate caspase 9, which in turn initiates the activation of caspase 3 and the cleavage of numerous cellular substrates leading to apoptosis (Fadeel *et al.*, 2005; Wolf & Green, 1999). In addition, Smac and DIABLO promote apoptosis by binding to the apoptotic inhibiting proteins that neutralise the activation of caspase 3 (Du *et al.*, 2000; Verhagen *et al.*, 2000).

The regulation of apoptosis by sHSPs has been the subject of several reviews (Arrigo, 2005; Beere, 2004; Beere, 2005; Parcellier *et al.*, 2005; Samali *et al.*, 1998). Overexpression of HSP27 and HSP20 prevented the cytochrome *c* activation of caspase 9 (Fan, Chu & Kranias, 2005a; Garrido, Bruey, Fromentin, Hammann, Arrigo & Solary, 1999). sHSPs have also been demonstrated to interfere with downstream effector caspases. Muscle cells expressing αβ-crystallin inhibited the activation of caspase 3 during myogenic differentiation (Kamradt, Chen, Sam & Cryns, 2002).

It was proposed that the anti-apoptotic activity of αβ-crystallin is mediated by their phosphorylation state. The Ser^{59} phosphorylation of αβ-crystallin led to the inhibition of caspase 3 activation in cardiac myocytes during hypoxic and hyperosmotic stress (Morrison, Hoover, Thuerauf & Glembotski, 2003). In addition, phosphorylated

HSP20 associated with pro-apoptotic proteins such as Bax and 14-3-3, which led to the restriction of cytochrome *c* release and thus, the repression of caspase 3 activation (Fan *et al.*, 2005a). Conversely, a missense mutation in αβ-crystallin negates its anti-apoptotic function leading to greater mitochondrial permeability and cytochrome *c* release to the cytoplasm. This consequently results in the activation of caspase 9 and unregulated caspase-dependent cascade apoptosis associated with desmin-related cardiomyopathy (Maloyan *et al.*, 2005). In general, these studies show that sHSPs are able to interfere with chemical signals that activate initiator and effector caspases, regulating caspase-dependent apoptotic cell death.

6. The emerging role of sHSPs in meat quality

The possible role of sHSPs in meat quality was initially presented in reviews by Ouali *et al.* (2006) and Herrera-Mendez *et al.* (2006) who proposed that muscle cells inevitably engage in apoptosis *post mortem*. In response, sHSPs are variably recruited to impede the onset of apoptotic cell death and to chaperone unfolding muscle proteins in an attempt to maintain cellular homeostasis. The protective function of sHSPs delays the rate of muscle ageing and attenuates myofibrillar protein degradation. In this section, the current knowledge of the expression of sHSPs in *post mortem* muscle and the possible mechanisms by which they may be crucial in the development of meat quality is discussed.

6.1 Muscle cells undergo apoptotic cell death post mortem

Muscle increasingly becomes ischemic and acidifies *post mortem* due to the termination of the oxygen and nutrient supply to muscle cells. As ischemic conditions and nutrient withdrawal are known to induce apoptosis, muscle cells inevitably engage towards apoptotic cell death preceding *rigor mortis* during the conversion of muscle to meat (Herrera-Mendez *et al.*, 2006; Ouali *et al.*, 2006). Upon the initiation of apoptosis, apoptotic cells are isolated from neighbouring cells by the inversion of the oppositely charged inner and outer leaflet of the cellular membrane.

In a study to establish the apoptotic death of *post mortem* muscle cells, g*astrocnemius* and *plantaris* muscles were excised from rats at 1 h *post mortem* then stored at 4°C for up to 3 days (Becila *et al.*, 2010). The inversion of phosphatidylserine from the inner to the outer membrane leaflet, cell shrinkage and actin degradation were observed early *post mortem*. The shrinkage of muscle cells in rat *longissimus* muscle

post mortem was also confirmed by Ouali *et al.* (2007). These hallmarks of apoptosis confirm that muscle cells indeed undergo apoptotic cell death *post mortem*.

6.2 sHSPs as biomarkers for meat quality attributes

Predicting the ultimate outcome of a given quality attribute early *post mortem* is advantageous for the meat industry. These attributes are protein mediated. Thus, application of proteomics to meat science should allow researchers to determine new biomarkers for meat quality. Bernard *et al.* (2007) reported the negative correlation between HSP27 and αβ-crystallin concentrations and meat tenderness, juiciness and flavour. In addition, HSP27 concentration was also negatively correlated with colour quality (L* and a* values) in beef (Kim *et al.*, 2008). These findings suggest the potential for the development of statistical models based on the expression of these biomarkers to enable the meat industry to predict the ultimate outcome for any given meat quality attribute early *post mortem*. However, the mechanisms by which sHSPs are involved in the development of various meat quality attributes are largely unknown.

6.3 Meat tenderness and proteases

Meat ultimate pH (pH_u) is an indicator of tenderness, and on average, intermediate pH_u (pH 5.8 to 6.19) meat is tougher compared with low ($pH_u \leq 5.79$) and high pH_u ($pH \geq 6.2$) meat (Bouton, Harris & Shorthose, 1971; Jeremiah, Tong & Gibson, 1991; Lomiwes, Farouk, Frost, Dobbie & Young, 2011). Intermediate pH_u meat is also more inconsistent in tenderness and the biochemical reasons causing this are largely unknown (Pulford, Fraga Vazquez, Frost, Fraser-Smith, Dobbie & Rosenvold, 2008).

It is widely accepted that *post mortem* meat tenderisation is an enzymatic process. Currently, μ-calpains and cathepsins are two protease systems that have been strongly implicated in the hydrolysis of myofibrillar proteins resulting in meat tenderisation. μ-Calpains are Ca^{2+}-dependent proteases that are optimally active at physiological pH. Early *post mortem*, the accumulation of Ca^{2+} into the muscle sarcoplasm activates μ-calpain which in turn destabilises the intact myofibrillar structure by degrading myofibrillar proteins including titin, filamin, troponin-T and desmin (Hopkins & Taylor, 2004; Huff-Lonergan, Mitsuhashi, Beekman, Parrish, Olson & Robson, 1996). Upon activation, μ-calpain increasingly autolyses and ultimately becomes inactive (Maddock, Huff-Lonergan, Rowe & Lonergan, 2005). In addition, as muscle pH acidifies *post mortem*, μ-calpain activity becomes increasingly limited.

Further to inactivity through autolysis and pH fall, µ-calpain activity is also moderated by the enzyme's inhibitor, calpastatin (Goll, Thompson, Li, Wei & Cong, 2003). Studies have reported the positive correlation between calpastatin activity and meat toughness (Kemp, Sensky, Bardsley, Buttery & Parr, 2010; Shackelford, Koohmaraie, Cundiff, Gregory, Rohrer & Savell, 1994) with delayed meat tenderisation possibly attributed to the higher ratio of calpastatin in relation to calpain activity (Koohmaraie, Whipple, Kretchmar, Crouse & Mersmann, 1991).

Cathepsins are a group of serine (cathepsin G), cysteine (cathepsin B, H and L) and aspartic (cathepsins D and E) proteases confined within the lysosomes (Sentandreu, Coulis & Ouali, 2002), and are active between the pH range of 3.0 and 6.0 (Etherington, 1984). As muscle pH decreases *post mortem*, lysosomal membranes become increasingly destabilised, resulting in the release of cathepsins to the sarcoplasm and access to hydrolyse myofibrillar proteins. The contribution of cathepsins to meat tenderisation was largely discounted due to minimal degradation of actin and myosin, the primary substrates of cathepsins in muscle (Hopkins *et al.*, 2004). However, cathepsin activity has been associated with the destabilisation of Z-lines (Ouali, Garrel, Obled, Deval, Valin & Penny, 1987) and has been demonstrated to hydrolyse titin and troponin-T (Dutson, 1983; Zeece & Katoh, 1989), myofibrillar proteins that are also associated with meat tenderness. Thus, it is proposed that meat tenderness, as observed in low pH_u meat, is due to the synergistic activities of endogenous proteolytic enzymes in muscle (Jiang, 1998; Ouali, 1992). Initial meat tenderisation appears to be due to µ-calpain, which is optimally active at physiological pH. As pH declines in *post mortem* muscle and µ-calpain activity becomes limited, cathepsins become increasingly active and hydrolyse myofibrillar proteins associated with the tenderisation of meat *post rigor* (Lomiwes, Farouk, Wu & Young, submitted-b).

It is important to note that other proteases endogenous to skeletal muscle tissue have been implicated with meat tenderisation by either directly hydrolysing myfibrillar proteins or regulating biochemical processes associated with meat tenderisation. Among these lesser known proteases are proteosomes, caspases, and matrix metallopeptidases (Aubry, Sentandreu, Levieux, Ouali & Dutaud, 2006; Kemp, Bardsley & Parr, 2006; Kemp *et al.*, 2010; Ouali *et al.*, 2006; Sentandreu *et al.*, 2002). More research is required to characterise the proteolytic and functional activities of these enzymes in *post mortem* muscle.

6.4 sHSPs chaperone the degradation of myofibrillar proteins during ageing leading to meat toughness

sHSPs are abundant and are dynamically expressed in muscle after slaughter (Jia *et al.*, 2007; Jia, Hollung, Therkildsen, Hildrum & Bendixen, 2006; Pulford *et al.*, 2008). As previously discussed, sHSPs regulate actin polymerisation and actin-myosin interactions (Miron *et al.*, 1991; Mounier *et al.*, 2002) and are involved in managing the proper assembly of intermediate filaments of muscle (Feasson *et al.*, 2002; Perng *et al.*, 1999). Furthermore, sHSPs stabilise and prevent the aggregation of model proteins during periods of stress (Jakob *et al.*, 1993; Rao *et al.*, 1993). As meat tenderisation is attributed to the proteolytic hydrolysis of myofibrillar proteins, the constitutive role of sHSPs in regulating muscle filament assembly and their protective function in stabilising myofibrillar components during periods of stress have been implicated sHSPs in the development of tenderness in beef.

Earlier proteomic studies to discover new biomarkers for meat quality have consistently found variable expression of sHSPs between tough and tender meat. Currently there are two conflicting views on the relationship between sHSPs expression in muscle *post mortem* and meat tenderness. Proteomic analyses have revealed a significant positive correlation between HSP27 concentration and toughness in beef (Kim *et al.*, 2008) and pork (Hwang *et al.*, 2005). Due to the chaperone function of sHSPs in maintaining protein integrity, the down-regulation of HSP27 and αβ-crystallin in tender meat was suggested to favour the proteolytic degradation of actin and myosin leading to more tender meat (Bernard *et al.*, 2007; Kim *et al.*, 2008). In contrast, Morzel *et al.* (2008) reported a significant negative relationship between HSP27 expression and beef toughness. HSP27 and HSP20 levels were also reported to be significantly higher in bull *M. longissimus thoracis* than in *M. semitendinosus* (Guillemin, Jurie, Cassar-Malek, Hocquette, Renand & Picard, 2011a; Guillemin, Jurie, Micol, Renand, Hocquette & Picard, 2011b). Although shear force measurements were not reported these in studies, meat from *M. longissimus thoracis* is considered more tender than *semitendinosus* muscle (Calkins & Sullivan, 2007). Protein aggregation that occurs during meat ageing purportedly changes the chemical and physical recognition sites of skeletal myofibrillar proteins and decreases their susceptibility to proteolytic degradation (Morzel, Gatellier, Sayd, Renerre & Laville, 2006). Higher concentrations of sHSPs in *M. longissimus thoracis* was proposed to prevent the formation of protein aggregates thereby facilitating in the proteolytic degradation of myofibrillar proteins leading to tender meat (Guillemin *et al.*, 2011a; Morzel *et al.*, 2008).

The high incidence of intermediate pH_u beef, particularly in bulls (Graafhuis & Devine, 1994), has largely been attributed to the pre-slaughter handling of animals. Stresses during transport and lairage collectively contribute to the depletion of muscle glycogen, inevitably resulting in meat with an elevated pH_u (McVeigh, Tarrant & Harrington, 1982). Considering that stress is known to up-regulate sHSP expression, meat with elevated pH_u may have higher concentrations of sHSPs. Interestingly, the expression of sarcoplasmic sHSPs is pH_u related such that detected concentrations of HSP20, HSP27 and αβ-crystallin in the muscle sarcoplasm declined with decreasing pH_u (Pulford *et al.*, 2008) Based on these findings, it was hypothesised that toughness in intermediate pH_u beef is due to a combination of sub-optimal endogenous proteolytic activity and high levels of bio-available sHSPs that stabilise unfolded myofibrillar proteins, preventing their further hydrolysis (Pulford, Dobbie, Fraga Vazquez, Fraser-Smith, Frost & Morris, 2009).

Studies in our laboratory to determine the kinetics of HSP20, HSP27 and αβ-crystallin in bovine *M. longissimus dorsi* during ageing showed that sHSPs declined to minimum concentrations within 2 days *post mortem*. However, the extent of sHSP decline was pH_u related such that high pH_u meat had sustained higher levels of sHSPs post rigor followed by intermediate then low pH_u meat (Lomiwes, Farouk, Frost, Dobbie & Young, submitted-a). The rapid disappearance of sHSPs with decreasing pH levels is likely due to the precipitation of these proteins as muscle pH declines far below their respective isoelectric points (Pulford *et al.*, 2008). Although high pH_u meat had higher concentrations of sHSPs compared with other pH_u groups, sHSPs in high pH_u meat were progressively degraded during ageing, possibly by μ-calpain which is optimally active in high pH_u (Lomiwes *et al.*, 2011). This suggests that sHSPs in this group are less bioactive due to their susceptibility to extensive proteolytic degradation.

In a separate but complementary study, αβ-crystallin was demonstrated to increasingly associate with skeletal myofibrils as muscle pH declined from 7.4 to 6.5, suggesting that the disappearance of sHSPs from the sarcoplasm as muscle pH declined (Pulford *et al.*, 2008) was concomitant with their association to muscle myofibrils (Figure 8A). Moreover, αβ-crystallin added exogenously to myofibrillar extracts reduced the proteolytic hydrolysis of desmin and titin. Further analysis revealed that αβ-crystallin can be a substrate of μ-calpain and was able to competitively inhibit μ-calpain activity (Lomiwes *et al.*, submitted-c). Overall, protein aggregation and proteolytic hydrolysis of sHSPs effectively nullify the chaperoning function of sHSPs in low and high pH_u meat, respectively (Figure 8B). It is proposed sHSPs protect the

structural integrity of muscle in two ways. First, sHSPs bind to muscle myofibrils and stabilise unfolded proteins thereby maintaining the integrity of muscle structure. Finally, myofibril-bound sHSPs become alternative substrates for μ-calpain and reduce the hydrolysis of myofibrillar proteins.

The chaperone and apoptotic functions of sHSPs upon the onset of stress are mediated by the phosphorylation and disassembly of large sHSP complexes to smaller oligomers. Additionally, the chaperone ability of these oligomers is regulated by their substructure. The majority of studies linking the proteomic profile of muscle to meat quality attributes employed two-dimensional SDS-PAGE combined with MALDI-TOF mass spectrometry for protein identification (Hwang *et al.*, 2005; Jia *et al.*, 2007; Laville *et al.*, 2009). These studies have identified that HSP20, HSP27 and αβ-crystallin can be present in various isoforms in muscle as indicated by their p*I*. As phosphorylation of sHSPs is known to affect their p*I*, it is proposed that the various isoforms of sHSPs in muscle reported in these studies are phosphorylated monomers. Due to the denaturing conditions of the SDS-PAGE assay, this analytical tool is limited to the semi-quantitative determination of sHSP monomers and their various isoforms. Subtleties of phosphorylation and quaternary structure cannot be seen. Thus to verify the chaperone and anti-apoptotic role of sHSPs in determining meat quality, further work is required to define the oligomeric substructures of these sHSPs during the conversion of muscle to meat.

6.5 Delayed onset of apoptosis and meat tenderness

The identification of apoptotic hallmarks in muscles immediately *post mortem* suggests that apoptosis, and not *rigor mortis*, is the initial phase in the conversion of muscle to meat (Herrera-Mendez *et al.*, 2006; Ouali *et al.*, 2006). Thus, cellular changes associated with apoptosis during the conversion of muscle to meat are believed to be intrinsically linked with meat quality attributes, particularly meat tenderness (Ouali *et al.*, 2007).

Caspases, noted earlier, are proteolytic enzymes that are endogenous in muscle tissue and have also been linked with myofibrillar protein degradation leading to meat tenderness. However, it is becoming clear that caspases do not significantly contribute to meat tenderness by means of proteolytic degradation (Huang, Huang, Zhou, Xu & Xue, 2011; Mohrhauser, Underwood & Weaver, 2011; Underwood, Means & Du, 2008). Considering the critical role of caspases in apoptotic cell death, it is proposed that the involvement of caspases in meat tenderisation is in their ability to mediate

apoptosis rather than myofibrillar protein hydrolysis. In a study characterising the kinetics of caspases in bull *M. longissimus dorsi* early *post mortem*, Pulford *et al.* (2009) reported high initial activities of initiator caspases 8 and 9 early *post mortem*, with no significant differences between pH_u groups. Interestingly, the activation of effector caspase 3/7 was delayed in intermediate pH_u meat compared with high and low pH_u meat. Considering that sHSPs are known inhibitors of caspase activation, high concentrations of bioactive sHSPs in intermediate pH_u meat is proposed to interfere with effector caspase 3/7 activation, slowing the process of apoptotic death and leading to the delayed tenderisation observed in this pH_u group.

The rate of pH decline during the conversion of muscle to meat affects meat tenderness (Hwang & Thompson, 2001). Characterisation of pH kinetics in muscle has shown that the pH does not decline continuously early *post mortem*. Rather, pH decline within the first eight hours *post mortem* is characterised by the occurrence of one or two stability steps between pH 6.4 and 6.2, where pH momentarily plateaus before resuming its decline (Herrera-Mendez *et al.*, 2006; Ouali *et al.*, 2006). The frequency of these stability steps has been associated with meat tenderness such that muscles with a single stability step were significantly more tender compared with muscles displaying two steps early *post mortem*. A distinguishing feature of apoptosis is the simultaneous inversion of acidic phosphatidylserine groups from the internal to the external leaflet and the basic phosphatidylcholine and phosphatidylethanolamine groups from the external to the internal leaflet of the cellular membrane (Becila *et al.*, 2010; Martin *et al.*, 1995). The inversion of phospholipid groups in the cellular membrane modifies the buffering capacity and charge distribution within muscle cells, and has been suggested to counteract muscle acidification resulting in the polyphasic pH decline of muscle early *post mortem* (Ouali *et al.*, 2007). Phospholipid inversion in cellular membranes is inhibited by the anti-apoptotic protein BCl-2 (Martin *et al.*, 1995). Considering that sHSPs are also anti-apoptotic proteins, they may similarly impede or inhibit phospholipid inversion during apoptosis consequently resulting in the multiple pH stability steps or delayed rate of pH decline, and consequently, tougher meat.

The reasons for the large variation of meat tenderness in intermediate pH_u meat are still an enigma to meat science. However, the variability in tenderness in this pH_u category may be owing to the optimal binding of sHSPs to myofibrillar proteins combined with the low proteolytic activities of μ-calpain and cathepsins. With the established correlation between sHSP expression and meat tenderness (Bernard *et al.*, 2007; Kim *et al.*, 2008), we propose that inconsistent tenderness in intermediate pH_u

meat may also correspond with the variation of sHSP expression in this group. Thus, intermediate pH_u meat with high concentrations of sHSPs will be tough and the inverse will be true for tender intermediate pH_u meat (Figure 9). As sHSP chaperone activity is regulated by their oligomeric sub-structure, the variable substructure of sHSPs in intermediate pH_u meat is proposed to contribute to the high variation of meat tenderness in this group.

6.6 sHSPs and other meat quality attributes

Looking beyond meat tenderness, proteomic analysis has consistently revealed a strong association between sHSP expression and meat colour. Proteomic analysis revealed higher levels of HSP27 and αβ-crystallin in porcine (Sayd et al., 2006) and bovine (Kim et al., 2008) muscles that yielded meat that were dark in colour. These associations were confirmed in another study, where the expression of αβ-crystallin, and to a lesser extent HSP27, were significantly negatively related with Hunter L^* values (Hwang *et al.*, 2005).

The effect of pH_u on the colour of meat is widely known. Generally there is a significant negative correlation between meat lightness (L^* value) and pH_u in pork (Dransfield, Nute, Mottram, Rowan & Lawrence, 1985) and beef (Page, Wulf & Schwotzer, 2001). Thus, the unappealing dark colour in meat is associated with higher pH_u meat. This is proposed to be due to the enhanced mitochondrial activity in higher pH_u meat (Tang, Faustman, Hoagland, Mancini, Seyfert & Hunt, 2005) which maintains myoglobin in its dark deoxymyoglobin state rather than the more appealing cherry red oxymyogobin (Faustman & Cassens, 1990). Pre-slaughter stresses favour high pH_u meat and induce the up-regulation of sHSPs. Although the role of sHSPs in meat colour development is unclear, it has been proposed that the anti-apoptotic function of sHSPs impedes the rate of pH fall by mechanisms discussed in the previous section, thus favouring darker meat colour (Sayd et al., 2006). However, the effect of stress and pH_u on sHSP expression were not investigated in the studies by Hwang *et al*. (2005), Kim *et al.* (2008) and Sayd *et al.* (2006). Therefore, it is speculated that higher levels of sHSPs in darker meat may be due their up-regulation in stressed animals, as indicated by their dark colour, rather than the direct affect of sHSPs on *post mortem* biochemical mechanisms driving the development of meat colour.

The expression of sHSPs has also been related with several other meat quality traits. Higher drip loss in pork *M. longissimus dorsi* was associated a decrease in

HSP27 and αβ-crystallin concentrations. Conversely, Kwasiborski *et al.* (2008) report a significant positive correlation between αβ-crystallin and cooking loss. Furthermore, the down regulation of αβ-crystallin and HSP27 in bull *M. longissimus thoracis* was associated with increasing juiciness and flavour as determined by trained panellists (Bernard *et al.*, 2007). As most of the current work on sHSPs and meat quality have primarily focussed on the contribution of these proteins to meat tenderness, the implications of sHSP expression on other meat attributes including colour, cooking loss, juiciness and flavour is less defined.

7. Conclusion

The application of proteomics in meat science has revealed the variable expression of sHSPs in muscle cells early *post mortem* and during ageing. These proteins assist in mediating muscle contraction and enhancing cell survival by chaperoning destabilised proteins and negatively regulating apoptotic cell death during periods of stress. Due to the role of these proteins in diseases, much research regarding this group of proteins is in the field of human pathology (Clark & Muchowski, 2000; Sun *et al.*, 2005a).

Muscle cells are hypothesised to undergo apoptotic cell death proceeding slaughter. Further, a phase of apoptosis is believed to precede *rigor mortis* during the conversion of muscle to meat (Herrera-Mendez *et al.*, 2006; Ouali *et al.*, 2006). This is supported by the appearance of several apoptotic hallmarks in muscle after slaughter (Becila *et al.*, 2010). Due to the biochemical and structural consequences of apoptosis in muscle cells, mechanisms that mediate apoptosis influence the development of meat quality.

Studies searching for predictive meat quality markers have consistently reported a significant association between sHSP expression and several meat quality attributes. Considering the abundance of these proteins in muscle tissue and their chaperoning and anti-apoptotic function, the role of sHSPs beyond being mere indicators of meat quality is proposed. In this paper, increasing evidence for the role of sHSPs in meat quality, particularly meat tenderness, has been reviewed. Briefly, they are proposed to attenuate myofibrillar degradation and to impede the process of apoptotic cell death.

At present the knowledge of sHSPs in meat science is restricted to the semi-quantitative analysis of the expression and presence of various isoforms of monomeric sHSPs. As the chaperoning and anti-apoptotic role of sHSPs are reliant upon the degree of phosphorylation, disassembly of large complexes to smaller sHSP oligomers and the

substructure of these oligomers, the elucidation of sHSP expression and oligomeric structure in *post mortem* muscle is required. Furthermore, characterisation of the biological role of sHSPs during the conversion of muscle to meat is necessary to verify their proposed role in meat quality.

This review progresses from the novel hypothesis of the apoptotic death of muscle cells *post mortem* by Ouali *et al.* (2006) and Herrera-Mendez *et al.* (2006) to the implications of apoptosis in meat quality. It is proposed that sHSPs in muscle mediate the progression of apoptotic cell death and are thus implicated in the development of meat quality. Furthermore, the expression of sHSP which are up-regulated during periods of stress as indicated by pH_u, may explain the high variability of quality attributes in meat. We hope that this hypothesis will lead new scientific perspectives and experimental approaches to examine meat quality in light of apoptotic cell death and the proteins involved in this cellular process.

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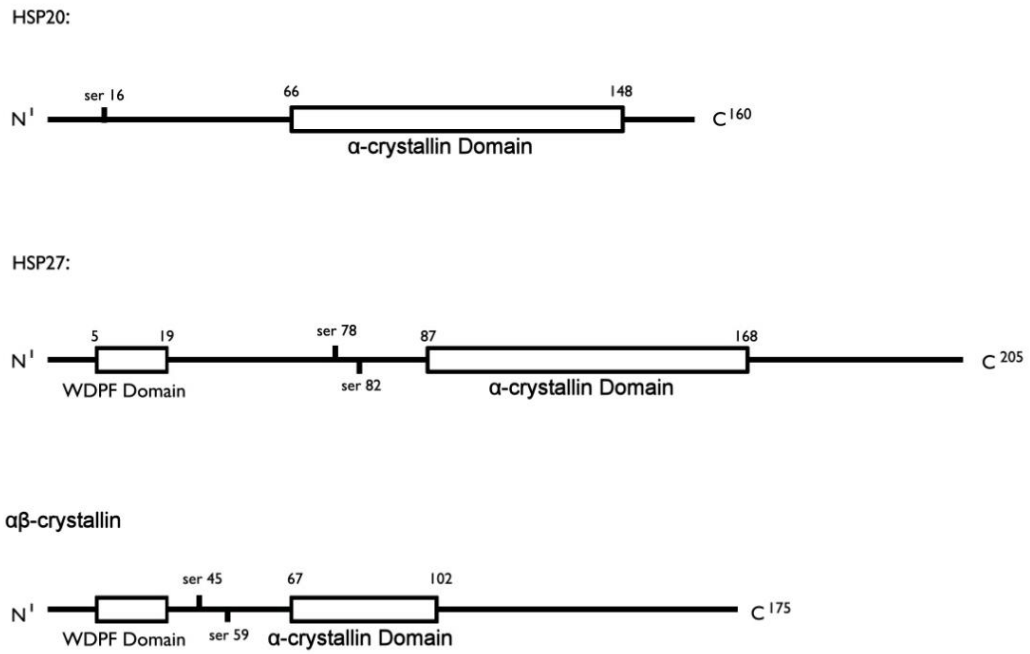

Figure 1: Comparison of the schematic structures of HSP20, HSP27 and αβ-crystallin monomers adapted from Gusev *et al.* (2002), Haslbeck (2002) and Mymrikov *et al*. (2011) with modifications. The α-crystallin and WDPF domains and known serine residues that are phosphorylated by protein kinases are depicted.

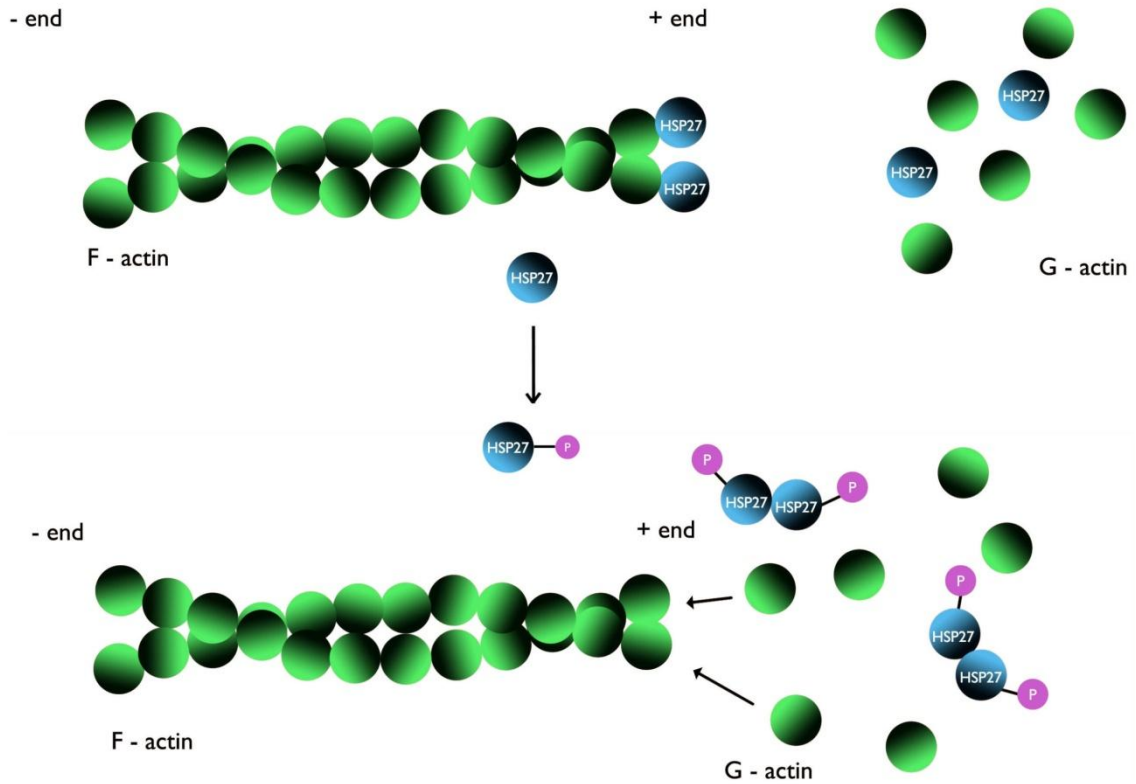

Figure 2: The regulation of actin polymerisation by sHSPs adapted. HSP27 inhibits the polymerisation of actin by mimicking monomeric G-actin and binding to the plus end of F-actin thereby inhibiting further polymerisation. Phosphorylation of HSP27 results in their detachment from F-actin, favouring the actin polymerisation.

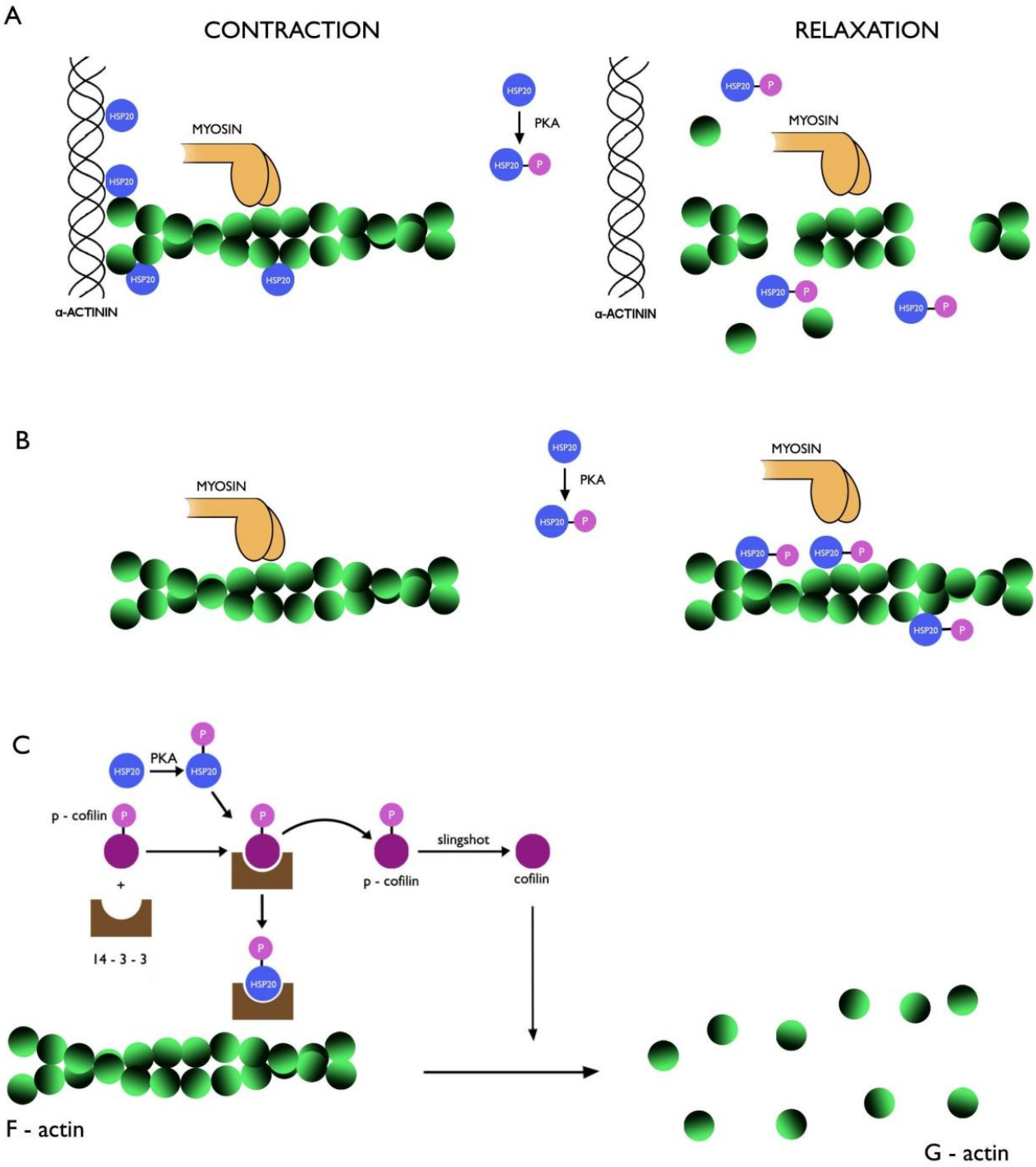

Figure 3: Schematic diagrams depicting the proposed function of sHSPs in regulating smooth muscle contraction adapted from Mymrikov *et al.* (2011) with modifications. Phosphorylation of HSP20 is proposed to favour the association of HS20 from F-actin to G-actin, disrupting the binding of actin with other contractile proteins and inducing muscle relaxation (A). Alternatively, phosphorylated HSP20 is proposed to bind to F-actin thereby inhibiting its association with myosin and inducing muscle relaxation (B). Finally, phosphorylated HSP20 is suggested to replace cofilin in the cofilin-14-3-3 protein complex. In turn, the depose cofilin catalyses the depolymerisation of F-actin to G-actin, inducing muscle relaxation (C).

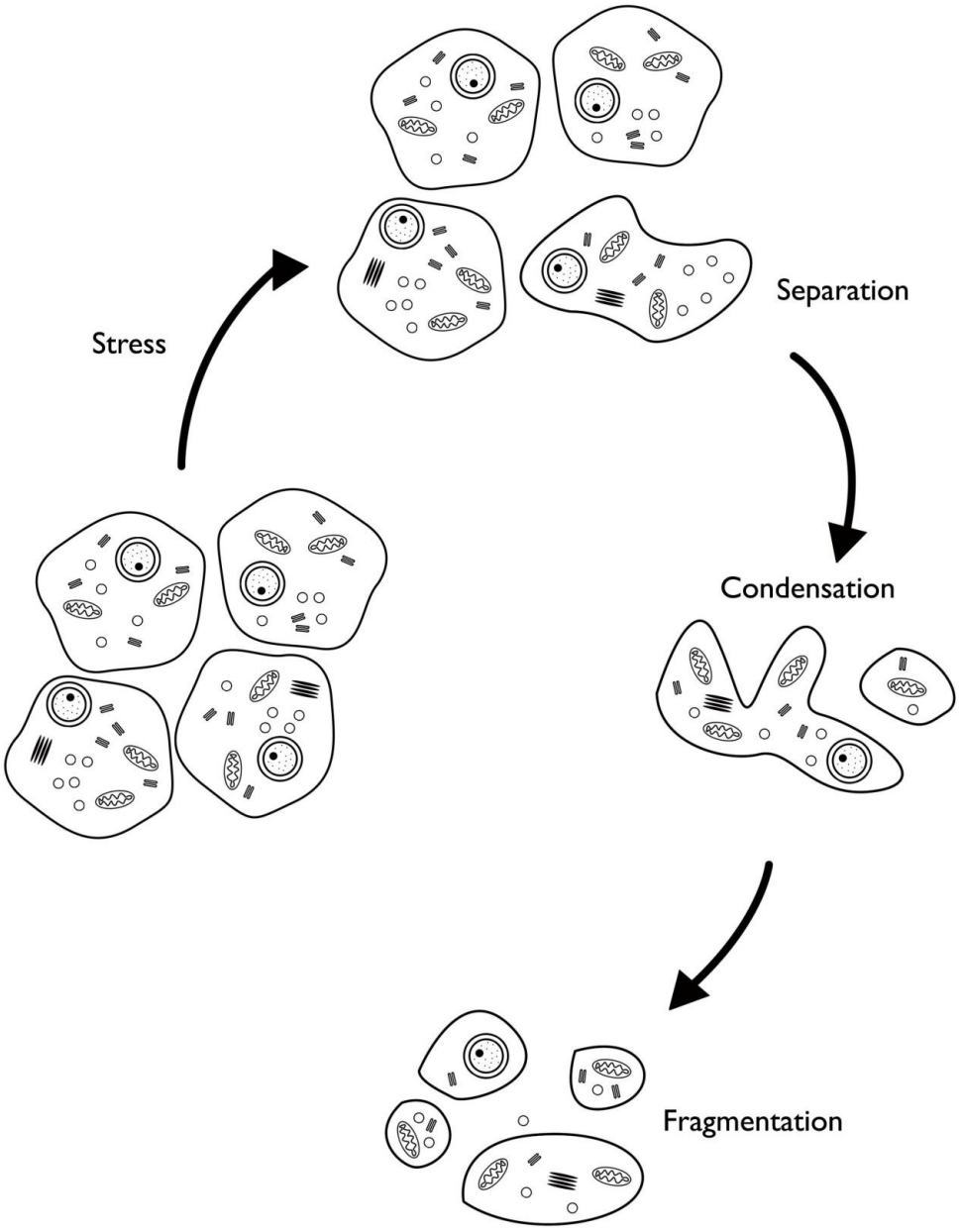

Figure 4: An illustration of depicting the process of apoptosis. Upon the onset of apoptosis, apoptotic cells are separated from surrounding healthy cells by the inversion of cellular membrane phospholipids. Proceeding cell separation, apoptotic cells undergo condensation, then fragmentation into smaller apoptotic bodies that are subsequently ingested by phagocytes or neighbouring cells.

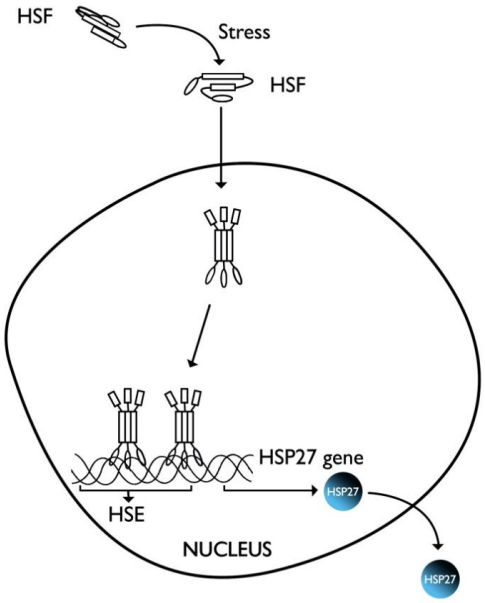

Figure 5: The transcriptional up-regulation of HSP27 in response to stress. Illustration adapted from Santoro (2000) with modifications. Stress signals activate heat shock factors (HSF) which are translocated to the cell nucleus where they form trimers and bind to heat shock elements of the HSP27 gene resulting in the synthesis of HSP27.

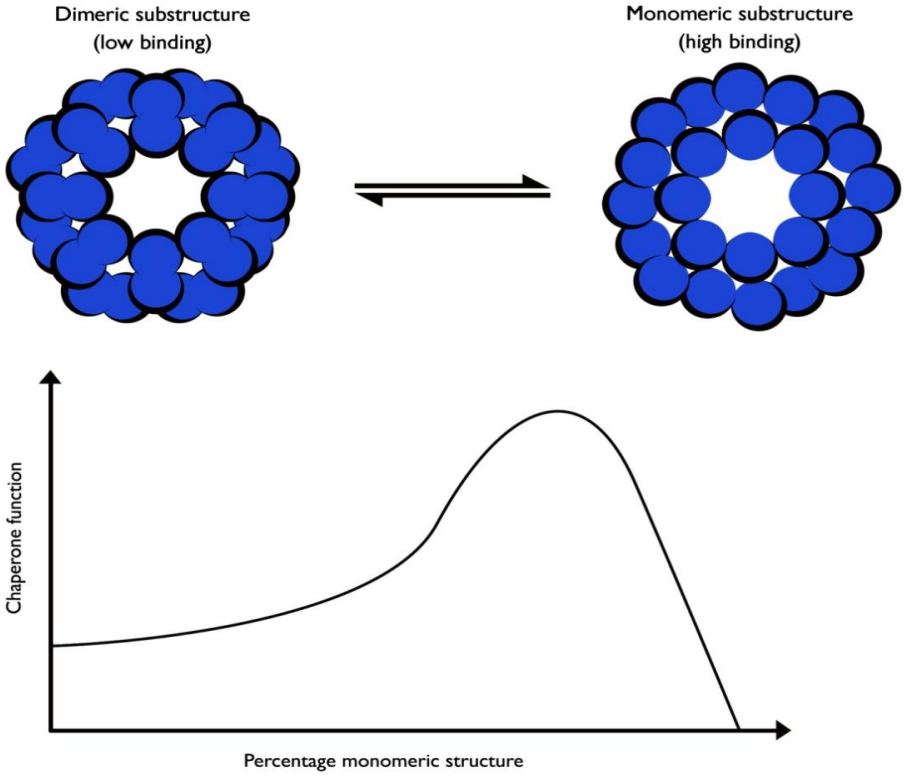

Figure 6: The relationship between small heat shock protein substructure and chaperone function adapted from Benesch *et al.* (2008) with modifications. Oligomers with a monomeric substructure have little chaperone ability due to their high affinity to bind and co-precipitate with substrate proteins. Thus, optimal chaperoning ability is defined by the correct balance of monomeric and dimeric substructure of sHSP oligomers.

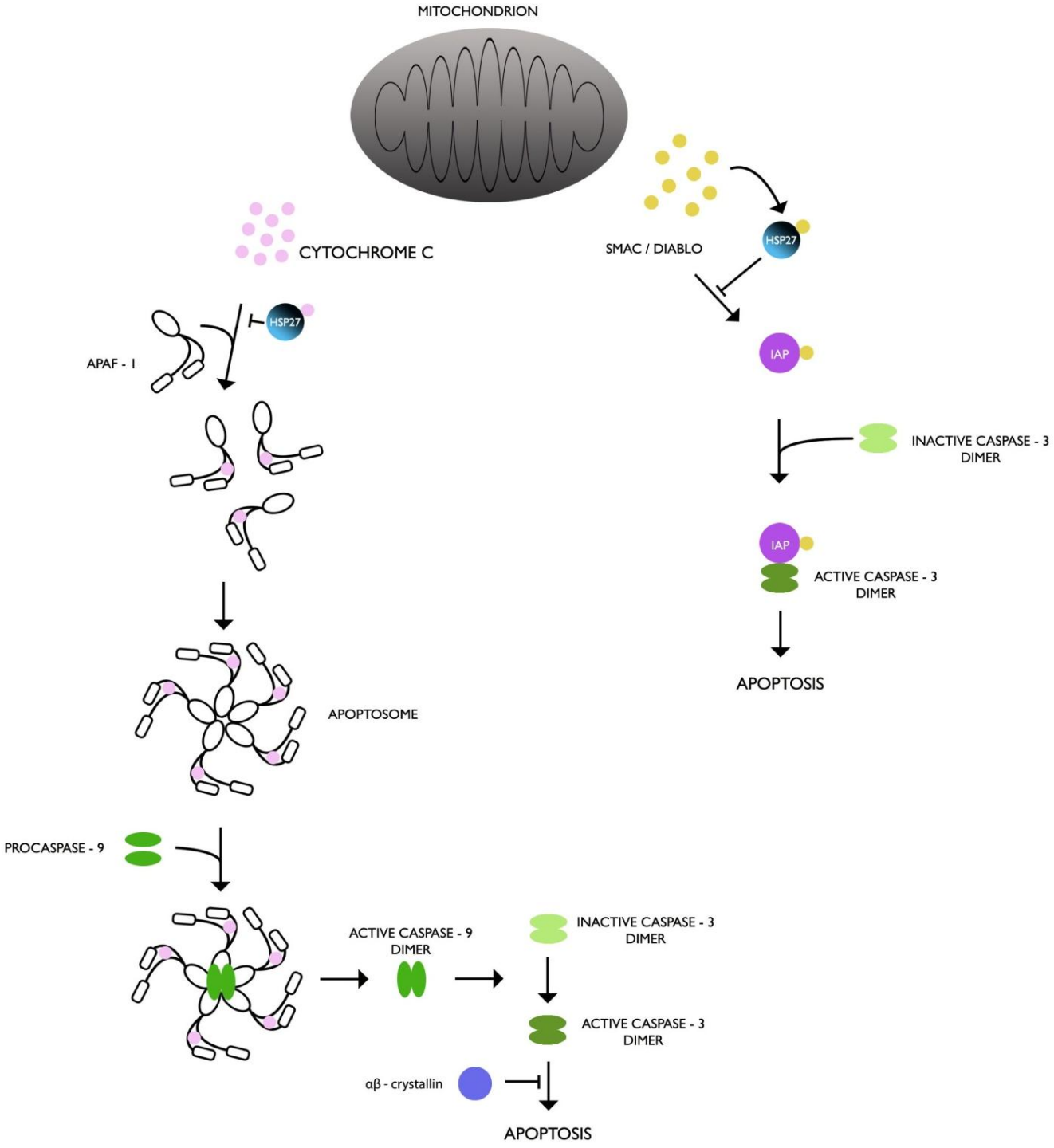

Figure 7: An illustration depicting cellular apoptosis adopted from Beere (2004; 2005) and Mymrikov *et al.* (2011) with modifications. Increasing mitochondrial permeability induced by cell stress results in the release of the apoptotic factors cytochrome *c*, Smac and DIABLO into the sarcoplasm. Cytochrome *c* triggers the formation of apoptosomes which in turn activate caspase-9 and caspase-3 dimers subsequently initiating apoptosis. In addition, Smac/DIABLO bind to apoptotic inhibiting proteins (IAP), allowing the activation of caspase-3 and the onset of apoptosis. Small heat shock proteins regulate apoptosis by binding to cytochrome *c* and inhibiting the formation thereby inhibiting the activation of downstream caspases. αβ-Crystallin was also demonstrated to inhibit the activation o caspase-3. Furthermore, HSP27 is able to bind to Smac/DIABLO, favouring the inhibition of caspase-3 activation by IAP.

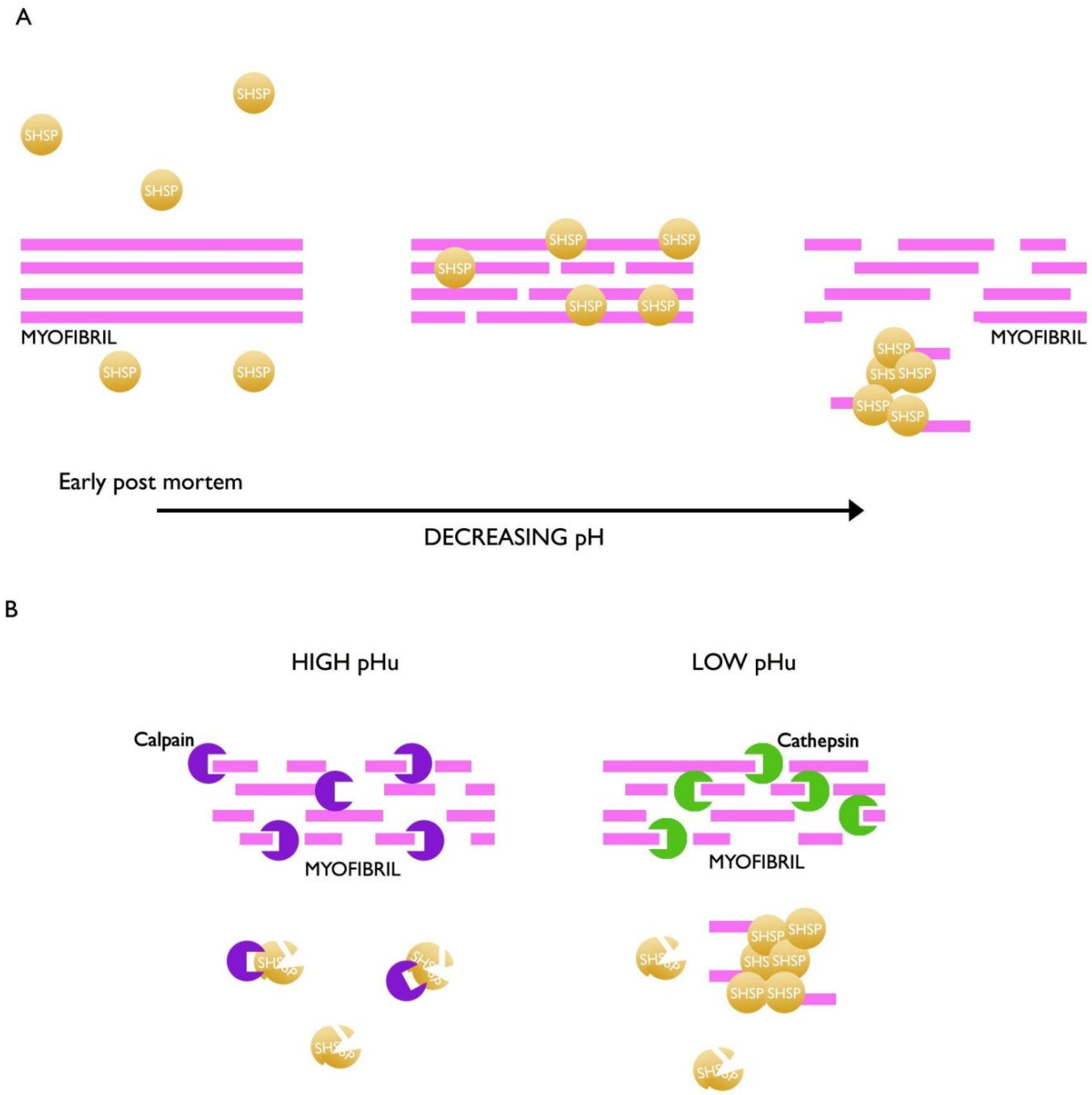

Figure 8: The association of sHSPs with myofibrils early in the intramuscular pH decline. sHSPs increasingly associate with myofibrils as pH declines from physiological levels. However, as the pH of muscle acidifies beyond the isoelectric points of sHSPs, they aggregate and precipitate into the muscle sarcoplasm (A). sHSPs chaperone activity is nullified in high and low pH_u meat in the proposed model (B). sHSPs do not associate with myofibrils in high pH_u meat, but act as an alternative substrate to the µ-calpain. In low pH_u meat, sHSP aggregate due to the acidic pH in this meat category. The high activity of µ-calpain in high pH_u meat and the combined activities of µ-calpain and cathepsin B in low pH_u meat result in tender meat in both these pH_u groups.

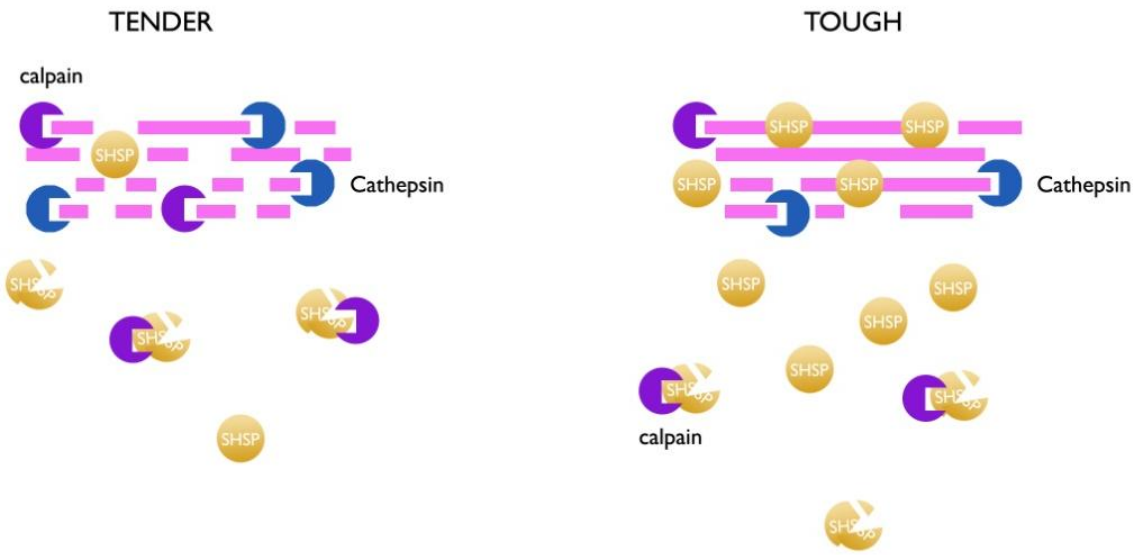

Figure 9: A proposed model of the chaperone function of sHSPs in intermediate pH_u meat resulting tenderness variability in this group. Although µ-calpain and cathepsin activity is least optimal in intermediate pH_u meat, there is still sufficient proteolytic activity to degrade myofibrillar proteins resulting in acceptable meat tenderness. However, high levels of sHSPs in intermediate pH_u meat result in meat toughness by further inhibiting the proteolytic action of µ-calpain by acting as an alternative substrate and the preventing extensive degradation of myofibrils.

IV

The protection of bovine skeletal myofibrils from proteolytic damage *post mortem* by small heat shock proteins[2]

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Abstract

This study aimed to determine how small heat shock proteins (sHSP) protect myofibrillar proteins from μ-calpain degradation during ageing. Immunoprecipitation experiments with *M. longissimus dorsi* (LD) from Angus heifers (n = 14) examined the interaction between αβ-crystallin, desmin, titin, HSP20, HSP27 and μ-calpain. Results showed that αβ-crystallin associated with desmin, titin, HSP20, HSP27 and μ-calpain. Exogenous αβ-crystallin reduced desmin and titin degradation in myofibrillar extracts and attenuated μ-calpain activity. In a second experiment, bull LD (n = 94) were aged at -1.5°C for up to 28 days *post mortem*. μ-Calpain autolysed faster in high ultimate pH (pH_u) meat ($pH_u \geq 6.2$) and this was concomitant with the more rapid degradation of titin and filamin in this pH_u group. Desmin stability in intermediate pH_u meat (pH_u 5.8 to 6.19) may be due to the protection of myofibril-bound sHSP combined with the competitive inhibition of μ-calpain by sHSP.

Keywords: Small heat shock proteins, μ-calpain, αβ-crystallin, desmin, beef

Introduction

The integrity of the myofibrillar framework within a sarcomere is essential to the function of skeletal muscle. The intracellular environment surrounding myofibrils within a working muscle is in a constant state of flux, which may expose myofibrillar structural proteins to possible damage. During muscle trauma, muscle homeostasis is disrupted which can lead to the fragmentation of myofibrillar structural proteins and loss of sarcomere integrity and muscle function (Gissel & Clausen, 2001; Zhang, Yeung, Allen, Qin & Yeung, 2008).

Under normal conditions, intracellular calcium concentrations are maintained at low levels in the sarcoplasmic reticulum by calcium ATPase pumps. However, muscle stress, such as strenuous exercise may lead to the accumulation of intracellular calcium and result in irreversible damage (Gissel *et al.*, 2001). A consequence of elevated calcium concentration is the potential activation of the endogenous calcium-dependent protease μ-calpain (Belcastro, Shewchuk & Raj, 1998). Because the myofibrillar structural proteins desmin and titin are known μ-calpain substrates and are integral to Z-disk myofibrillar structure (Baron, Jacobsen & Purslow, 2004; Raynaud *et al.*, 2005), the degradation of these proteins may result in the destabilisation of the sarcomere.

In response to the detrimental changes taking place within a muscle cell during stress, a series of compensatory cellular mechanisms are activated to limit damage. These include inflammatory/repair responses (Tidball, 2005) and an increased expression of selective stress proteins (McArdle, Vasilaki & Jackson, 2002). Small heat shock proteins (sHSP), also known as chaperone proteins, have been shown to contribute to the maintenance and/or remodelling of structural as well as functional proteins during stress (Sun & MacRae, 2005; Tytell & Hooper, 2001). The role of sHSP at maintaining muscle structural integrity due to intracellular stresses such as changing pH has significant implications for meat quality. For example, studies have shown that variation in *post mortem* muscle ultimate pH affects the redistribution of sHSP in the muscle and the nature of this intracellular distribution together with the intracellular pH is correlated with meat tenderness (Pulford, Fraga Vazquez, Frost, Fraser-Smith, Dobbie & Rosenvold, 2008).

The sHSP αβ-crystallin is an important chaperone in preserving the infrastructure of various cells. In skeletal and cardiac muscle, αβ-crystallin associates with myofibrillar proteins in both the Z-disc and I band by interacting with exposed hydrophobic residues of myofibrillar structural proteins like desmin and titin (Bennardini, Wrzosek & Chiesi, 1992a; Raman & Rao, 1994).

In *post mortem* muscle, calcium accumulates in the sarcoplasm, activating μ-calpain and initiating the proteolytic degradation of myofibrillar proteins leading to meat tenderisation (Koohmaraie, 1992b). To prevent the inevitable apoptotic death of muscle cells *post mortem*, it has been proposed that sHSP proteins are induced and recruited to maintain cell homeostasis (Ouali *et al.*, 2006). The contribution of sHSP to meat quality is not yet understood. However, studies have demonstrated the expression of αβ-crystallin and HSP27 in muscle are correlated with shear force and colour (Bernard, Cassar-Malek, Le Cunff, Dubroeucq, Renand & Hocquette, 2007; Kim *et al.*, 2008).

This study first explores the ability of the sHSP αβ-crystallin to maintain myofibrillar protein structure following the activation of exogenous μ-calpain. Second, the relationship between sHSP expression, ultimate pH (pH_u) and myofibrillar protein degradation was investigated to determine whether the interactions of sHSP with myofibrillar proteins *in vitro* is mimicked in muscle during *post mortem* ageing.

Materials and Methods

Experiment 1

Animals and sample collection

M. longissimus dorsi (LD) from Angus heifers (n = 14) all slaughtered stunned by captive bolt then slaughtered on the same day. Carcasses were not electrically stimulated. After the LD muscles were hot-boned from the carcass, they were packed in vacuum bags and held in a 37°C water bath. Muscle pH was monitored using a combination puncture electrode pH meter (Mettler-Toledo GmbH, Switzerland). Approximately 10 g of muscle samples were collected from each LD at pH 7.4, 7.2, 7.0, 6.8 and 6.5 and used for subsequent biochemical analyses.

Extraction of muscle fractions and protein measurement

For the extraction of the soluble muscle fraction, 2 g of muscle was homogenised in soluble fraction extraction buffer (50 mM Tris-HCl, 10 mM EDTA, 1 mM DTT, 0.1 mM phenylmethanesulfonyl fluoride (PMSF) with COMPLETE™ protease inhibitors (Roche, 11 836 170 001); pH 7.4). The homogenate was then

centrifuged at 3000 g for 10 minutes at 4°C and the soluble muscle fraction stored at -80°C until analysed.

Bovine myofibrillar fractions with associated proteins were prepared as described by Wang *et al.*(1988). Muscle fibres were teased apart in myofibrillar extraction buffer (50 mM Tris-HCl, 10 mM EGTA, 2mM $MgCl_2$, 0.1 mM PMSF, 0.1% Triton X-100; pH 7.0) and stored in the buffer at 4°C overnight. The muscle fibres were then gently homogenised in the same buffer and centrifuged at 3000 g for 5 minutes. The supernatant was discarded and the myofibrillar pellet was washed three times in myofibril extraction buffer without Triton X-100. The myofibrillar fraction was stored in 50 percent glycerol in a standard salt solution (20 mM NaH_2PO_4:Na_2HPO_4, 100 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM NaN_3; pH 6.8) at -20°C until analysed.

For the preparation of stripped myofibrillar fractions, 0.5 g of muscle sample was teased apart in stripped myofibrillar extraction buffer (20 mM NaH_2PO_4:Na_2HPO_4, 100 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 0.1 PMSF. 0.1 mg mL^{-1} soybean trypsin inhibitor, 0.5% Triton X-100; pH 7.0), finely chopped with scissors then agitated at 200 rpm in an oscillating shaker in extraction at room temperature for 1 hour. The resulting suspension was centrifuged at 2000 g at 4°C for 5 minutes. The supernatant was discarded and the pellet was washed four times with BASS buffer (0.1 M NaH_2PO_4:$NaHPO_4$, 25 mM KCl, 39 mM boric acid, 0.1 mM PMSF, 0.1 mg mL^{-1} soybean trypsin inhibitor; pH 7.0). The pellet was then resuspended in BASS buffer containing 0.5 mg mL^{-1} collagenase type 1 (Roche, 04 834 606) and 0.1 mM $CaCl_2$ and digested at 25°C for 30 minutes before redispersing the digested pellet in the buffer followed centrifugation at 2000 g for 5 minutes. The resulting pellet was washed four times with BASS buffer and resuspended in ice cold phosphate buffer (0.1 M NaH_2PO_4:Na_2HPO_4; pH 7.0) followed by centrifugation at 300 g for 5 minutes. Myofibrils remaining in the suspension were pelleted from the supernatant by centrifugation at 3000 g for 5 minutes. Some myofibrils were then 1: stripped further using phosphate buffer containing 0.5 M KCl for 10 minutes on ice, washed three times phosphate buffer, suspended in phosphate buffer containing 0.1 mM PMSF and 50% glycerol then stored at -20°C (this treatment removed all myofibril associated proteins, but maintained the myofibrillar structural protein integrity) or 2: resuspended in phosphate buffer (these myofibrils had most associated proteins removed, including μ-calpain, but still had αβ-crystallin attached).

Sample protein concentrations for the soluble and myofibrillar fractions from the first trial were determined using a Bradford Protein Assay kit (BioRad, 500-0202).

Purification of bovine αβ-crystallin

Bovine LD muscle was homogenised in extraction buffer (10 mM Tris-HCl, 10 mM EDTA, 2 mM DDT; pH 7.5) then filtered through a fine wire mesh to remove large particles. The suspension was centrifuged at 15,000 g and the supernatant was stored at -80°C until purified.

Protein purification was conducted at 4°C using GE Pharmacia FPLC system, as described by Atomi *et al.*(1991) and Schoenmakers *et al.*(1969). Briefly, the soluble muscle suspension was initially passed through a Sephacryl S-200 size exclusion column (Amersham Biosciences, 17-0584-10), and the fractions containing αβ-crystallin, as determined by immunoblot were passed through a DEAE Sepharose fast flow FPLC column (Amersham Biosciences, 17-0907-01). Finally, αβ-crystallin were passed through a SP Sepharose fast flow column (Amersham Biosciences, 17-0729-01) and αβ-crystallin was eluted using a linear salt gradient (0 to 0.5 M KCl), with αβ-crystallin eluting at approximately 0.3 M KCl. αβ-Crystallin fractions were pooled and dialysed against a buffer (10 mM NaH_2PO_4:$NaHPO_4$; pH 7.0), then concentrated to approximately 10 mg mL^{-1} using Centricon-10 filters (Millipore, 4206) and stored at -80°C. Purity and renaturation of the bovine αβ-crystallin was verified by sodium dodecyl gel electrophoresis (SDS-PAGE) (data not shown).

Gel electrophoresis and Western Blotting

Protein samples (10 µg) were mixed with Laemmli sample loading buffer with reductant (mercaptoethanol) ((Laemmli, 1970), heated in a boiling water bath for 5 minutes before resolution on a 10% SDS-PAGE gel. For the separation of titin fragments, the myofibril fractions were suspended in sample loading buffer devoid of reductant (50 mM Tris-HCl, 0.2% SDS, 0.2 % bromophenol blue), heated at 50°C for 15 minutes then resolved on 5% SDS-PAGE gels.

Proteins were subsequently blotted onto Immobilon-P PVDF membranes (Millipore, IPVH00010) and then blocked overnight at 4°C in 5% non-fat dry milk powder in PBS-Tween (0.08 M Na_2HPO_4, 0.02 M NaH_2PO_4, 0.1 M NaCl, 0.1% Tween). The blots were probed with anti-αβ-crystallin (Abcam, Ab13496), anti-HSP20 (Hytest, HSP20-11), anti-HSP27 (Hytest, #4HSP27), anti-µ-calpain (Thermo Scientific, MA3-940), anti-desmin (Sigma, D1033) or anti-titin (Sigma, 9D10) monoclonal antibodies for 1 hour at ambient temperature. All monoclonal antibodies were derived from mouse cells. αβ-Crystallin, HSP20 and HSP27 primary antibodies were diluted

1:10000 in PBS-Tween. Desmin, titin and μ-calpain antibodies were diluted 1:5000, 1:200 and 1:5000, respectively, in PBS-Tween. Membranes were washed four times for 10 minutes with PBS-Tween, then incubated with goat anti-mouse IgG (H+L) horseradish peroxidase (BioRad, #172-1011) diluted to 1:5000 in PBS-Tween. Following four washes with PBS-Tween, as previously described, membrane bound antibody was detected with ECL Western Blot substrate kit (Pierce, 32106). Western Blot images were captured with a G:Box Chemi HR16 image capture instrument (Syngene, Cambridge, UK).

Immunoprecipitation of myofibrillar and soluble proteins

αβ-Crystallin antibody (10 μg mL^{-1}) was covalently bonded to 100 μL Dynabeads® Protein G magnetic beads (Invitrogen, 100-03) according to the manufacturer's protocol. Immunoprecipitation of soluble and myofibrillar proteins were performed in 1 mL immunoprecipitation buffer (50 mM Tris-HCl, Complete™ protease inhibitor (Roche, 11 836 170 001), 10 mM EDTA, 10 mM EGTA; pH 7.4) containing either 1: partially solubilised (0.5% Triton X-100) isolated bovine LD myofibrils or 2: soluble muscle proteins. Lysates were cleared by incubation with 100 μL magnetic beads for 1 hour at 4°C in an oscillating shaker. Unbound proteins were incubated with αβ-crystallin/magnetic bead complex overnight at 4°C. The protein suspension was then placed in a magnet, the supernatant discarded, and the proteins bound to the magnetic bead complex were washed four times for 5 minutes with 1 mL PBS containing 0.1% Triton X-100. Proteins were eluted from the complex with 0.1 M citric acid, pH 5.2, and then immediately neutralised by addition of 1 M Tris-HCl to pH 7.4. Eluted proteins were diluted in Laemmli sample loading buffer then analysed by Western Blotting.

A mixture of 2.5 μg mL^{-1} of purified μ-calpain (Calbiochem, #208712) and 10 μg of purified αβ-crystallin (Abcam, ab74441) were incubated for 5 minutes at 37°C in 50 mM Tris-HCl (pH 7.4) containing either 10 mM EGTA or 10 μM $CaCl_2$. αβ-Crystallin was immunoprecipitated overnight 4°C as previously described. Eluted proteins were diluted in Laemmli sample loading buffer then analysed by Western Blotting.

Measurement of μ-calpain activity

μ-Calpain activity was determined according to Mittoo *et al.*(2003). Briefly, μ-calpain (Calbiochem, #208712) was diluted in 100 μL Calpain Assay Buffer (50 mM Tris-HCl; pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.05% ($^{v}/_{v}$) 2-

mercaptoethanol) and dispensed into a black-walled 96 well plate (Nunc, #265301) together with 1 µM fluorogenic calpain-1 substrate (Calbiochem, #208748). The reaction was started with the addition of 5 mM $CaCl_2$. A change in fluorescence was measured over 60 minutes with a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany) using Ex 490 nm and Em 520 nm filters. When appropriate, varying amounts of αβ-crystallin (0.5 – 10 µg), 1 µg calpastatin (Calbiochem, #208900), 1 g casein or 1 g bovine serum albumin were added to the reaction mixture 5 minutes prior to the addition of µ-calpain.

µ-Calpain induced proteolysis of stripped myofibrils

Stripped myofibrils (10 µg) prepared as described earlier were resuspended in binding buffer (pH 7.4) excluding Complete™ protease inhibitor and incubated at 37°C in the presence or absence of µ-calpain at 3 hours. The myofibril preparation was centrifuged at 3000 g at 4°C for 5 minutes then the pellet was washed three times with the same buffer. The resulting pellet was denatured in Laemmli sample loading buffer and αβ-crystallin, desmin, and titin were detected by electrophoresis followed by Western Blotting.

Experiment 2

Animals and sample collection

Bull LD (n = 94) from animals that were slaughtered over 2 consecutive days were used in this study. All animals were head-only electrically stunned before slaughter and low voltage electrical stimulation was applied to all carcasses preceding standard dressing processes. The left side LD was hot-boned and about 10 g of the muscle was sub-sampled and frozen in liquid nitrogen (Day 0). The frozen muscle fragments were then stored at -80°C until analysed. The rest of the muscle was vacuum packed and stored at -1.5°C for 24 hours. At 24 hours *post mortem*, each LD was equally divided into 5 sub-samples, then randomly allocated an ageing timepoint of 1, 2, 7, 14 and 28 days *post mortem*, vacuum packed then stored at -1.5°C. Ten grams were excised from all sub-samples at each timepoint, frozen in liquid nitrogen and all frozen muscle fragments were stored at -80°C until required for analysis.

pH measurements

The pH of all sub-samples from the second trial was measured with Testo® 230 meter (Lenzkirch, Germany).

Extraction of muscle fractions and protein measurement

Sarcoplasmic fractions were prepared from frozen muscle fragments of all samples at all timepoints as described by Pulford *et al.*(2008). Total muscle extracts were also prepared by homogenising 0.5 g of frozen muscle fragments in total muscle extraction buffer (50 mM Tris-HCl; pH 5.8, 10% glycerol, 2% SDS and 2% 2-mercaptoethanol), centrifuged at 10000 g for 5 minutes at 4°C and the supernatant collected.

The protein content of sarcoplasmic fractions and total muscle extracts were determined using a DC Protein Assay (BioRad Laboratories, Hercules, CA) and RC-DC Protein Assay kits, respectively. Sample protein concentrations were expressed as bovine serum albumin equivalents from standard curves.

Gel electrophoresis and Western Blotting

Total muscle extracts were diluted with an equivalent volume of Laemmli sample loading buffer with reductant (2% mercaptoethanol). After mixing, the samples were heated at 50°C for 20 minutes then centrifuged at 10000 g for 10 minutes. Protein (40 µg) from total muscle extracts were loaded onto 7.5% Tris-HCl (BioRad Laboratories, 345-0006) gels for desmin and µ-calpain, and then resolved on a BioRad Criterion Cell system at 120 V at room temperature. Desmin and µ-calpain determination by Western Blots were conducted as previously described.

For the analysis of larger molecular weight proteins (>100 kDa) during ageing, 100 µg of protein was loaded onto pre-cast 5% Tris-HCl gels (BioRad) and separated at 10 mA for 17 hours in a BioRad Criterion cell system. The gels were stained with colloidal Coomassie blue (17% ammonium sulphate, 2% phosphoric acid, 30% methanol, 0.04% Coomassie blue G-250). The gels were then washed with distilled water and scanned with a GS700 densitometer (BioRad).

Quantitative measurement of small heat shock proteins

Individual sHSP was measured by indirect enzyme-linked immunosorbent assay (ELISA) as described by Pulford *et al.* (2008), with some modifications. Sarcoplasmic fraction was adjusted to concentrations of 4 µg mL^{-1} protein with coating buffer (10

mM Na_2HPO_4, 15 mM NaCl; pH 7.4). Protein standards were prepared from purified (Hytest, 8HSP20), HSP27 (Hytest, 8HSP25) and αβ-crystallin (Sigma, C7858) diluted in coating buffer. An aliquot (100 µL) of samples and standards were dispensed into 96 well Costar® high binding polystyrene plates (Corning Inc., 3590) in duplicate. Plates were placed on an oscillating shaker overnight at 4°C. Following coating, the contents of all wells were discarded then all wells were blocked with 1% BSA in wash buffer (10 mM Na_2HPO_4, 15 mM NaCl, 0.1% Tween 20; pH 7.4) then washed four times with wash buffer. Following washing, 100 µL of mouse monoclonal antibody (HSP20, Hytest 4HSP20; HSP27, Hytest 4HSP27 and αβ-crystallin, Abcam ab74441) each diluted to 1:10,000 with 1% BSA in assay buffer then washed as previously described. Goat anti-mouse IgG (H+L) HRP conjugate (Bio-Rad Laboratories, #172-1011) was diluted to 1:5,000 in assay buffer (1% BSA) and 100 µL aliquots of this solution was dispensed into all wells and left to incubate for 1 h at room temperature. Following washing, 100 µL 3,3',5,5'-tetramethybenzidine substrate (Sigma, T0440) was dispensed into each well and left to incubate for 30 minutes. The reaction was stopped by dispensing 100 µL of 2 N H_2SO_4 into all wells. The absorbance of each well was measured at 450 nm with a Fluostar Optima plate reader (BMG Labtech, Ortenberg, Germany).

Measurement of µ-calpain activity

Measurement and extraction of µ-calpain activities in bull muscle samples was determined according to procedures previously described by Wiklund *et al.*(2010) using an assay adopted to muscle tissue.

Data analysis

Statistical analysis of the data was conducted using a mixed model smoother (Upsdell, 1994). The model used was time *post mortem* × pH_u and time effect (a random term describing the variability between ageing timepoints). The curves in the plots are estimated means of the measured parameters plotted against pH_u and at increasing times *post mortem*. The bands are 95% least significant intervals for testing differences between and within groups. Points between and within groups that do not overlap are significantly different from each other.

Results

Association of αβ-crystallin with soluble and myofibrillar proteins in bovine LD muscle

In order to identify other components that may support the role of αβ-crystallin as a skeletal muscle chaperone, αβ-crystallin was immunoprecipitated with associated proteins from either soluble muscle fractions or partially solubilised myofibrils using αβ-crystallin antibody covalently attached to magnetic beads (Figure 1). αβ-Crystallin not only associated with desmin, but was also found to associate with HSP20, HSP27 and µ-calpain. However, HSP27 only associated with αβ-crystallin from the soluble muscle fraction.

Interaction of αβ-crystallin with µ-calpain

To confirm any interaction between αβ-crystallin and µ-calpain, purified αβ-crystallin and µ-calpain were combined *in vitro*. Immunoprecipitation of αβ-crystallin with either 0.5 µg or 1 µg of µ-calpain showed direct interaction between purified αβ-crystallin and intactµ-calpain in the presence of EGTA that was used to bind intact Ca^{2+}. A weak association between αβ-crystallin and autolysed µ-calpain was also observed following the activation of the enzyme with 10 µM calcium chloride ($CaCl_2$) (Figure 2). These results indicated that αβ-crystallin has the ability to associate with both intact and autolysed µ-calpain.

The effectiveness of αβ-crystallin as an alternative substrate of µ-calpain was investigated by adding exogenous µ-calpain to αβ-crystallin. This was conducted with bovine myofibrillar preparations that retained attached αβ-crystallin but were stripped of µ-calpain. Rapid proteolysis of αβ-crystallin occurred by 15 minutes at 37°C after the addition of calpain in the presence of calcium (Figure 3A).

To further investigate µ-calpain induced degradation of αβ-crystallin, µ-calpain catalysis of a fluorogenic calpain-specific substrate in a competition assay was monitored. Fluorescence was highest when only µ-calpain was incubated with the fluorogenic substrate. However, fluorescence emission from the assay was attenuated to varying degrees when µ-calpain was incubated with αβ-crystallin, casein and calpastatin. Both casein and calpastatin demonstrated competitive inhibition of µ-calpain when using a calpain-specific fluorescent reporter, which were significantly greater than for αβ-crystallin at the same concentration ($p < 0.05$). Although αβ-

crystallin consistently reduced the μ-calpain catalysis of the fluorogenic substrate, this was not significantly different from the μ-calpain control (Figure 3B).

Protection of the myofibrillar proteins desmin and titin by αβ-crystallin from μ-calpain induced degradation

To determine if αβ-crystallin protects myofibrillar proteins against μ-calpain-induced degradation, the temporal effect of added μ-calpain on desmin and titin stability in the presence and absence of αβ-crystallin was monitored. Stripped bovine myofibrils were incubated with μ-calpain at 37°C to 180 minutes in the absence and presence of excess αβ-crystallin. Myofibrillar proteins were immunoblotted for desmin, titin and αβ-crystallin with their corresponding monoclonal antibodies. Simultaneous incubation of myofibrils with αβ-crystallin and μ-calpain showed a rapid relocalisation of αβ-crystallin to the myofibrils at 15 minutes, which once attached, became rapidly degraded (Figure 4A). μ-Calpain-induced desmin and titin in myofibrils in the absence of αβ-crystallin was detected within 15 and 5 minutes, respectively. However, in experiments using myofibrils with attached αβ-crystallin, μ-calpain proteolysis of both desmin (Figure 4B) and titin (Figure 4C) was markedly reduced. These results support the notion that the relocalisation of αβ-crystallin to myofibrils during muscle damage or *post mortem* changes serve to protect structural myofibrillar proteins such as desmin and titin (Figure 4C).

The effect of pH on αβ-crystallin binding to stripped bovine LD myofibrils

At pH 7.4, low concentrations of αβ-crystallin were associated with stripped myofibrils *in vitro*. However, as the pH decreased to pH 7.2, an increase in myofibril associated αβ-crystallin (Figure 5) was observed. This trend continued as the pH progressively decreased to pH 6.5 and suggests that the association of αβ-crystallin with myofibrils increased as the *in situ* muscle pH declined *post mortem*.

Small heat shock protein kinetics in bovine LD sarcoplasm early post mortem

To investigate whether the association of αβ-crystallin with myofibrillar proteins *in vitro* is also observed in muscle during ageing, concentrations of αβ-crystallin and HSP20 from the sarcoplasm of bull LD aged at -1.5°C was determined. sHSP concentrations at Day 0 were relatively similar for both sHSPs along the pH_u continuum. At 1 day *post mortem*, a strong positive relationship between sHSP levels and pH_u was observed such that elevated levels of sHSP were maintained at higher pH_u

samples (Figure 6). At 7 days *post mortem*, sHSP levels slightly decreased throughout the pH_u continuum. However, high pH_u samples maintained higher levels of sHSP.

μ-Calpain kinetics in the early post mortem period in bull LD

To determine the autolysis of μ-calpain in bull beef along the pH_u continuum, Western blots of total muscle extracts from 16 bull LD were conducted. The majority of high pH_u muscles had already undergone μ-calpain autolysis at Day 0 *post mortem* (Figure 7). In contrast, no autolysis was detected in any samples with a pH_u below 6.3 at that timepoint. Early autolysis of μ-calpain in high pH_u meat indicates the activation of μ-calpain in this pH_u group, and that μ-calpain is still inactive at more acidic pH_u levels early *post mortem.* At 1 day *post mortem*, autolysis was detected in most samples, but to a lesser degree in low pH_u samples. μ-Calpain had completely autolysed to 76 kDa in the majority of the samples at 7 days *post mortem*.

μ-Calpain activity in total muscle extracts eluted using a salt gradient was determined using a μ-calpain specific fluorogenic substrate. μ-Calpain activities generally decreased with increasing pH_u at 0 and 1 day *post mortem* (Figure 8). Relative μ-calpain activity levels decreased during ageing such that at 7 days *post mortem*, enzyme activities were minimal across the pH_u spectrum.

Degradation of myofibrillar proteins in bovine LD during ageing

The degradation of myofibrillar proteins was determined in total muscle extracts to investigate if variability in the degradation of key myofibrillar proteins is responsible for the variable tenderisation rates attained in low, intermediate and high pH_u meat. Western blots probed with desmin antibody revealed that desmin degradation was initially detected at 2 and 7 days *post mortem* in the representative high and low pH_u samples, respectively (Figure 9). Desmin degradation became increasingly extensive in these samples at subsequent ageing timepoints. In contrast, desmin degradation in the representative intermediate pH_u sample was minimal throughout the ageing period.

To show the degradation of higher molecular weight proteins, proteins from total muscle extracts were resolved in 5% polyacrylamide gels then stained with colloidal Coomassie blue solution. It was clear that the rate of titin degradation in muscle early *post mortem* varied across the pH_u spectrum. The detection of the titin degradation product T2 was observed in high pH_u muscles at Day 0 *post mortem* (Figure 10). In contrast, T2 was initially detected in low pH_u at Day 1 *post mortem* with intact titin still

detectable in low pH_u muscle. Intact titin was no longer detectable in any muscles at Day 2 *post mortem*.

Discussion

sHPS are intracellular protein stabilisers that respond to changes in the cell's microenvironment, with increased expression particularly associated with cellular restructuring events such as differentiation and apoptosis (Arrigo, 2005; Benjamin, Shelton, Garry & Richardson, 1997). Repetitive cell stress can result in more sustained expression of sHSP. For example, αβ-crystallin and HSP27 have been observed to accumulate in muscle after repeated eccentric exercises (Feasson *et al.*, 2002; Neufer & Benjamin, 1996). In addition, the expression of αβ-crystallin is higher in slow twitch muscle fibres compared to fast twitch muscle fibres (Atomi *et al.*, 1991). Since slow twitch muscle are exposed to a high degree of cellular activity and/or restructuring, the increase in αβ-crystallin indicates the importance of the chaperone role of these proteins in preserving overall muscle integrity.

Due to the protective function of sHSP in muscle cells, it has been proposed that these proteins may protect myofibrillar proteins from degradation in *post mortem* muscle, leading to variable meat quality (Bernard *et al.*, 2007; Pulford, Dobbie, Fraga Vazquez, Fraser-Smith, Frost & Morris, 2009). Under the ischemic conditions of *post mortem* muscle, muscle cells inevitably undergo apoptosis, a type of programmed cell death (Herrera-Mendez, Becila, Boudjellal & Ouali, 2006). In response to impending cell death, sHSP are speculated to be induced and recruited to critical regions within the cell to prevent the irreversible denaturation of key muscle proteins and thus maintain cellular homeostasis (Ouali *et al.*, 2006).

This study first sought to investigate whether sHSP offered any protection to the proteolytic degradation of myofibrillar proteins and aimed to characterise the mechanisms driving this. Immunoprecipitation of soluble and myofibrillar protein fractions in muscle with αβ-crystallin antibody found that αβ-crystallin associated with other sHSP. However, αβ-crystallin only associated with HSP27 from the soluble muscle fraction. Additionally, αβ-crystallin also associated with desmin and μ-calpain. Although the direct interaction between αβ-crystallin with titin is not shown in this study, αβ-crystallin has been found to associate with titin in the Z-disc and I-band regions of the myofibril (Golenhofen, Perng, Quinlan & Drenckhahn, 2004) and our results demonstrated that αβ-crystallin attenuated titin degradation upon exposure to μ-

calpain. Furthermore, since the Z-disc region is susceptible to mechanical and enzymatic damage the presence of sHSP, such as αβ-crystallin, in these regions may serve to stabilise myofibrillar proteins undergoing denaturation. Thus, the localisation of other sHSP, especially HSP20 which we show to relocalise (like αβ-crystallin) to myofibrils *post mortem*, to specific structural regions of the myofibrils should also be considered.

The rapid association of αβ-crystallin with structural proteins susceptible to damage is an essential function of a chaperone protein. Bennardini *et al.*(1992b) found that increased αβ-crystallin binding affinity to cardiac myofibrils in acidic buffers prevented the aggregation of both actin and desmin. Furthermore, αβ-crystallin binding to a specific region within the myofibril may prevent extensive damage during cell stress by stabilising its structural integrity until other mechanisms can be activated to regain homeostasis. Our results demonstrated that a slight decrease in muscle pH induced rapid binding of αβ-crystallin to the myofibril. However, the binding of sHSP to the myofibrillar fraction of muscle was only characterised from pH 6.5, and the nature of this association at more acidic values such as in the low pH_u range requires further investigation especially in its relationship to meat tenderness.

The accumulation of intracellular calcium in muscle cells leads to the activation of μ-calpain. μ-Calpain has been located within the Z-disk region of the myofibrils, making this region susceptible to proteolytic degradation (Raynaud *et al.*, 2005). Immunoprecipitation results showed the interaction between αβ-crystallin and μ-calpain in both soluble and myofibrillar proteins. This observation suggests that αβ-crystallin may act as an alternative substrate and may therefore be a competitive inhibitor of μ-calpain induced proteolysis of myofibrils.

The hypothesis that αβ-crystallin is a substrate for μ-calpain in skeletal muscle is supported by the results of this study in that the presence of αβ-crystallin in the μ-calpain induced catalysis of the fluorogenic calpain specific substrate was reduced, confirming that αβ-crystallin can act as an inhibitor of μ-calpain. However, this study revealed that myofibril-bound αβ-crystallin became degraded when incubated with activated exogenous μ-calpain. Although this clearly demonstrates that αβ-crystallin is a substrate for μ-calpain, it is not clear whether degraded αβ-crystallin retains its property and thus, myofibrillar chaperoning ability.

The association with myofibrils of αβ-Crystallin at various *pre rigor* pH increased, thereby supporting the hypothesis that the relocalisation of αβ-crystallin during damage

may serve to protect myofibrillar structural proteins (Bennardini *et al.*, 1992b; Bullard *et al.*, 2004) by acting as an alternative substrate from activated µ-calpain.

The second experiment of this study investigated whether the protection of myofibrillar proteins by sHSP from proteolytic degradation observed *in vitro* was mimicked in *post mortem* muscle aged at -1.5°C. Quantitative analysis of αβ-crystallin and HSP20 during ageing showed that the levels of these sHSP in the muscle sarcoplasm were pH_u related, with higher levels of sHSP remaining in the sarcoplasm at higher pH_u. The greater loss of sHSP from the sarcoplasm at more acidic pH_u levels supports the model of increasing association of sHSP with myofibrils as they transition from the soluble sarcoplasmic phase to the myofibrillar fraction. The low association of sHSP with myofibrils at elevated pH levels suggests that high pH_u meat is more susceptible to proteolytic degradation during ageing. In addition, non-associated sHSP may precipitate from the sarcoplasm of low pH_u meat as the inherent muscle pH declines to levels below the isoelectric points of each protein (pH 6.49 and 6.76, respectively for HSP20 and αβ-crystallin) (Pulford *et al.*, 2008).

The accumulation of intracellular calcium in *post mortem* muscle leads to the activation of µ-calpain (Goll, Thompson, Li, Wei & Cong, 2003; Jeacocke, 1993; Koohmaraie, 1992a). Upon activation, µ-calpain undergoes autolysis, which enhances its proteolytic activity, but also promotes the aggregation of µ-calpain leading to enzyme inactivation(Edmunds, Nagainis, Sathe, Thompson & Goll, 1991). The onset of *rigor mortis* is naturally faster in high pH_u meat due to the depleted glycogen stores at slaughter (Fernandez & Tornberg, 1994). Thus, the early activation and autolysis of µ-calpain in high pH_u meat may be facilitated by the rapid accumulation of calcium due to failure of calcium pumps early *post mortem*. Furthermore, high pH_u is indicative of *ante mortem* stress (McVeigh, Tarrant & Harrington, 1982; Mounier, Dubroeucq, Andanson & Veissier, 2006). As intracellular calcium has been found to accumulate in stressed muscle (Belcastro *et al.*, 1998; Murphy, 2010), there is a possibility that µ-calpain had already been activated in high pH_u samples pre-slaughter.

µ-Calpain is optimally active near neutral pH (Koohmaraie & Geesink, 2006). However, the results presented in this study show that relative µ-calpain activity at 0 and 1 day *post mortem* generally decreased as pH_u increased. This disparity may be explained by the earlier activation and autolysis of the enzyme in high pH_u samples. Thus, it is hypothesised that the activation of µ-calpain at near neutral pH_u levels of high pH_u meat is ideal for µ-calpain activity resulting in the rapid degradation of titin and filamin in this pH_u group. In contrast, it is speculated that once µ-calpain had been

activated in intermediate and low pH_u meat, the pH of the muscle in these pH_u groups had declined to levels that are not optimal μ-calpain activity. Furthermore, soluble sHSP levels increase with increasing pH_u. Hence, it is proposed that less extensive degradation of myofibrillar proteins as is observed in intermediate pH_u beef is due to lower μ-calpain activity combined with the competitive inhibition of the enzyme by sHSPs like αβ-crystallin.

Earlier *in vitro* experiments showed that μ-calpain induced degradation of desmin and titin was attenuated in myofibrils incubated in excess αβ-crystallin. In addition, αβ-crystallin was shown to be a competitive inhibitor of μ-calpain. The variable activity of μ-calpain and the effects of sHSP on myofibril proteolysis were also evident in the degradation of titin and desmin in *post mortem* muscle. Lower μ-calpain activity in high pH_u samples could be due to the faster autolysis of the enzyme to the inactive 84 kDa isoform. However, the degradation of titin early *post mortem* in high pH_u samples indicates that this enzyme is not completely inhibited and still capable of actively degrading myofibrillar proteins. Rapid titin degradation in high pH_u samples was concomitant with μ-calpain autolysis, supporting the role of μ-calpain in titin proteolysis. The delayed autolysis of μ-calpain at 1 day *post mortem* coincided with slower rate of titin degradation in the low pH_u samples. This delay may simply be due to the slower onset of *rigor mortis* and slower activation of μ-calpain in low pH_u beef. However, the association of sHSP with myofibrils at decreasing pH_u levels may also function in impeding the proteolysis of titin, as indicated by the presence of intact titin in low pH_u meat at 1 day *post mortem*.

The slower rate of desmin degradation in the representative low pH_u sample compared with the high pH_u may be due to the protective effects of myofibril-bound sHSP at decreasing pH_u levels. Increasing degradation of desmin with time in the low pH_u muscle is attributed to cathepsin activity, which is active at acidic pH and released from the lysosomes *post rigor* (Ertbjerg, Henckel, Karlsson, Larsen & Moller, 1999; Sentandreu, Coulis & Ouali, 2002). Minimal desmin degradation was observed in the intermediate pH_u sample. This is proposed to be due to the combination of sHSP protection and the sub-optimal protease activity in the intermediate pH_u range.

The degradation of desmin and titin has been implicated in the tenderisation of beef during ageing (Huff-Lonergan, Mitsuhashi, Beekman, Parrish, Olson & Robson, 1996; Young, Graafhuis & Davey, 1980). The stability of myofibrillar proteins in intermediate pH_u beef during ageing is accountable for the unacceptable toughness commonly found in this group. This was confirmed in our results which showed the

lack of desmin degradation in the intermediate pH_u sample even after 28 days *post mortem*. The increased stability of myofibrils in the intermediate pH_u range is hypothesised to be due to the protective effects of myofibril-bound sHSP and inhibition of proteolytic activity by the sub-optimal pH levels and sHSP. To prove this hypothesis, further work is required to characterise how variation in sHSP levels, proteolytic enzyme activity and myfibrillar protein degradation relate to meat quality traits such as tenderness in *post mortem* beef.

Conclusion

Immunoprecipitation of αβ-crystallin from the sarcoplasmic and myofibrillar fractions of *pre rigor* beef LD demonstrated the interaction of αβ-crystallin with desmin, titin, HSP20, HSP27 and µ-calpain. Exogenous αβ-crystallin was found to protect myofibrillar proteins from µ-calpain proteolysis by binding to myofibrils and stabilising denaturing proteins. Furthermore, αβ-crystallin reduced µ-calpain activity by functioning as a competitive inhibitor of the enzyme.

These *in vitro* interactions were also observed in *post mortem* bull LD aged at -1.5°C. The greater decline of sarcoplasmic sHSP in low pH_u beef supports the increasing transition of sarcoplasmic sHSP to the myofibrils as pH_u decreased. Higher levels of myofibril-bound sHSP in low pH_u beef suggest that myofibrillar protection by sHSP from proteolytic degradation is potentially greater in low pH_u beef. Low levels of µ-calpain activity in high pH_u beef early *post mortem* is due to the early autolysis of the enzyme combined with its inhibition by elevated levels of sHSP in this pH_u group. *Post rigor* desmin degradation in low pH_u beef may be due to cathepsin activity. The lack of desmin degradation in intermediate pH_u beef may be due to the protection of myofibrils by sHSP and low protease activity. Further characterisation of sHSP interactions with myofibrils and endogenous proteases during ageing is required to confirm the protection of myofibrils by sHSP and how this relates to meat quality.

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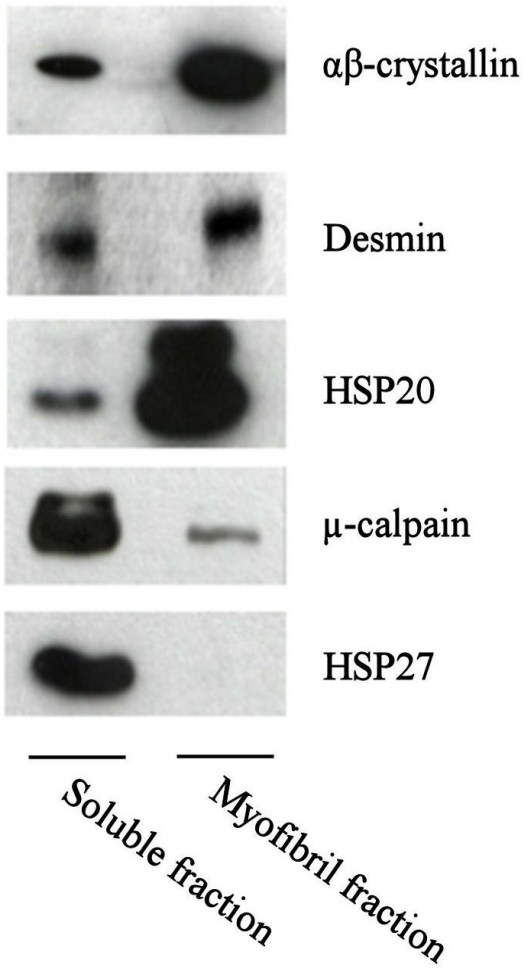

Figure 1: Association of αβ-crystallin with desmin, HSP20, μ-calpain and HSP27 in soluble and myofibril fractions of bovine LD muscle. αβ-Crystallin antibody covalently attached to magnetic beads was used to immuprecipitate αβ-crystallin in soluble and myofibril skeletal LD fractions. Eluted proteins were separated by SDS-PAGE and analysed by Western blotting using antibodies for αβ-crystallin, desmin, HSP20, μ-calpain and HSP27.

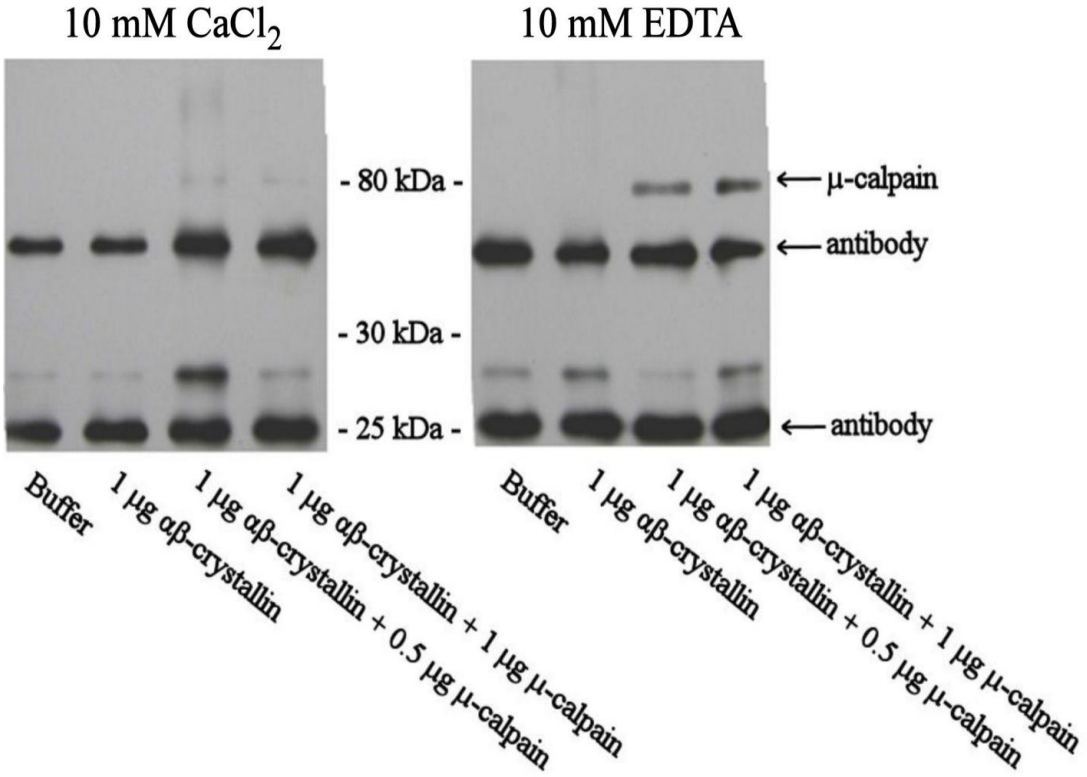

Figure 2: Interaction of αβ-crystallin with intact and autolysed μ-calpain. αβ-Crystallin was immunoprecipitated with αβ-crystallin antibody covalently attached to magnetic beads in buffer containing either 10 mM $CaCl_2$ or 10 mM EDTA for 5 minutes at 37°C. Reactions included αβ-crystallin antibody in buffer only and αβ-crystallin in varying concentrations of μ-calpain.

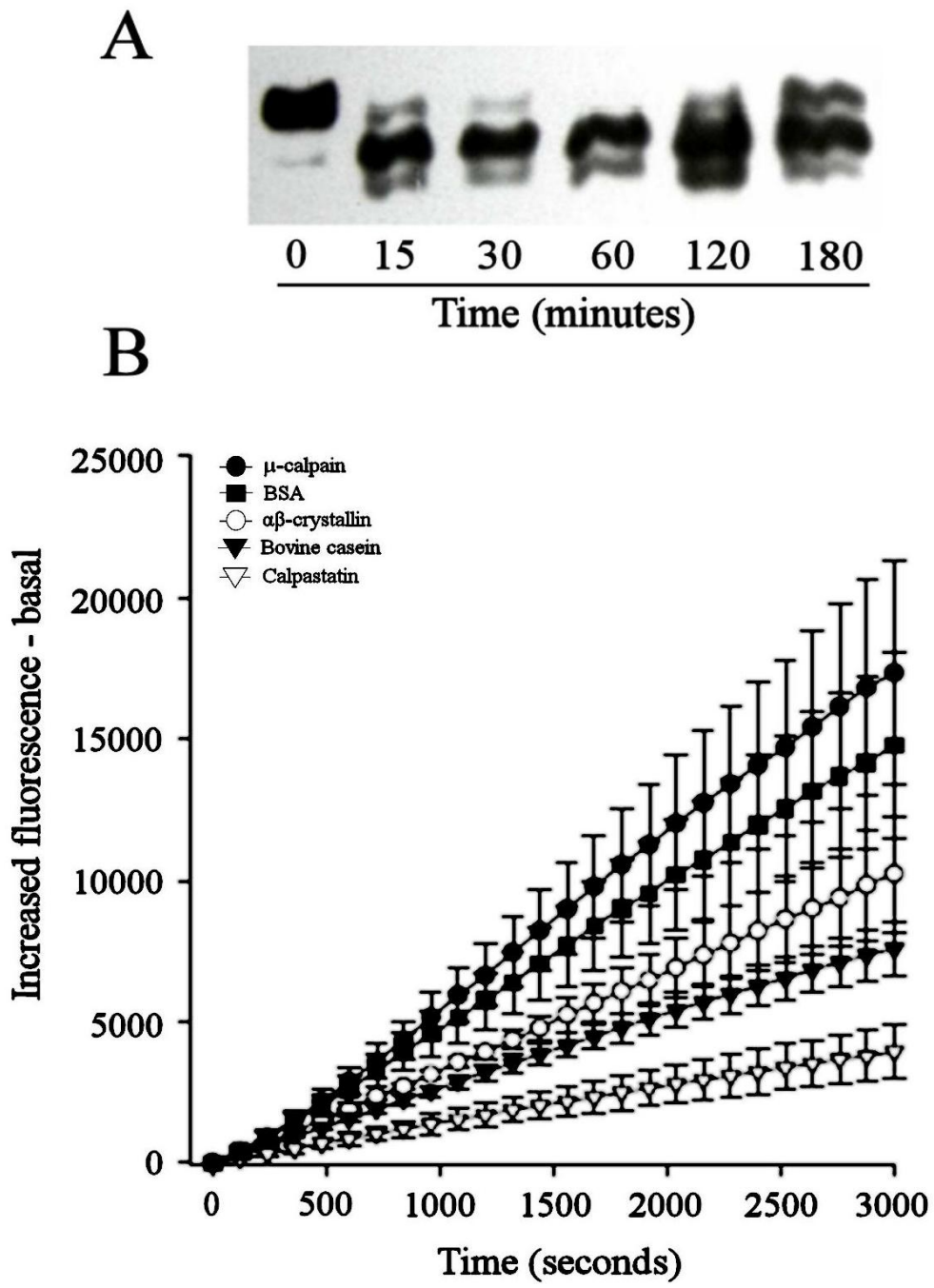

Figure 3: The increasing degradation of αβ-Crystallin in the presence of activated μ-calpain (A) and the efficacy of αβ-crystallin as a competitive inhibitor of μ-calpain by reducing the μ-calpain catalysis of the fluorogenic μ-calpain substrate (B).

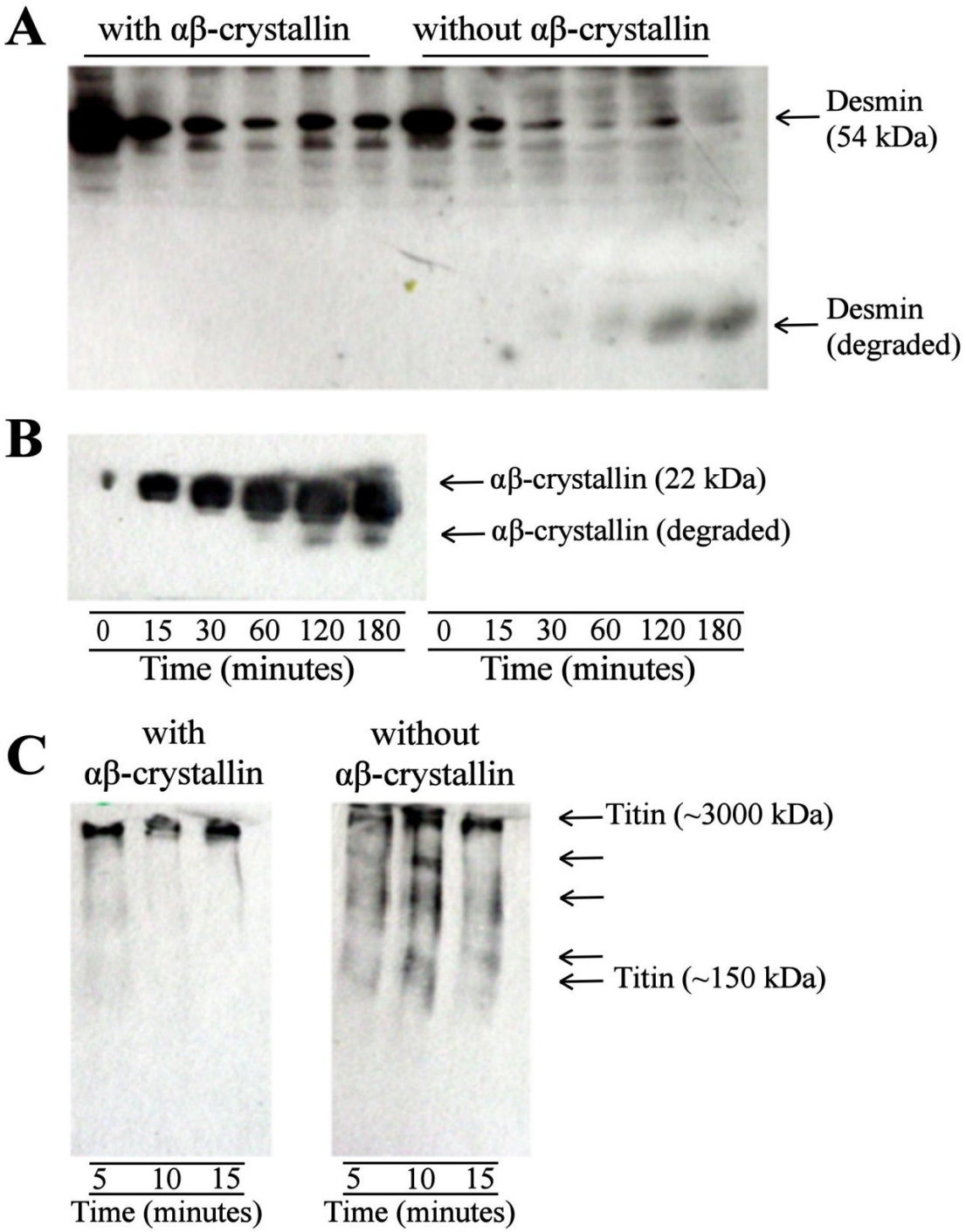

Figure 4: The efficacy of αβ-crystallin in reducing μ-calpain induced desmin and titin degradation. Stripped bovine LD myofibrils with no associated αβ-crystallin and μ-calpain were incubated with exogenous μ-calpain in the presence or absence of αβ-crystallin and incubated at 37°C for up 180 minutes. Myofibril fractions were subsequently analysed by Western blot using antibodies specific for desmin (A), αβ-crystallin (B) and titin (C).

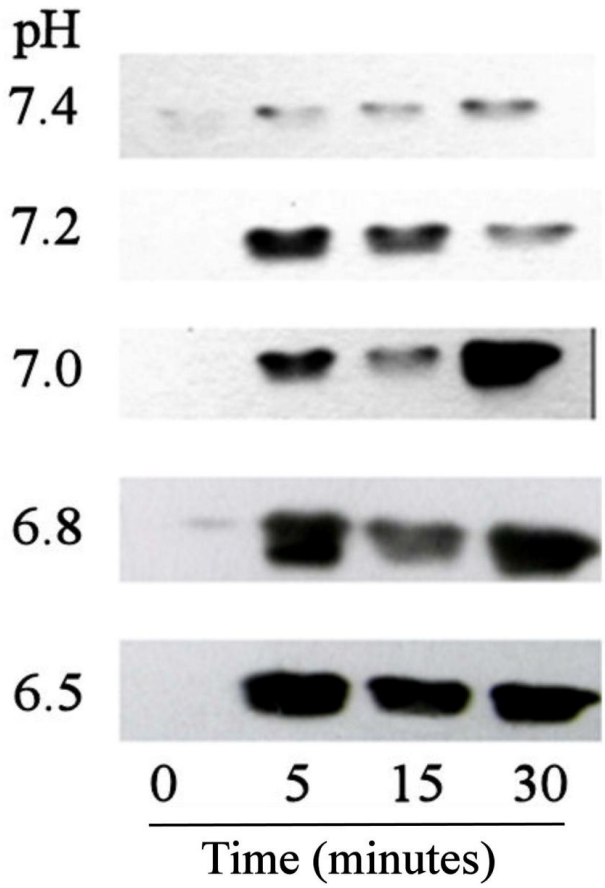

Figure 5: The increasing association of αβ-crystallin with the myofibril fraction of LD muscle at decreasing muscle pH. Myofibril fractions from LD muscle with incubated with purified bovine αβ-crystallin at 37°C then immunoblotted with αβ-crystallin monoclonal antibody.

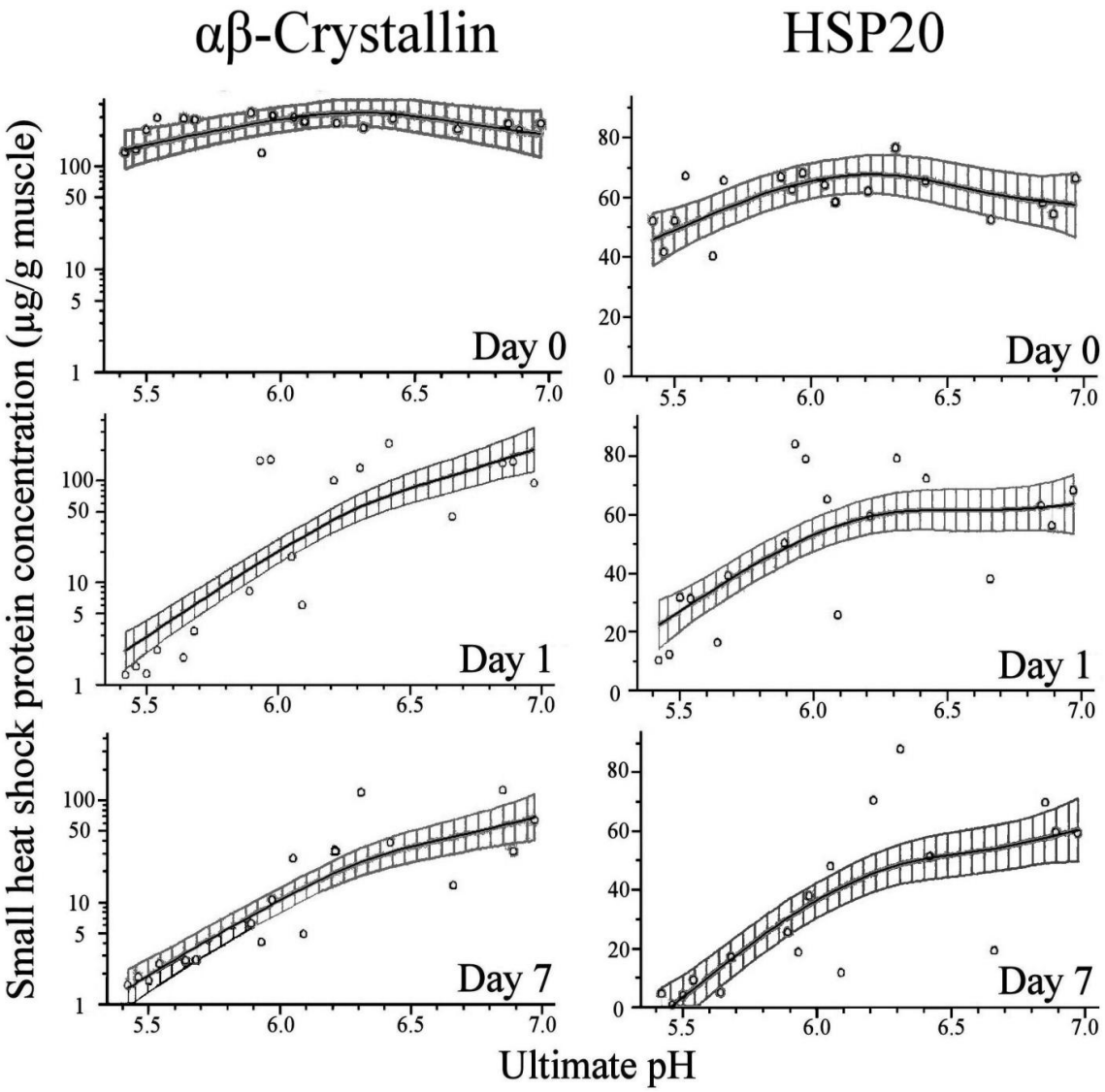

Figure 6: αβ-Crystallin and HSP20 levels in muscle sarcloplasm along the pH_u spectrum at 0, 1 and 7 days *post mortem*. Splines are least significant intervals at 95% significance.

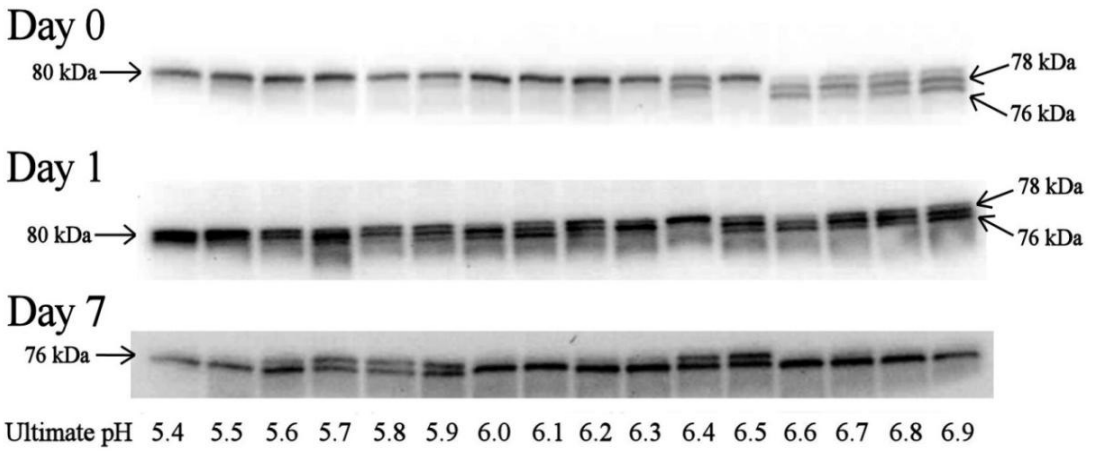

Figure 7: μ-Calpain autolysis in representative samples across the pH_u spectrum at 0, 1 and 7 days *post mortem*. Intact μ-calpain (80 kDa) was autolysed to the intermediary 78 kDa fragment, then to the inactive 76 kDa fragment.

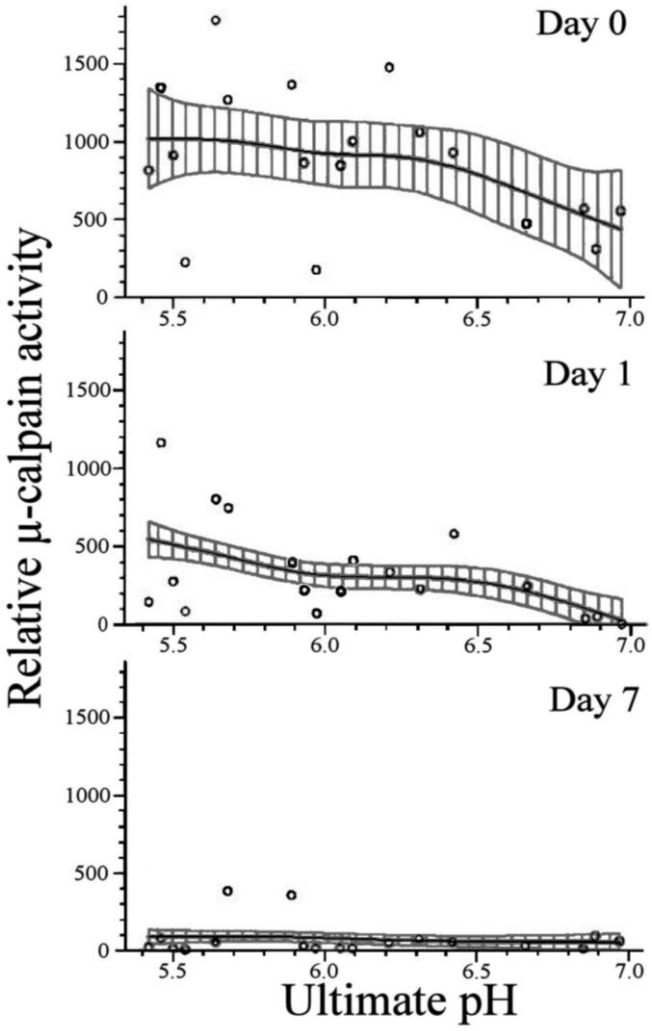

Figure 8: The relative activity of μ-calpain measured across the pH_u spectrum during ageing at 0, 1 and 7 days *post mortem*. Splines are least significant differences at 95% significance.

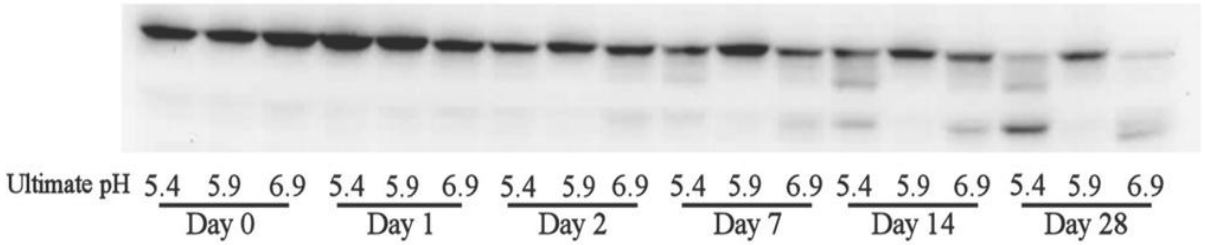

Figure 9: The degradation of desmin in representative low, intermediate and high pH_u samples at all ageing timepoints.

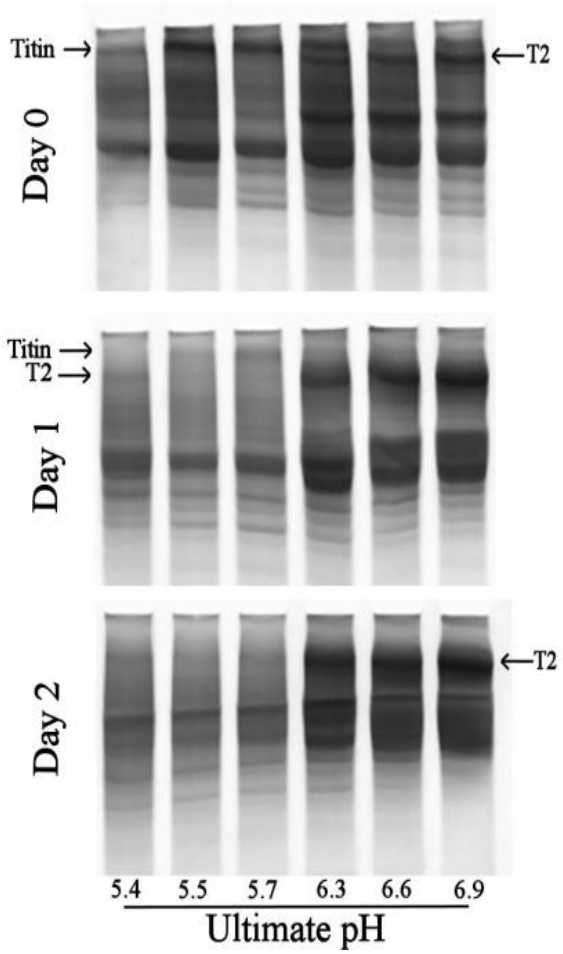

Figure 10: The degradation of titin in representative low and high pH_u beef samples. The degradation of titin was indicated by the detection of the T2 degradation product.

V

Small heat shock proteins and toughness in intermediate pH_u beef[3]

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Abstract

Bull *M. longissimus dorsi* (n = 94) categorised into high (pH ≥ 6.2), intermediate (pH 5.8 -6.19) and low (pH ≤ 5.79) ultimate pH (pH_u) were aged at -1.5°C for 28 days. Shear force was higher and more variable ($p < 0.05$) in intermediate pH_u samples during ageing. Titin, filamin and desmin degradation was also less extensive in intermediate pH_u samples compared to the other two pH categories. The extent of the decline of HSP20, HSP27 and αβ-crystallin concentrations during *post mortem* ageing was pH_u related such that high pH_u meat maintained the highest concentration of small heat shock proteins followed by intermediate and low pH_u meat. μ-Calpain autolysis was slowest in intermediate pH_u and cathepsin B activities remained consistently low during ageing in this group ($p < 0.05$). Meat toughness in the intermediate pH_u group may be attributed to the combination of a larger pool of sHSP with a sub-optimal cathepsin B activity and intermediary μ-calpain activities.

Keywords

Small heat shock proteins, intermediate pH, tenderness, αβ-crystallin, HSP20, HSP27

Introduction

There is a curvilinear relationship between meat ultimate pH (pH_u) and tenderness in beef and lamb (Bouton, Harris & Shorthose, 1971; Devine, 1994; Jeremiah, Tong & Gibson, 1991) and on average, meat with the lowest tenderness have intermediate pH_u – that is, meat with a pH_u between 5.8 and 6.19. A recent survey of New Zealand beef found that 18 percent of bull meat attained intermediate pH_u (Lomiwes, Wiklund, Farouk, Frost, Dobbie & Young, submitted; Wiklund, Farouk, Stuart, Dobbie, Lomiwes & Frost, 2009) accounting for much of the variation and suboptimal meat quality of bull beef.

Meat tenderisation is caused by the hydrolysis of cytoskeletal and myofibrillar proteins by proteases resulting in the structural degradation of muscle fibres. Three proteolytic systems have been recognised as being potentially involved in *post mortem* proteolysis leading to meat tenderness. These are lysosomal cathepsins (Ouali, 1992), calpains – particularly µ-calpain (Huff-Lonergan, Mitsuhashi, Beekman, Parrish, Olson & Robson, 1996; Koohmaraie & Geesink, 2006) and the multicatalytic proteinase complex (Sentandreu, Coulis & Ouali, 2002). There are several views as to which of these protease systems are variably involved in meat tenderisation, and the matter still remains contentious among meat scientists.

The marked biochemical changes taking place in muscle *post mortem* purportedly triggers apoptosis of muscle cells (Ouali *et al.*, 2006). Apoptosis is a process of programmed cell death and is induced when a cell is damaged, infected or exposed to adverse environmental conditions. In this process, the metabolic insult triggers a cascade of biochemical reactions leading the cell to self destruct without damaging surrounding healthy cells. Ouali *et al.* (2006) suggested that after slaughter, muscle cells inevitably tend towards apoptosis due to the loss of nutrients and oxygen supply to the muscles. In response to impending cell death, small heat shock proteins (sHSP) are synthesised to prevent unnecessary apoptosis and thus attempt to maintain cell homeostasis (Beere, 2004).

sHSP belong to a large group of chaperone proteins that typically have a molecular weight ranging from 15 and 43 kDa (Arrigo, 2005). Studies have found that sHSP have a protective role in cells, and their expression is particularly prominent where cells are exposed to harmful conditions such as hyperthermia (hence the name heat shock), hypoxia and harmful oxidants. They are also known to be involved in protein repair and maintain cell survival by keeping proteins from fatally aggregating in stressed cells (Soti, Sreedhar & Csermely, 2003).

Small heat shock proteins are abundant in skeletal muscle (Kato, Goto, Inaguma, Hasegawa, Morishita & Asano, 1994) and are believed to be implicated in the apopotic processes in *post mortem* muscle that consequently affect meat quality (Ouali *et al.*, 2006). However, very little work has been conducted to investigate the contribution of sHSP with regards to meat quality, and their role in the conversion of muscle to meat is still an enigma to meat science.

In a study to determine the kinetics of sHSP early *post mortem* in bull beef, it was demonstrated that the concentration of total sHSP in the soluble phase of meat *post rigor* was determined by the muscle's pH_u (Pulford, Fraga Vazquez, Frost, Fraser-Smith, Dobbie & Rosenvold, 2008) due to the precipitation of sHSP from the soluble phase at pH_u values 6.2 and lower. In addition, the proteolytic activity of calpain and cathepsin enzyme systems were found to be pH-related with the reduction of µ-calpain activity concomitant with the rise of cathepsin B activity as pH_u decreased (Pulford, Dobbie, Fraga Vazquez, Fraser-Smith, Frost & Morris, 2009). Authors from these studies hypothesised that the protective effect of total soluble sHSP and sub optimal proteolytic activities of calpains and cathepsins observed at intermediate pH_u levels may explain higher toughness observed in intermediate pH_u beef.

This study sought to test the hypothesis that a combination of the protective function of sHSP and low enzyme activity in intermediate pH_u meat maintains the integrity of the muscle structure resulting in meat toughness or delayed ageing as observed in intermediate pH_u beef.

Materials and Methods

Animals and muscle sample collection

Bulls (n = 94) slaughtered at a commercial abattoir were used in this study. Animals were head-only electrically stunned as is routine for the Halal slaughter of beef in New Zealand. Upon reaching the hot boning floor (< 1 hour *post mortem*), the *M. longissimus dorsi* (LD) from the left side of each carcass was excised and 10 g muscle samples were cut from the anterior end of each muscle, frozen in liquid nitrogen then stored at -80°C. The remaining LD was packaged in vacuum bags then stored at -1.5°C for 24 h.

After 24 hours, each LD was cut into six equally sized sub-samples and vacuum packed. The six sub samples from each LD were randomly allocated an ageing

timepoint and were stored -1.5°C for 1, 2, 7, 14 and 28 days *post mortem*. At each timepoint, 10 g was excised from the muscle sample, frozen in liquid nitrogen and stored at -80°C for subsequent analysis. The rest of the sub sample was used for pH and shear force measurements.

pH and shear force measurements

The pH of each loin at all ageing timepoints was measured with a Testo® 230 meter (Lenzkirch, Germany). The pH meter was calibrated at pH 7.0 and 4.0 with buffers (Mallinckrodt Chemicals, USA) stored at room temperature (20°C).

Meat tenderness was determined by cooking the loins in weighted plastic bags in a water bath at 100°C until the internal temperature of the loin reached 75°C as measured by a thermocouple. The cooked loins were immediately chilled in an ice water bath and cooled to below 10°C. The shear force from 10 mm × 10 mm cross sections (n = 10) was determined for each loin using a MIRINZ tenderometer. Shear force values for each loin were expressed as kgF.

Meat sample preparation and protein determination

Sarcoplasmic fractions were prepared from the frozen muscle samples for all timepoints as described by Pulford *et al.* (2008).

Whole muscle protein (WMP) fractions were prepared for all timepoints from frozen muscle samples. Muscle, 0.4 g, was powdered with a mortar and pestle then homogenised in WMP extraction buffer (10 mM sodium phosphate buffer, 0.2 % SDS; pH 7.0). The homogenate was centrifuged at 1500 g for 15 minutes at 25°C and the resulting supernatant was recovered.

Protein concentration of sarcoplasmic and WMP fractions at all timepoints were determined as described by Lowry, Rosenbrough, Farr & Randall (1951) using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA).

Coomassie blue sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Approximately 0.5 g frozen muscles from all timepoints were crushed and homogenised in 5 mL of extraction buffer (50 mM Tris-HCl (pH 5.8), 10% glycerol, 2% SDS and 2% 2-mercaptoethanol) followed by centrifugation at 10,000 g for 5 min at 4°C. An aliquot of the supernatant was collected for protein determination by RC-DC protein kit (BioRad).

For SDS-PAGE, an aliquot of the supernatant was mixed with an equivalent volume of reduced sample buffer (62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.02% bromophenol blue), then heated for 20 min at 50°C. After centrifugation at 10,000 for 10 min, a total of 100 µg of protein from each sample was loaded onto wells of 5% Tris-HCl gels (BioRad Laboratories) and separated in a BioRad Criterion Cell system at room temperature at 10 mA for 17 h. Gels were subsequently stained with colloidal Coomassie blue (17% ammonium sulphate, 2% phosphoric acid, 30% methanol, 0.04% Coomassie G-250) and scanned with a GS700 calibrated densitometer scanner (BioRad Laboratories).

Immunoblots

Immunoblots for sHSP were conducted on sarcoplasmic fractions that were adjusted to a concentration of 4 mg mL^{-1} with sarcoplasmic extraction buffer. A total of 20 µg protein were loaded onto 12% Bis-Tris gels (BioRad Laboratories, 345-0118) then separated at 120 V at room temperature. For µ-calpain, desmin and myosin immunoblots, WMP fractions were adjusted to a concentration of 4 mg mL^{-1} with WMP extraction buffer and a total of 40 µg protein were loaded onto 7.5% Tris-HCl gels (Bio-Rad Laboratories, 345-0006) and resolved in a Bio-Rad Criterion Cell system at 120 V at room temperature.

Following SDS-PAGE, proteins were transferred onto Immobilon-P PVDF membranes (Millipore, IPVH00010) and blocked by incubating the membrane with 5% non-fat dry milk powder diluted with PBS-Tween (0.08 M Na_2HPO_4, 0.02 M NaH_2PO_4, 0.1 M NaCl, 0.1% Tween) overnight at 4°C. Membranes were then washed three times with PBS-Tween then incubated with the chosen primary antibody for one hour at room temperature. Primary (monoclonal) antibodies from mouse included αβ-crystallin (Abcam, ab74441) HSP20 (HyTest, 4HSP20) and HSP27 (HyTest, 4HSP27) and were diluted to 1:10,000 in PBS-Tween. Desmin (Sigma, D1033), Troponin T (Sigma, T6277) and µ-calpain (Thermo Scientific, MA3-940) monoclonals were diluted to 1:5000, 1:10000 and 1:5000, respectively in PBS-Tween. After washing, membranes were subsequently incubated with goat anti-mouse IgG (H+L) HRP conjugate (BioRad, #172-1011) diluted to 1:5000 in PBS-Tween at room temperature for 1 hour. The membranes were then washed as previously described and the bound antibody was detected using ECL Western Blot substrate kit (Pierce, 32106). Western Blot images were captured with a G:Box Chemi HR16 image capture instrument (Syngene, Cambridge, UK).

Cathepsin B and μ-calpain activities

Isolation and measurement of cathepsin B activity was determined as described by Caballero *et al.* (2007) with minor modifications. Crushed frozen muscle fragments (2 g) were suspended in ice cold homogenisation buffer (10 mM K_2HPO_4, 50 mM NaCl, 250 mM sucrose, 1 mM EDTA; 7.4). Standard curves were prepared from purified Cathepsin B (Sigma, C6286) and diluted with incubation buffer (100 mM $C_2H_3NaO_2$, 1 mM EDTA, 5 mM DTT, 0.1% Brij; pH 5.5). Sample lysates and standards were dispensed into 96 well fluorescent plates (Nunc, #265301) and mixed with 70 μL of incubation buffer then warmed to 37°C for 10 minutes. An aliquot (5 μL) of 40 μM Cathepsin B Fluorogenic Substrate III (Calbiochem, 219392) was then dispensed into each well. Fluorescent measurements were collected every minute for 1 h at 37°C with a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany) fitted with 360 nm excitation and 460 nm emission filters.

Calpain extraction and activities were determined as described by Wiklund *et al.* (2010).

Quantitative determination of small heat shock proteins

For the quantitative measurement of small heat shock proteins in muscle sarcoplasm, sarcoplasmic extracts were adjusted to a concentration of 4 μg mL^{-1} protein with coating buffer (10 mM Na_2HPO_4, 15 mM NaCl; pH 7.4). Aliquots (100 μL) of each sample were dispensed into 96 well Costar® High Binding EIA/RIA polystyrene plates (Corning Inc., 3590) in duplicate. Individual sHSP was measured by indirect enzyme-linked immunosorbent assay (ELISA) according to the procedures described by Pulford *et al.* (2008), with some modifications. Protein standards for each sHSP were prepared from purified HSP20 (Hytest, 8HSP20), HSP27 (Hytest, 8HSP25) and αβ-crystallin (Sigma, C7858) diluted in coating buffer. Wells were blocked with 1% BSA in wash buffer (10 mM Na_2HPO_4, 15 mM NaCl, 0.1% Tween 20; pH 7.4) then washed four times with wash buffer. Following washing, wells were recursively incubated with 100 μL of mouse monoclonal antibody (HSP20, Hytest 4HSP20; HSP27, Hytest 4HSP27 and αβ-crystallin, Abcam ab74441); each diluted to 1:10,000 with 1% BSA in assay buffer then washed as previously described. A 100 μL aliquot of goat anti-mouse IgG (H+L) HRP conjugate (Bio-Rad Laboratories, #172-1011) diluted to 1:5,000 with 1% BSA in assay buffer was dispensed into each well and left to incubate for 1 h. Secondary antibody bound to the wells were detected by incubating all wells with 100

µL of 3,3',5,5'-tetramethybenzidine substrate (Sigma, T0440) at room temperature for 30 min and the reaction was stopped by dispensing 100 µL of 2N H_2SO_4 into each well. The optical density of each well was measured with a Fluostar Optima (BMG Labtech, Ortenberg, Germany) plate reader and the absorbance at 450 nm was measured.

Data analysis

Statistical analysis of the data was conducted using the REML directive of Genstat (Genstat, 2008).

To test the significance between days and pH_u groups for each of the parameters measured, the data was analysed using a mixed model smoother (Upsdell, 1994). The model used was time *post mortem* × pH_u and time effect. Time effect was a random term describing the variability between ageing timepoints. The curves derived from the model show the estimated means of the parameters when grouped into time *post mortem* or ultimate pH_u. The splines are 95% least significant intervals for testing differences between groups. Thus, two groups are significantly different from each other if the splines do not overlap.

Results

Tenderness as affected by pH_u and ageing

Tenderness was pH_u related with shear force values increasing as pH_u increased, peaking at approximately pH_u 5.9, and then decreased as pH_u continued to increase thereafter (Figure 1). With the upper limit of acceptable tenderness in beef at 11 kgF (Bickerstaffe, Bekhit, Robertson, Roberts & Geesink, 2001), high pH_u meat was already acceptably tender at 1 day *post mortem* and remained more tender than low and intermediate pH_u meat throughout the ageing period (Table 1). Low pH_u meat was initially tough, but with a sharp decline in shear force attained acceptable tenderness levels at 14 days *post mortem*. In contrast, the decline in shear force was slower in intermediate pH_u meat which attained acceptable tenderness only at 28 days *post mortem*. In addition, shear force values in the intermediate pH_u group were numerically more variable compared with the low and high pH_u groups at all timepoints (Table 1).

Myofibrillar protein degradation during ageing

The degradation of titin and filamin was most rapid in high pH_u meat with T2 and F2 degradation products detected at 0 and 1 day *post mortem*, respectively (Figure 2). In addition, intact titin and filamin were no longer detectable in high pH_u meat at 2 and 7 days *post mortem*, respectively. Titin and filamin degradation products were detected in low pH_u at 1 and 2 days *post mortem*, respectively. Protein degradation in intermediate pH_u was much more delayed, with T2 and F2 degradation products initially detected at 2 and 28 days *post mortem*, respectively. Intact titin and filamin bands were still detectable in intermediate pH_u meat at 7 and 28 days *post mortem*, respectively. It was difficult to ascertain the degradation nebulin in high pH_u meat due to the diffusion of protein bands in the area of the gel where nebulin is resolved. These bands may be further breakdown products of larger proteins. However, nebulin degradation in low and intermediate pH_u meat was detected at 2 days *post mortem*. Any nebulin breakdown at latter ageing periods was difficult to establish due to the diffusion of protein bands as seen in the high pH_u gels.

Desmin degradation was initially detected at 1 and 2 days *post mortem* in high and low pH_u meat, respectively (Figure 3). The extensive degradation of desmin in low pH_u meat was indicated by the increasing detection of b1 and b2 degradation products, and was concurrent with the disappearance of intact desmin during ageing. Desmin in the high pH_u sample was not degraded to the same extent as low pH_u meat. However, the intensity of b1 degradation product in high pH_u increased with ageing, suggesting increased desmin degradation, the density of the intact desmin band remained constant throughout ageing. Minimal degradation of desmin in the intermediate pH_u was observed, with b1 faintly detected at 7 days *post mortem* (Figure 3).

μ-Calpain and cathepsin B activities

In high pH_u meat, μ-calpain activities greatly decreased within 2 days *post mortem*, and the extent of decline in μ-calpain levels was more marked early *post mortem* than in low and intermediate pH_u meat. Activity of μ-calpain in high pH_u meat was consistently numerically lower than for low and intermediate pH_u groups up to 7 days *post mortem*. μ-Calpain activity was numerically highest in low pH_u meat at 1, 2, 7 and 14 days *post mortem* (Table 2).

Autolysis of μ-calpain in the high pH_u samples had already occurred at 0 day *post mortem* as indicated by the presence of the 78 kDa subunit (Figure 4). μ-Calpain progressively autolysed with time such that intact μ-calpain was completely degraded to

76 kDa at 28 days *post mortem*. μ-Calpain autolysis in low pH_u meat was initially detected at 1 day *post mortem* and by 14 days, only the 76 kDa subunit was detected. In comparison, μ-calpain autolysis in intermediate pH_u meat was more delayed than other pH_u groups such that the 78 kDa subunit was still detectable at 28 days ageing.

Cathepsin B activities were higher in low pH_u meat throughout the ageing period compared with the intermediate and high pH_u groups. Sarcoplasmic cathepsin B activities in low pH_u meat progressively increased with ageing such that highest cathepsin B activities were attained at 28 days *post mortem* (Figure 5). In comparison, cathepsin B activities in intermediate and high pH_u meat were significantly lower compared with low pH_u meat and remained relatively unchanged throughout the ageing period.

The kinetics of sHSP in post mortem muscle sarcoplasm

There is a positive linear relationship between sHSP concentration and pH_u, with sHSP concentrations increasing with increasing pH_u (Figure 6). Sarcoplasmic HSP20, HSP27 and αβ-crystallin concentrations were at their peak at 0 day *post mortem* at all pH_u values then markedly decreased subsequently (Table 2). All sHSPs in the low pH_u group declined considerably faster. sHSP concentrations at 1 day *post mortem* and subsequent ageing timepoints were pH_u related with sHSP concentrations highest in the high pH_u group followed by intermediate then low pH_u groups. HSP20 and HSP27 concentrations were also more dynamic in high pH_u, increasing and decreasing during ageing.

Immunoblots confirmed that the rate of disappearance of each sHSP from the muscle sarcoplasm is pH_u related. Intact HSP20, HSP27 and αβ-crystallin bands increasingly disappeared with ageing. However, band intensities were greater in high pH_u followed by intermediate and low pH_u meat at all timepoints. Degradation products of all sHSP in high pH_u meat intensified with ageing and were concomitant with the weakening densities of the intact sHSP bands. This suggests the progressive degradation of intact sHSP to lower molecular weight proteins in the high pH_u group. Very faint HSP27 degradation products were also detected in low pH_u meat at 1 and 2 days *post mortem*. No sHSP degradation was detected in intermediate pH_u meat.

Discussion

This study was conducted to determine the contribution, if any, of sHSP to the inconsistent tenderness found in intermediate pH_u meat. The shear force trends reported are consistent with previous results that found intermediate pH_u meat to be tougher (Jeremiah *et al.*, 1991; Purchas & Aungsupakorn, 1993) and tenderise at a slower rate than low and high pH_u meat (Bouton *et al.*, 1971; Pulford *et al.*, 2008; Watanabe, Daly & Devine, 1996a). To exploit the full financial value of intermediate pH_u meat, the challenge for the meat industry is to mitigate its toughness and to collapse the high variability in this pH_u group.

Meat tenderisation has been attributed to the disruption of the highly organised muscle structure by the degradation of key myofibrillar proteins. The degradation of titin, nebulin, filamin and desmin was determined to investigate the role that these proteins may contribute to meat tenderness. Our results concur with previous studies that reported the faster degradation of titin and nebulin degradation in high pH_u meat (Watanabe & Devine, 1996b) and the delayed fragmentation of nebulin and desmin in the intermediate pH_u group (Pulford *et al.*, 2009; Watanabe *et al.*, 1996b). Thus, the rate and extent at which these proteins were degraded at the conditions at which they were aged is pH_u related.

It is widely accepted that the *post mortem* degradation of myofibrillar proteins is due to the proteolytic activity of endogenous enzymes found in muscle. Calpains, specifically μ-calpain, and cathepsins are two enzyme systems most implicated with the proteolysis of myofibrillar proteins, and their specific activity in *post mortem* muscle has been comprehensively reviewed (Goll, Thompson, Li, Wei & Cong, 2003; Koohmaraie *et al.*, 2006; Ouali, 1990). μ-Calpain proteolyis may be restricted to the first 48 hours *post mortem* as their activity naturally declines with ageing time and is limited by the acidification of *post mortem* muscle. As cathepsins are naturally confined within the lysosomes, their role in meat tenderisation has been discounted (Koohmaraie, 1992b; Whipple, Koohmaraie, Dikeman, Crouse, Hunt & Klemm, 1990). However, the susceptibility of lysosomes to acidic pH (Dutson, 1983) or the failure of the lysosomal membrane ionic pumps *post mortem* (Sentandreu *et al.*, 2002) is speculated to compromise the integrity of lysosomes leading to the release of cathepsins into the sarcoplasm.

Extractable levels of μ-calpain and cathepsin B were measured to determine whether the variable degradation of myofibrillar proteins could be related to their activities. μ-Calpain activity is optimal at near neutral pH (Koohmaraie, 1992a) and

declines with ageing in *post mortem* muscle, as was found in this study. Therefore, myofibrillar proteolysis by µ-calpain is restricted to early *post mortem* when the inherent pH of muscle is near physiological levels. Contrary to the results reported by Pulford *et al.* (2009), we found µ-calpain levels in high pH_u meat were generally lower than in low and intermediate pH_u groups in the first 7 days *post mortem*. This disparity may be due to the variation in the extraction and assay procedures used by the researchers.

µ-Calpain immunoblots were conducted to determine enzyme autolysis, which is an indicator of µ-calpain activity and subsequent inactivation. Lower quantifiable activities of µ-calpain in high pH_u meat may be explained by the early activation of the enzyme, as indicated by autolysis. Autolysis reduces enzyme activity in that µ-calpain is inactive in meat when the large sub-unit has autolysed to 76 kDa (Li, Thompson & Goll, 2004). Additionally, µ-calpain increasingly associates with myofibrils upon activation (Boehm, Kendall, Thompson & Goll, 1998) and is proteolytically active until further autolysis. However, their activities are often not represented in quantitative assays, because bound µ-calpain is difficult to extract and often does not react with exogenous substrates used to measure µ-calpain activity (Delgado, Geesink, Marchello, Goll & Koohmaraie, 2001). Thus, lower quantifiable activities of µ-calpain early *post mortem* may be interpreted as an indicator of µ-calpain activation and autolysis. Although non-intuitive, this model is supported by our data, which demonstrated that the immediate autolysis of µ-calpain in high pH_u meat corresponded with low quantitative levels of the enzyme. This in turn was concomitant with the immediate hydrolysis of titin and the rapid tenderisation of the meat in this pH_u group. Conversely, elevated µ-calpain levels measured in low pH_u meat at 0 day *post mortem* corresponded with the absence of enzyme autolysis and protein degradation at that timepoint.

To determine relationships if any, between cathepsins and myofibrillar degradation, analysis of cathepsin B activity was conducted at all ageing times. The marked rise of cathepsin B activities in low pH_u meat at 2 days *post mortem* indicates its release from the lysosomes as the muscle pH declined to acidic values. Cathepsin B are optimally active at pH values ranging from 5.0 and 6.0 (Ouali, 1992), and the increased activity of this enzyme coincided with the further degradation of desmin and decline in shear force in low pH_u meat. Due to the delayed release of cathepsin B into the sarcoplasm, it is speculated that its contribution to meat tenderness must take place at latter ageing periods, as demonstrated in this study. Low cathepsin B activities in intermediate and high pH_u meat may be due to the increased stability of lysosomal

membranes owing to the higher pH in these groups compared with low pH_u meat (Ertbjerg, Henckel, Karlsson, Larsen & Moller, 1999). Nonetheless, cathepsin B activity in the high pH_u meat is limited by the near neutral pH of the muscle, which does not favour its activity (Ertbjerg *et al.*, 1999), and although cathepsin B activities gradually increased *post rigor* in intermediate pH_u meat, it was significantly lower than in high and low pH_u meat. Depleted cathepsin B activity as observed in intermediate pH_u meat also corresponded with the minimal degradation of desmin and delayed fragmentation of filamin and titin.

The results in this study suggest that the rapid degradation of high molecular weight proteins such as titin in high pH_u meat is due to the early activation of μ-calpain, which is optimally active at physiological pH. According to this model, rapid tenderisation in high pH_u meat is due to the fragmentation of intra-myofibrillar linkages in the I-band where titin has been found to degrade (Boyer-Berri & Greaser, 1998) and in the N_2 lines where titin and nebulin are co-located (Taylor, Geesink, Thompson, Koohmaraie & Goll, 1995). Desmin and filamin degradation, which were concomitant with tenderisation in low pH_u meat, implies that tenderness in this pH_u group is due to the disruption of the inter-myofibrillar linkages and costameres within muscle cells (Huff Lonergan, Zhang & Lonergan, 2010; Young & Davey, 1981). Delayed tenderisation and toughness in intermediate pH_u meat may be attributed to the minimal fragmentation of key myofibrillar proteins owing to the intermediate and low levels of calpain and cathepsin B, respectively, throughout ageing.

The termination of oxygen and nutrient supply to the muscle *post mortem* leads to the inevitable apoptotic death of muscles cells (Herrera-Mendez, Becila, Boudjellal & Ouali, 2006; Ouali *et al.*, 2006). In response to the initiation of apoptosis, sHSP proteins are up-regulated in an attempt to maintain cell homeostasis. sHSP act as molecular chaperones by binding to damaged or unfolding proteins preventing their irreversible damage and aggregation within the cell (Sun & MacRae, 2005). Studies have linked the up-regulation of the sHSP HSP27 and αβ-crystallin with higher shear force values (Bernard, Cassar-Malek, Le Cunff, Dubroeucq, Renand & Hocquette, 2007; Hwang, Park, Kim, Cho & Lee, 2005; Kim *et al.*, 2008). This is likely to be due to the protective function of sHSP, whose up-regulation has been associated with delayed desmin degradation in low and intermediate pH_u meat (Pulford *et al.*, 2009).

Stresses experienced by an animal immediately pre-slaughter leads in the depletion of muscle glycogen consequently resulting in intermediate or high pH_u meat (Lahucky, Palanska, Mojto, Zaujec & Huba, 1998; McVeigh, Tarrant & Harrington,

1982). Due to the emerging evidence implicating sHSP with meat quality, quantitative and qualitative analysis of HSP20, HSP27 and αβ-crystallin was conducted over an extended ageing period to investigate any role they may have in meat toughness. Sarcoplasmic sHSP immediately declined and reached minimum concentrations after 2 days *post mortem* in all pH_u groups. This suggests that any role that sHSP may contribute to meat toughness is restricted to their interactions with muscle cells either *pre rigor* or even pre-slaughter, where sHSP are known to be up-regulated in stressed muscle (Paulsen *et al.*, 2007). The variable rate of decline of HSP20, HSP27 and αβ-crystallin concentrations at each pH_u group is most likely to be associated with their respective isoelectric points (pI) of pH 6.49, 5.98 and 6.76 (Pulford *et al.*, 2008). The rapid sHSP decline in low pH_u meat is possibly due sHSP precipitation as muscle pH declines to concentrations far below the pI of each sHSP (Pulford *et al.*, 2008). Conversely, higher sustained concentrations of sHSP in high pH_u meat are likely due to the pI of HSP20 and αβ-crystallin being within the high pH_u range.

The loss of sHSP from the sarcoplasm was confirmed by the immunoblots, which established the faster disappearance of all three sHSP in low pH_u meat. The blots also revealed the progressive degradation of these proteins in high pH_u meat during ageing. It is speculated the degradation of these proteins is due to the proteolytic action of enzymes such as calpains and cathepsins. Nevertheless, this suggests that although high pH_u meat inherently sustained higher quantitative concentrations of sHSP throughout ageing, a significant portion is degraded and may no longer be bioactive and capable of protecting myofibrillar proteins from proteolytic damage. sHSP in intermediate pH_u meat does not precipitate to the degree observed in low pH_u meat as the pH of this group does not decline to the same extent. Additionally, sHSP does not undergo degradation in intermediate pH_u meat as observed in high pH_u meat. With the lower proteolytic activity of μ-calpain and cathepsin B, combined with a potentially larger pool of bio-active sHSP in intermediate pH_u meat, any prospective role for sHSP to maintain the integrity of myofibrillar proteins that may lead to meat toughness is proposed to be optimal in the intermediate pH_u range. Moreover, it is hypothesised that the inconsistent tenderness in intermediate pH_u meat may be due to the variable concentrations of sHSP in muscles that fall within this category, so that a muscle with inherently high sHSP concentrations will be unacceptably tough.

Conclusions

The rapid decline of sHSP concentration in muscle *post mortem* suggests the protective effect of sHSP is restricted to timepoints preceding *rigor mortis*. The tenderness of low and high pH_u meat is not affected by sHSP due to the respective precipitation and degradation of sHSP in these pH_u groups. Therefore, the delayed and less extensive degradation of myofibrillar proteins as observed in intermediate pH_u meat may be due to the combination of the protective effects of sHSP on myofibrillar proteins combined with the low proteolytic activity in this pH_u group. It is also suggested that the variable concentrations of sHSP in muscle that eventually yield intermediate pH_u meat accounts for the high variability in tenderness in this group such that intermediate pH_u meat with a high concentration of sHSP *pre rigor* will be unacceptably tough. However, further work is required to prove this hypothesis.

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Table 1: Mean shear force and variance of low, intermediate and high pH bull beef during ageing. Mean values without a common superscript letter within the same day are significantly different ($p < 0.05$). Variance test was conducted using Levene's test for variance.

Time *post mortem* (days)	Mean shear force (kgF)			Variance (kgF)		
	Low	Intermediate	High	Low	Intermediate	High
1	16.5[a]	16.4[a]	8.8[b]	10.5	19.3	11.6
2	15.0[a]	16.3[a]	8.4[b]	12.1	17.9	13.3
7	11.5[a]	14.0[b]	7.0[c]	12.1	24.3	11.1
14	9.7[a]	11.9[b]	5.6[c]	7.8	18.2	6.9
28	8.6[a]	10.1[b]	5.8[c]	9.5	12.7	6.5

Table 2: Mean relative μ-calpain activity, HSP20, HSP27 and αβ-crystallin concentrations in low, intermediate (int.) and high pH_u bull beef during ageing. Means of each protein without a common superscript letter within the same day are significantly different ($p < 0.05$).

Time *post mortem* (days)	μ-calpain (relative activity) (±SEM)			HSP20 (± SEM) (ng/μg protein)			HSP27 (± SEM) (ng/μg protein)			αβ-crystallin (± SEM) (ng/μg protein)		
	Low	Int.	High	Low	Int.	High	Low	Int.	High	Low	Int.	High
0	841±45^{a}	863±64^{a}	719±58^{a}	12.7±0.4^{a}	10.47±0.3^{a}	10.1±0.3^{a}	12.2±1.7^{a}	13.1±2.3^{a}	12.0±1.9^{a}	22.5±2.8^{a}	14.2±1.5^{a}	23.0±2.8^{a}
1	554±58^{a}	416±46^{ab}	277±39^{b}	1.5±0.3^{a}	3.6±0.3^{b}	5.3±0.7^{c}	2.2±0.1^{a}	3.2±0.4^{b}	4.7±0.9^{c}	0.6±0.1^{a}	1.1±0.3^{b}	5.2±1.0^{c}
2	280±27^{a}	309±35^{a}	219±41^{a}	1.3±0.2^{a}	2.1±0.3^{a}	4.9±0.6^{b}	2.1±0.2^{a}	2.5±0.3^{a}	5.1±1.2^{b}	0.6±0.1^{a}	1.0±0.3^{b}	4.8±1.4^{c}
7	140±32^{a}	120±25^{a}	68±12^{a}	1.2±0.2^{a}	1.9±0.2^{b}	3.1±0.8^{c}	1.8±0.2^{a}	1.9±0.1^{a}	3.2±0.5^{a}	0.6±0.1^{a}	1.1±0.3^{b}	3.1±1.6^{c}
14	106±29^{a}	60±5^{a}	81±13^{a}	1.4±0.4^{a}	1.4±0.2^{a}	6.2±0.9^{b}	1.9±0.2^{a}	2.3±0.1^{b}	4.4±1.1^{c}	0.5±0.1^{a}	0.8±0.2^{b}	2.7±0.5^{c}
28	60±10^{a}	48±3^{a}	80±17^{a}	0.7±0.2^{a}	1.2±0.4^{b}	4.9±0.7^{c}	1.8±0.2^{a}	2.1±0.1^{a}	3.4±0.4^{b}	0.7±0.1^{a}	1.0±0.2^{b}	3.4±1.3^{c}

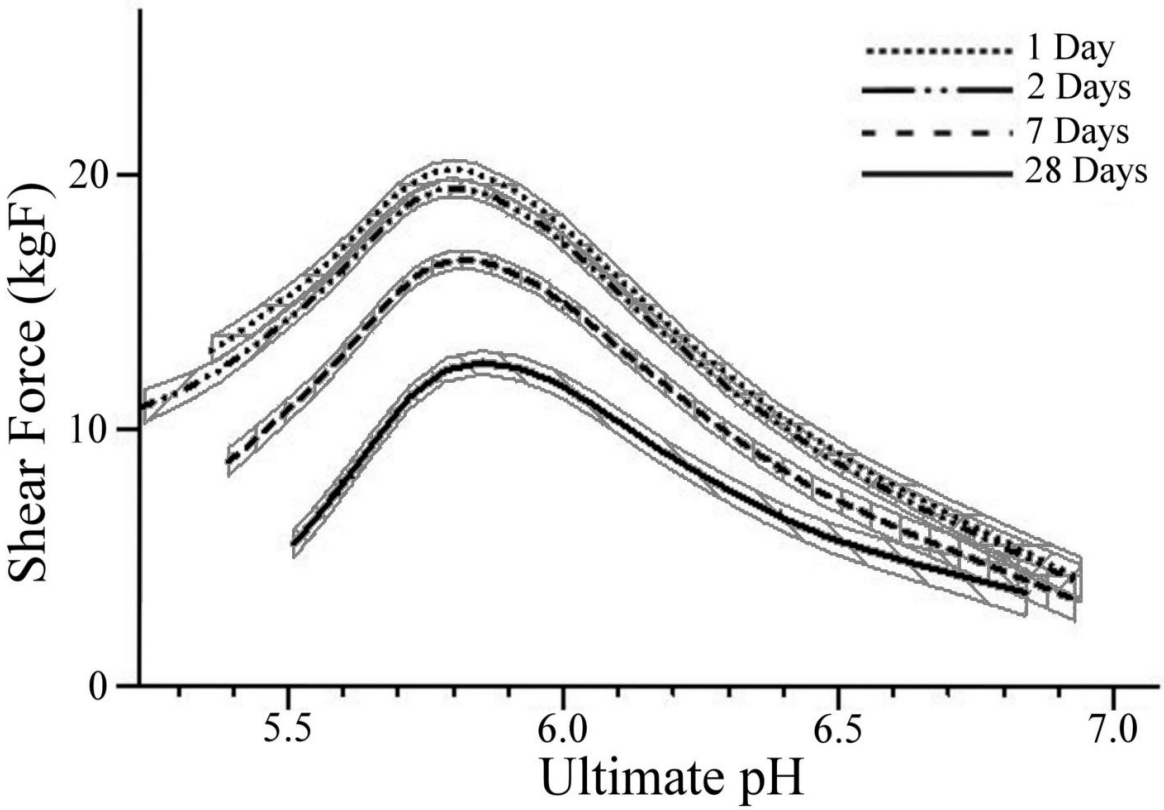

Figure 1: Shear force of low, intermediate and high pH_u meat during ageing. Splines are least significant intervals at the 95% level.

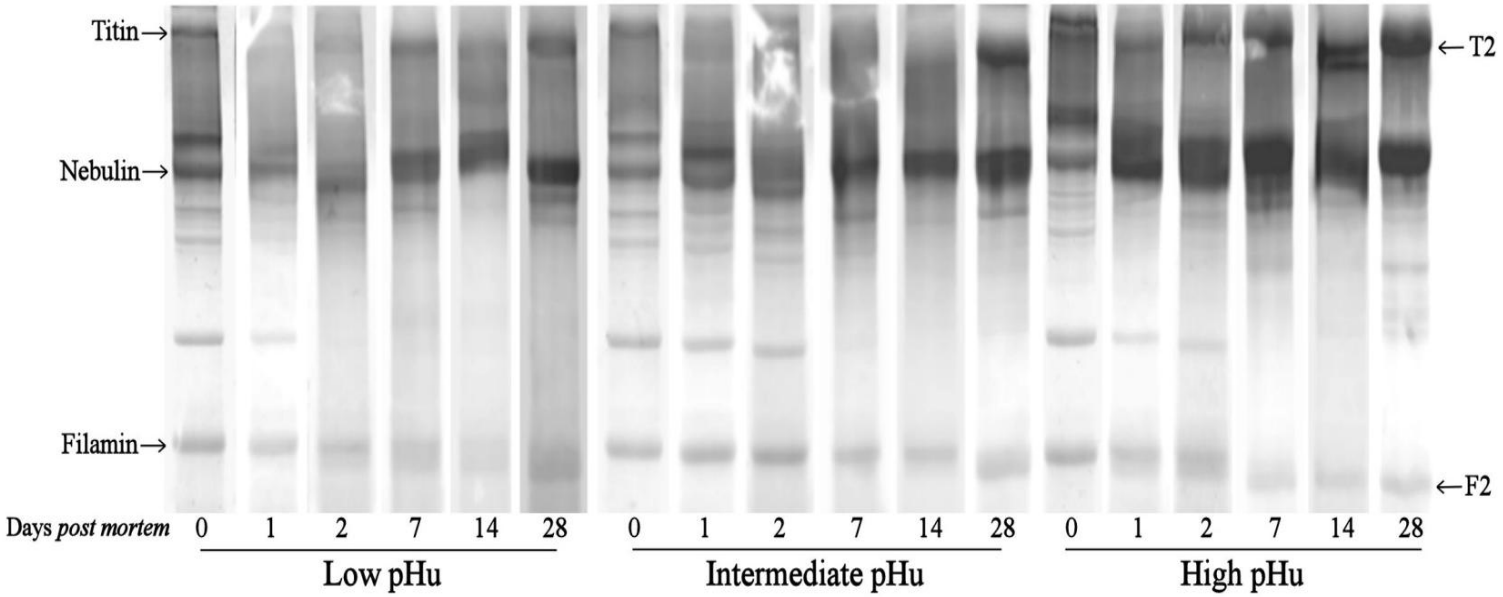

Figure 2: Representative SDS-PAGE gels for low, intermediate and high pH_u meat at each of the allocated ageing timepoints.

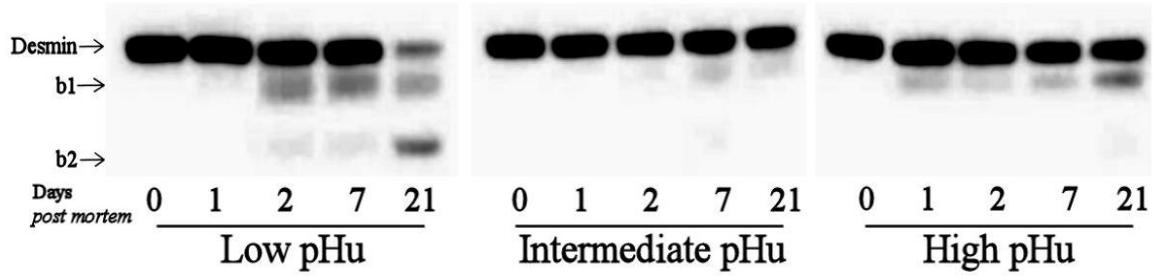

Figure 3: Desmin degradation in representative Western Blots for low, intermediate and high pH_u meat at each of the allocated timepoints.

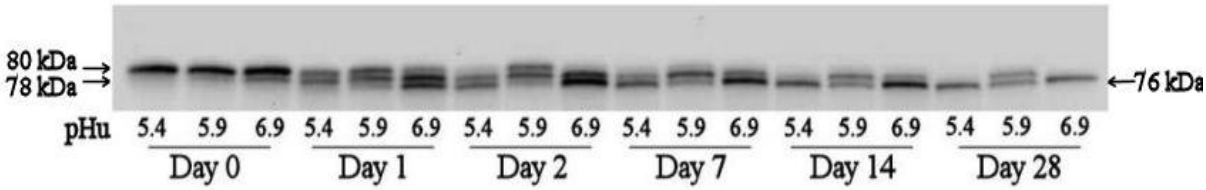

Figure 4: The autolysis of μ-calpain in representative low, intermediate and high pH_u meat. The autolysis of the 80 kDa sub-unit of μ-calpain to 76 kDa through the intermediary 78 kDa sub-unit occurred earlier in high pH_u meat.

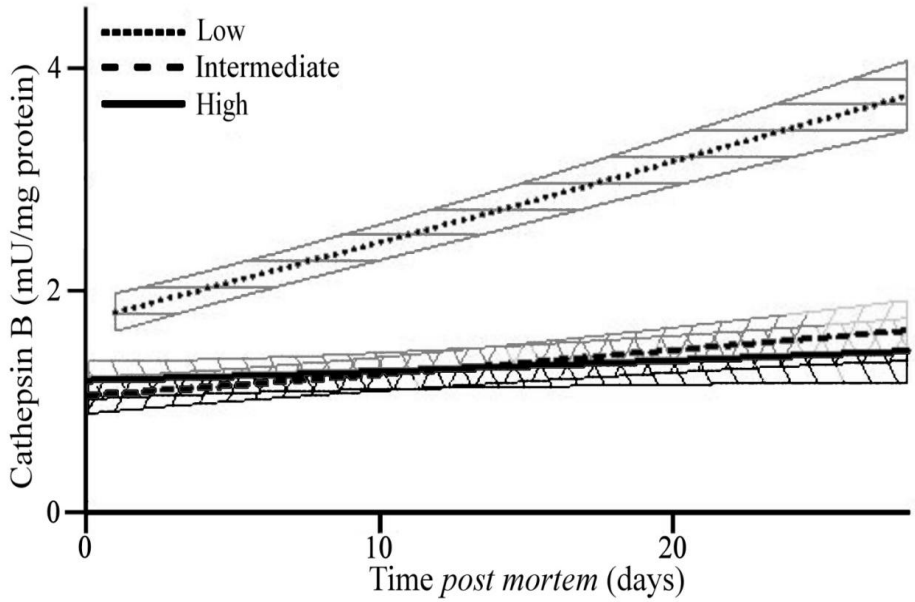

Figure 5: Cathepsin B activity in low, intermediate and high pH_u meat at all ageing timepoints. Splines are least significant intervals at the 95% level.

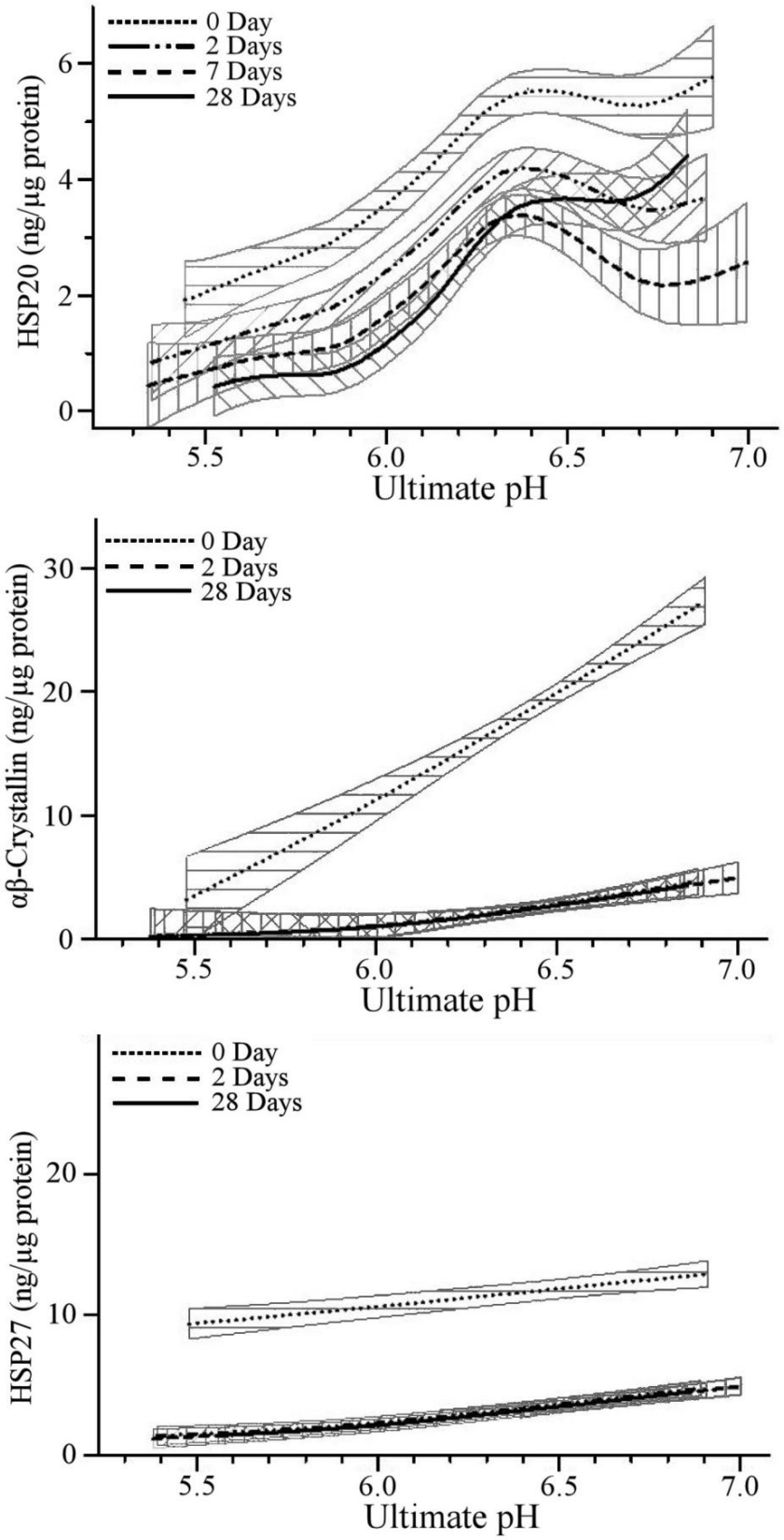

Figure 6: The concentration of sarcoplasmic HSP20, HSP27 and αβ-crystallin in bull *M. longissimus dorsi* during ageing across the pH_u spectrum. Splines are least significant intervals at the 95% level.

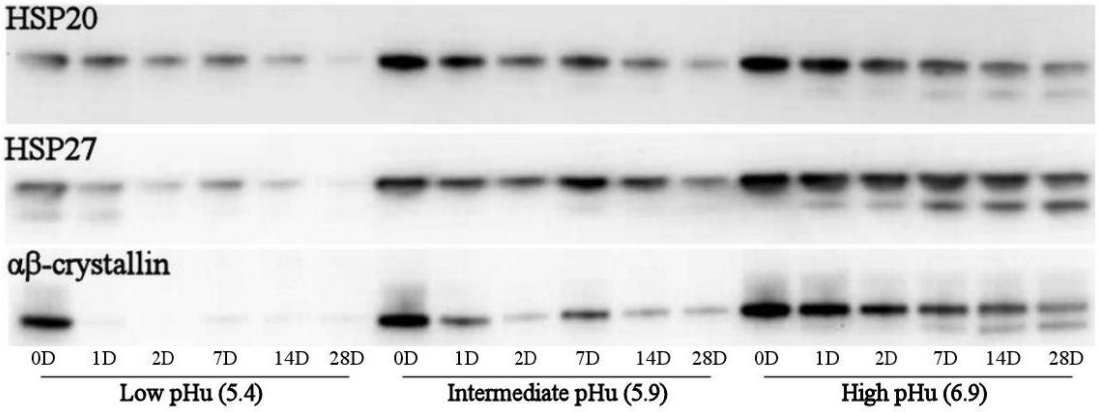

Figure 7: Representative Western Blots of sarcoplasmic HSP20, HSP27 and αβ-crystallin at all ageing timepoints.