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Effects of dietary beta-agonist treatment, Vitamin D_3 supplementation and electrical stimulation of carcasses on meat quality of feedlot steers

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DECLARATION

I declare that this thesis for the PhD (Animal Science) degree at the University of Pretoria has not been submitted by me for a degree at any other university.

Signed……

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ABSTRACT

In this study, 20 young steers received no beta-adrenergic agonist (C), 100 animals all received zilpaterol hydrochloride, with 1 group only receiving zilpaterol (Z) while the other 4 groups received zilpaterol and vitamin D_3 at the following levels and durations before slaughter: 7 million IU Vit D_3/animal/day for 3 days (3D7M); 7 million IU Vit D_3/animal/day for 6 days (6D7M); 7 million IU Vit D_3/animal/day for six days with 7 days no supplementation (6D7M7N) and 1 million IU Vit D_3/animal/day for 9 days (9D1M). Left carcass sides were electrically stimulated (ES) and the right side not electrically stimulated (NES). Samples were aged for 3 or 14 days *post mortem*. Parameters included Warner Bratzler shear force (WBSF), myofibril filament length (MFL), sarcomere length and calpastatin and calpain enzyme activities. For drip loss and instrumental colour measurements, samples were analysed fresh (1 day *post mortem*) or vacuum-aged for 14 days *post mortem*.

Both ES-treatment and prolonged aging reduced WBSF ($P < 0.001$). Treatments 6D7M, 6D7M7N and Z remained significantly tougher than C ($P < 0.001$), while 3D7M and 9D1M improved WBSF under NES conditions. ES was shown to be more effective at alleviating beta-adrenergic agonist induced toughness than high vitamin D_3 supplementation. Aging increased drip loss, lightness, redness and yellowness while ES increased drip loss. In general, Z showed increased drip loss, lighter meat, and reduced redness. Vitamin D_3 supplementation could not consistently overcome the adverse effects of zilpaterol hydrochloride in feedlot steers.

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CHAPTER 1: INTRODUCTION

1.1 PROJECT THEME

Meat Science focusing on meat tenderness.

1.2 PROJECT TITLE

Effects of dietary beta-agonist treatment, Vitamin D_3 supplementation and electrical stimulation of carcasses on meat quality of feedlot steers.

1.3 AIMS

The aims of this project were to:

1) To determine the effect of different levels and the duration of Vitamin D_3 supplementation on the tenderness, colour, drip loss and water holding capacity of beef from feedlot cattle treated with a beta-adrenergic agonist (zilpaterol) and slaughtered under different abattoir practices.
2) To determine the effect of different levels and the duration of Vitamin D_3 supplementation on the calpain proteolytic system of beta-adrenergic agonist (zilpaterol) treated and control feedlot animals.
3) To determine the interaction between the effects of supplemented Vitamin D_3, electrical stimulation and aging on the tenderness, colour, drip loss and water holding capacity of zilpaterol treated and control feedlot animals.

1.4 MOTIVATION

Consumer choice regarding meat quality is dependent on a number of factors, the most crucial being meat tenderness. The 1995 US National Beef Quality Audit indicated that the top two quality concerns in the industry were the low overall uniformity and consistency of beef products and inadequate tenderness (Vargas, Down, Webb, Han, Morgan & Dolezal,

1999). It has therefore become a top priority to solve the problem of inconsistent meat tenderness (Koohmaraie, 1996) in order to satisfy the consumer (Dransfield, 1994). Meat tenderness is a combined function of production, harvesting, post harvest processing, value adding and cooking method used to prepare the meat for consumption by the consumer. Failure of one or more links in the chain increases the risk of a poor eating experience for the consumer (Thompson, 2002).

Many physiological factors affect meat tenderness. These can occur both pre- and post slaughter and need to be taken into account when studying meat tenderness. Most South African feedlots (75% of meat produced in South Africa) supplement with a beta-adrenergic agonist, usually zilpaterol hydrochloride, during the final weeks of finishing. Beta-adrenergic agonists improve feed efficiency and increase carcass meat yield efficiency (Dikeman, 2007). Beta-adrenergic agonists however have a negative effect on meat tenderness by increasing the activity of calpastatin, an inhibitor to the calpains. The degree of these changes depends on the species, type of muscle, the particular beta-adrenergic agonist as well as the time and duration of supplementation (Dransfield, 1994).

Some post-slaughter factors include chilling rate and electrical stimulation and their effect on *post mortem* pH and temperature ratios. The optimum scenario is a pH/temperature relationship of greater than a pH of 6 for muscle temperatures greater than 35°C, and a pH of less than 6 for muscle temperatures less than 12°C. If this optimum relationship is not adhered to then heat and cold shortening can occur, as well as increased autolysis of the calpains and a decrease in meat tenderness (Thompson, 2002). Strydom, Osler, Leeuw & Nel (1999) found that electrical stimulation could reduce aging time to reach a certain level of tenderness. This was due to electrical stimulation causing a lower pH to occur at a relatively high temperature, thereby resulting in an earlier initiation of the aging process (Dransfield, 1994).

Various attempts have been made to overcome meat tenderness problems. In recent years, supplementation of very high levels of vitamin D_3 during the final days of finishing have been investigated (Montgomery et al., 2002 & 2004), the theory being that vitamin D_3 is needed for calcium absorption in the small intestine. Higher levels of vitamin D_3 could therefore eventually lead to higher concentrations of calcium in plasma resulting in more calcium available for the calcium dependant proteinase system. Results however have been inconsistent and no local trials (using beta-adrenergic agonist supplemented animals and electrical stimulation) have been done.

Another critical link in the consumer satisfaction process is the physical appearance of meat cuts during display, with the two most important factors being the colour of red meat as well as the amount of drip loss in packaging. It is well-known that processes like electrical stimulation and *post mortem* aging may affect colour (Ledward, 1985; Ledward, Dickinson, Powell & Shorthose, 1968; Renerre, 1990) and water holding capacity (Kristensen & Purslow, 2001; Den Hertog-Meischke, Smulders, Van Logtestijn & van Knapen, 1997; Devine, 2009) and that these procedures combined with beta-agonists may have additive effects on these parameters (Geesink, Smulders, Van Laack, Van der Kolk, Wensing & Breukink, 1993).

In this study we look at the possibility of high levels of vitamin D_3 supplementation being able to counteract the negative effects of beta-adrenergic agonist supplementation on meat tenderness, as well as the efficacy of this when compared to other cheaper, non-invasive methods such as electrical stimulation. We also investigate the effect that the combination of these factors has on other meat quality traits such as colour and water holding capacity.

1.5 HYPOTHESIS

Ho: Vitamin D_3 supplementation does not significantly influence meat quality of zilpaterol supplemented feedlot steers.

Ha: Vitamin D_3 supplementation does significantly influence meat quality of zilpaterol supplemented feedlot steers.

Meat tenderness focus/ characteristics:

- Warner Bratzler shear force
- Myofibril filament length
- Sarcomere length
- Enzyme activity (µ-calpain, m-calpain, calpastatin)

Colour focus/ characteristics:

- a* (green)
- b* (yellow)
- L* (lightness)
- Chroma
- Hue

Variables in Vitamin D_3 supplementation:

- Treatment (various levels and durations)
- Electrical stimulation
- Aging

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CHAPTER 2: LITERATURE REVIEW

2.1 MEAT TENDERNESS

The concept of meat tenderness is very complex since it is dependent on many physiological factors such as connective tissue characteristics (total collagen and collagen solubility)(Morton, Bickerstaffe, Kent, Dransfield & Keely, 1999; Monin, 1998), the energy status of the muscle, which influences the extent of muscle contraction (studied by measuring pH-temperature decline, glycolysis and sarcomere length) and meat tenderisation by means of the proteolytic degradation of cyto-skeletal proteins (studied by measuring ultimate pH, myofibril fragmentation, proteolytic calpain system levels etc.). These physiological factors are influenced by genetic factors (species and breed), pre-slaughter factors such as age, gender and feeding practices and factors related to processing conditions (electrical stimulation, chilling rate and cooking).

2.1.1 Baseline tenderness

Baseline meat tenderness is that which cannot be changed by any external practices or processes. It is determined by the amount and solubility of connective tissue (Koohmaraie & Geesink, 2006) which consists primarily of perimycium, endomycium and epimycium (Harper, 1999). These connective tissues consist of collagens and these have the ability to form cross links. As an animal matures these cross links become heat-stable. The more heat-stable cross links present in a muscle the tougher the meat will be (Bailey, 1989). In the case of feedlot production, the more common form of production in South Africa, this is not really a factor as animals are slaughtered young before the cross links of collagen can become an issue (for the more tender cuts such as the *M. gluteus medius* and *M. longissimus lumborum*).

2.1.2 Conversion of muscle to meat

When an animal is slaughtered, exsanguination takes place causing a drop in blood pressure. In an attempt to maintain blood pressure, the heart starts to pump faster and peripheral vasoconstriction takes place. This causes a stoppage of nutrient supply and removal of waste products to and from the muscle, stoppage of oxygen supply to the muscle and an increase in temperature of the carcass due to failure of the temperature control mechanism. Stored oxygen is depleted as myoglobin only stores small amounts of oxygen and ATP cannot be formed. This results in the onset of anaerobic glycolysis. There are two main sources of ATP produced from anaerobic glycolysis. The first is stored glycogen which is degraded to create energy for contraction of muscles and results in the production of lactic acid with the release of hydrogen ions, which accumulate in the muscle. The second is the transfer of phosphate from creatine phosphate to ADP to yield creatine and ATP. With no blood flow to remove the lactic acid, it is stored in the muscle resulting in a drop in pH (Pösö & Puolanne, 2005). The ultimate pH of the muscle depends on the amount of glycogen and creatine phosphate reserves in the muscle at the time of exsanguination, the buffering capacity of the muscle as well as extrinsic factors such as environmental temperature and the administration of drugs pre-slaughter (Pösö & Puolanne, 2005; Lawrie, 1985). Once no more ATP can be formed, the actin-myosin complex remains locked in permanent contraction called rigor mortis at a pH of 5.4 – 5.8 (Lawrie, 1985).

2.1.3 The pH temperature relationship

There are a number of factors which can influence the process of the conversion of muscle to meat and can therefore influence tenderness. The pH temperature relationship is one such factor. Normal pH drop should be from 7.0 to 5.6- 5.7 in 6 to 8 hours *post mortem* with an ultimate pH range of 5.3- 5.7 after 24 h. The rate of pH drop *post mortem* is inversely

related to meat tenderness, with a slower fall in pH yielding more tender meat (Hwang & Thompson, 2001b). This is all however also dependent on rate of decline of temperature. Locker and Hagyard (1963) showed that muscle shortening occurs when pre-rigor muscle is held at either low or high temperatures. At low temperatures cold shortening occurs which leads to increased toughness of the meat. In order for cold shortening to occur the muscle pH has to be greater than 6.0 at a temperature below 10ºC and still have ATP available for muscle contraction (Pearson & Young, 1989). Rigor or heat shortening is caused by a combination of a high temperature with a low pH. The low pH is usually due to a rapid pH drop causing early exhaustion of proteolytic activity (Dransfield, 1994; Simmons, Cairney & Daly, 1997). Both cold and heat shortening leads to decreased tenderness and increased drip loss (Thompson, 2002). A good relationship between pH and temperature seems to be a pH of more than 6.0 at temperatures above 35ºC and a pH below 6.0 for temperatures below 12ºC (Thompson,2002).

Electrical stimulation can have an effect on the pH/temperature relationship. Electrical stimulation can prevent cold shortening by causing a faster drop in pH in cases where carcasses are chilled rapidly or hot–deboning occurs (Hwang & Thompson, 2001a). Over stimulation however can lead to heat shortening and increased autolysis of calpains with the consequence of reduced aging potential. In addition increased drip loss could occur due to protein denaturation.

2.1.4 Electrical stimulation

Electrical stimulation is used as a means of accelerating the post- slaughter fall of pH and the onset of rigor. Electrical stimulation involves passing an electric current through the carcass after slaughtering. This stimulates the muscle to contract and utilize glycogen and ATP, thereby accelerating rigor mortis and causing a rapid decline in pH within the muscle (Taylor, 1981; Taylor, Perry & Warkup, 1995; O'Neill, Troy & Mullen, 2004; Strydom, Frylinck

& Smith, 2005). When the electrical current is interrupted, there is still sufficient glycogen and ATP in the muscle to enable the carcass to relax. Due to this low energy reserve, rigor mortis begins earlier while the muscle temperature is still high (Taylor, 1981). As a result, tenderization will start earlier at the prevailing temperature (Dransfield, 1994). When rigor mortis occurs in a relaxed muscle, the sarcomere lengths are not affected, allowing the meat to retain its inherent tenderness (Potter & Hotchkiss, 1995; Kerth, Cain, Jackson, Ramsey & Miller, 1999; Monson, Sanudo, Bianchi, Alberti, Herrera & Arino, 2007). Overstimulation however, can lead to a low pH at a high temperature resulting in heat shortening and therefore a decrease in tenderness. It has been shown that a good pH-temperature relationship seems to be a pH above 6.0 at high temperatures and a pH of below 6.0 at temperatures below 12ºC (Thompson, 2002). This means that under conditions where immediate chilling of the carcass occurs, it would be beneficial to implement electrical stimulation in order to cause a rapid drop in pH, thereby avoiding the potential of cold shortening. Electrical stimulation has also been shown to provide tender meat in half the aging time of non-stimulated meat but only under conditions of slow cooling (Dransfield, Etherington & Taylor, 1992). This corresponds with an experiment conducted by Strydom, Frylinck and Smith (2005), where *M. longissimus lumborum* muscles from electrically stimulated sides were more tender than the non-stimulated muscles at 2 days aging. At 14 days however, there was no significant difference between non-stimulated and stimulated muscles. This result coincided with decreased available µ-calpain activity at 24 h *post mortem*, meaning that initial tenderness was due to increased enzyme activity which was then exhausted.

Strydom, Osler, Leeuw and Nel (1999) found that electrical stimulation could reduce the aging time needed to reach a specific level of tenderness for meat of beta-adrenergic agonist supplemented animals and although it could not improve the tenderness to the same level as the control group, electrical stimulation could significantly reduce the difference between the two after prolonged aging. This could be explained by the ability of electrical

stimulation to advance the onset of rigor releasing calcium ions which activate µ-calpain causing muscle proteolysis and therefore tenderization (Ducasting, Valin, Schollmeyer & Cross, 1985). Likewise it can be attributed to electrical stimulation reducing the level of calpastatin activity leading to a lower inhibitory effect on µ-calpain (Ducasting, Valin, Schollmeyer & Cross, 1985). In agreement, Ferguson, Jiang, Hearnshaw, Rymill and Thompson (2000) obtained results showing that electrical stimulation increased µ-calpain and m-calpain activity as well as decreasing calpastatin activity, all leading to an improvement in tenderness in *Bos indicus* breeds of cattle. This situation could be regarded as being similar to beta-adrenergic agonist supplementation as an increase in *Bos indicus* content in a breed coincides with an increase in calpastatin activity and therefore a decrease in tenderness. Uytterhaegen, Claeys & Demeyer (1992) found no difference in µ-calpain and calpastatin activity at 1 h *post mortem* but did find a significant reduction in activity for stimulated samples of both compounds at 24 h which could confer accelerated aging. Hwang & Thompson (2001b) showed that rapid pH decline alone had no effect on enzyme activity when chilling was rapid, but that when chilling was slow, it caused a decrease in µ-calpain and calpastatin activity due to autolysis.

Hwang, Devine & Hopkins (2003) postulated that there were a number of possible explanations why stimulation would increase the activity of enzymes like the calpains. One is that the calpain/calpastatin ratios are affected by some intrinsic effect associated with the rapid pH decline that results in a low pH at increased temperatures, and a second could be due to a significant increase in the levels of ‘free’ calcium, leading to activation of the calpains, particularly µ-calpain. Dransfield (1994) predicted that calpain activity would be increased by a factor of six in rapidly glycolysing muscle compared to muscle with more normal rates of glycolysis. µ-Calpain however is likely to undergo autolysis under these conditions making the interplay with temperature and the levels of free calcium important (Hwang, Devine & Hopkins, 2003). Hwang, Devine and Hopkins (2003) also speculated that electrical stimulation may also protect those muscle fibres that enter rigor soon after

stimulation and therefore avoid prolonged pre-rigor exposure to high temperatures at a low pH, maintaining optimum calpain levels. Electrical stimulation also accelerates pH decline which is mirrored by an increase in 'free' calcium, suggesting that at the same temperature, stimulated muscle will be exposed to higher levels of 'free' calcium and this could lead to increased proteolysis (Hwang, Devine & Hopkins, 2003).

In general electrical stimulation has its advantages, in that it can counteract cold shortening where carcasses are chilled quickly. It can also result in tender meat at an early stage without the prolonged aging (Strydom, Frylinck & Smith, 2005). Electrical stimulation does not however improve inherently tender meat beyond baseline tenderness (Hwang, Devine & Hopkins, 2003).

2.1.5 Calcium dependant proteinases

Tenderisation during the storage of meat occurs by proteolysis of myofibrillar and cytoskeletal proteins (Dransfield, Etherington & Taylor, 1992). The calpains (calcium-activated neutral proteinases) degrade myofibrillar and cytoskeletal proteins while lysosomal acidic proteinases (cathepsins B, D, and L) also hydrolyse myofibrils and isolated proteins (Ouali, Garrel, Obled, Deval, Valin & Penny, 1987). Calpains appear to have primary involvement at a pH of more than 6 while the activity of cathepsins seems more important at pH's lower than this. In our study we focus on the calpains and their inhibitor calpastatin.

There are two isoforms of the large subunit of calpain, namely µ–calpain and m-calpain. Both are calcium dependant. The two subunits differ in the concentration of calcium required to induce their activity, with m-calpain requiring calcium concentrations in the millimolar range, and µ–calpain in the micromolar range (Geesink & Koohmaraie, 1999).

Calpain is a protease that is abundant in the cytoplasm of the cell, and can cleave many structural proteins. Calpain is tightly regulated by many mechanisms including calcium requirements and calpastatin. Calpastatin is a polypeptide that is specific for inhibiting the proteolytic activity of the calpains and does not inhibit any other proteolytic enzyme (Goll, Thompson, Taylor, Edmunds & Cong, 1995a).

The optimum pH for calpain activity is between 7.0 and 7.5 (Ouali, 1992), but is 20-25% active at the normal end-pH-value of *post mortem* muscle, around pH 5.5 (Geesink, Smulders, Van Laack, Van der Kolk, Wensing & Breukink, 1993). Calpains do not cause bulk degradation of the sarcoplasmic proteins, but they do however specifically degrade those structures and proteins that are responsible for maintaining the assembled myofibrillar proteins in the myofibril structure. The calpains can remove Z-disks (necessary to keep adjacent sarcomeres together) and degrade titin, nebulin (probably function as a scaffold that strengthens the myofibrillar structure) tropomysin, troponin and c-protein (Zeece, Robson, Lusby & Parrish, 1986a). Specific degradation of these structures would result in the release of thin and thick filaments from the surface of the myofibril.

Calpain activity is regulated by calcium concentrations as well as calpastatins (in the living animal there is not enough calcium in cells to activate the calpain system). It appears however, that active calpains generate only a limited amount of cleavages, but this limited proteolysis may nevertheless initiate myofibrillar protein breakdown (Béchet, 1995).

As well as being regulated by calcium concentration and calpastatins, the calpain system is also dependant on pH and temperature. At higher temperatures calpains are inactive, but activity increases with a drop in temperature, but below 10ºC inactivity increases with a drop in pH (Dransfield, 1994).The activity of µ–calpain decreases very quickly *post mortem*, while the activity of the m-calpain decreases very slowly during the aging period (Ducastaing, Valin, Schollmeyer & Cross, 1985; Koohmaraie, Seideman,

Schollmeyer, Dutson & Crouse, 1987). Active m-calpain can also degrade calpastatin, resulting in a decrease of calpastatin activity (Melloni, Salamino & Sparatore, 1992). The higher the activity of calpains, the more autolysis occurs and the more tender the meat becomes (Steen, Claeys, Uytterhaegen, De Smet & Demeyer, 1997).

2.1.5.1 Prolonged aging

Aging refers to the improvement in palatability that occurs as meat is held *post mortem* beyond the normal time taken for setting and cooling to enhance tenderness (Moran & Smith, 1929). Aging can therefore be seen as the later part of tenderization and can be measured. The extent of aging is largely related to the level of calpains at 24 h *post mortem* and varies according the initial levels and their inactivation during the development of rigor (Dransfield, 1992).

Temperature also plays an important role in governing aging, as once rigor is complete, time and temperature are the only variables which can be controlled (Dransfield, 1992). Freezing stops calpain activity but does not destroy the enzymes. This means that while the meat is frozen, enzyme activity remains halted, but is regained after thawing (Dransfield, 1992). Freezing doubles the rate of aging after thawing, when compared to aging of fresh samples, and this increased rate is probably due to cellular damage.

Aging has been proven to significantly reduce shear force values in *M. longissimus lumborum* muscles (Wulf, Tatum, Green, Morgan, Golden & Smith, 1996). Geesink, Koolmees, Smulders and Van Laack (1995) also found that shear force was reduced after 14 days of aging and Mitchell, Giles, Rogers, Tan, Naidoo and Ferguson (1991) found that aging significantly increased sensory tenderness up to 10 days, but with no further improvement at 21 days aging.

Rathmann et al. (2009), Hilton et al. (2009) and Kellermeier et al. (2009) all found that prolonged aging did improve WBSF in zilpaterol supplemented animals. In all three experiments however, the control groups were still more tender than the zilpaterol supplemented groups even after 21 days of aging. This is mainly attributed to the increase in calpastatin activity caused by beta-adrenergic agonists which retards *post mortem* aging (Geesink, Smulders, Van Laack, Van der Kolk, Wensing & Breukink, 1993).

2.2 BETA-ADRENERGIC AGONISTS

2.2.1 Mode of action of beta-adrenergic agonists

Growth rate and feed efficiency are both important traits in livestock production, and because consumers demanded leaner meat, more emphasis has been placed on carcass composition with less fat and more muscle (Monson, Sanudo, Bianchi, Alberti, Herrera & Arino, 2007). The introduction of beta-adrenergic agonists (hereafter referred to as beta-agonists) represents the latest use of pharmacologically active compounds which have opened up new prospects for improving efficiency and quality of meat products (Dransfield, 1992). The beta-agonist is added to feed for the purpose of promoting protein synthesis in muscle tissues and lipolysis in adipose tissue, resulting in a reduction of carcass fat and an increase of muscle mass of the carcass (Baker, Dalrymple, Ingle & Ricks, 1984). Beta-agonists achieve this by binding to certain beta-receptors on fat and muscle cell surfaces, thereby modifying the biochemical processes of tissue growth by increasing lipolysis, decreasing lypogenesis (Dunshea, 1993; Liu & Mills, 1989; Mersmann, 1998), decreasing protein degradation (Koohmaraie & Shakelford, 1991; Wheeler & Koohmaraie, 1992) and increasing protein synthesis (Eisemann, Huntington & Ferrell, 1988; Strydom, Frylinck, Montgomery & Smith, 2009). Beta-agonists significantly influence growth by improving lean content, reducing carcass fat and overall by having a positive effect on growth rate without there being a change in feed intake (Casey, 1998a).

Beta-agonists have the properties of a neurotransmitter and of a hormone. As a neurotransmitter, beta-agonists are closely related to norepinephrine. Norepinephrine is a naturally occurring catecholamine produced by tyrosine, and together with epinephrine, are the two neurotransmitters of the sympathetic nervous system. Beta-agonists are also related in their physiological effects to epinephrine and norepinephrine (and are accordingly analogues of these hormones) in that they stimulate glycogenolysis and lipoloysis (Casey, 1998b). Beta-agonists achieve this by binding to beta-adrenergic receptors (beta-AR). These receptors are similar to those that are responsive to epinephrine and norepinephrine. There are three subtypes of the receptors namely, $beta_1$-AR, $beta_2$-AR and $beta_3$-AR. All three receptors are present on most cells, but the distribution of subtypes and proportion of each varies between tissues in a given species. The beta-AR subtype distribution also varies within a given tissue between species. The pharmacological and physiological responses of an individual cell results from the particular mixture of the three beta-AR subtypes present on that cell. Amino acid sequence also causes modification of a given beta-AR subtype. The beta-AR subtype population may change with the stage of differentiation of a cell, but there tends to be more of a particular kind of beta-AR subtype in a particular kind of cell (Mersmann, 1998). In cattle, competitive ligand binding studies suggest that there are predominantly $beta_2$-AR on skeletal muscles cells and adipocytes (Sillence & Matthews, 1994). These factors together with the use of several different agonists make the mechanisms to produce the pharmacological effects observed with oral administration of a beta-agonist complex (Mersmann, 1998).

Beta-agonists have an affinity for either $beta_1$-AR or $beta_2$-AR receptors. Their efficacies are determined by their chemical structure, the number of receptors which need to be stimulated for an effect to occur as well as on the physiological effect of stimulating the respective beta-AR. A beta-agonist with a high efficacy would achieve a high response from a relatively small number of receptors, the situation however, can also be complicated if both types of receptors are present on an organ and both could be mediating a pharmacological

effect. Desensitising of beta-AR can also occur and the rate of this is determined by the intrinsic activity of the particular type of beta-agonist (Casey, 1998b).

The mechanism of action of beta-agonists is that they bind to the receptors in such a way that the agonist receptor complex activates the G_s protein (some compounds are antagonists and therefore bind to the receptor but do not activate the G_s protein and thus block the receptor function). The α-subunit of the G_s protein then activates adenylyl cyclase which is the enzyme that produces cyclic adenosine monophosphate (cAMP). cAMP is one of the major intracellular signalling molecules. cAMP then binds to the regulatory subunit of protein kinase A to release the catalytic subunit that then phosphorylates a number of intracellular proteins. Phosphorylation activates these proteins, some of which are enzymes such as hormone sensitive lipase (the rate-limiting enzyme for adipocyte triacylglycerol degradation). The cAMP response element binding protein (CREB) is phosphorylated by protein kinase A. The CREB binds to a cAMP response element in the regulatory part of a gene and then stimulates the transcription of that gene. Phosphorylation increases the transcriptional activity of the CREB, providing the mechanism for beta-agonist mediated transcription of a number of genes in the cell (Mersmann, 1998). Phosphorylation inactivates other enzymes such as acetyl-CoA carboxylase which is the rate-limiting enzyme for long-chain fatty acid biosynthesis (Mersmann, 1989a; Liggett & Raymond, 1993).

With beta-agonists mimicking the hormones of the sympathetic nervous system it is obvious that they would have an effect on the systems major activities namely, cardiac function, blood vessel tone, gut and bronchiole muscles as well as the metabolic systems already discussed (lipolysis and glycogenolysis). All organs have both $beta_1$-AR and $beta_2$-AR but $beta_1$-AR are more predominant in the heart and $beta_2$-AR more predominant in the other organs. Beta-agonists have the following effects on the cardiac system, namely, positive inotropy (increased contractility), positive chronotropy (increased heart rate) and positive dromotropy (increased conduction velocity). Beta-agonists also cause relaxation of

smooth muscles (vasodilation) and bronchial muscles (broncholdilation) as well as release of rennin from the kidneys, insulin from the pancreas and hepatic glycogenolysis (Morris, 1997).

The zilpaterol hydrochloride molecule is a physiologically highly active beta-adrenoreceptor agonist which acts on beta$_2$-AR on skeletal muscle, smooth muscle and adipose tissue and is intended for use in beef cattle as a repartitioning agent. The molecular structure of zilpaterol hydrochloride is (±)-trans-4,5,6,7-tetrahydro-7hydroxy-6-(isopropylamino) imidazo[4,5,1-jk]-[one] benzazepin-2(1H)-one hydrochloride and its formula is $C_{14}H_{19}N_3O_2$.HCL. Tests for interactions with various pharmacological agents indicate zilpaterol hydrochloride to be non-interactive, with the possibility of even complimenting selected pharmacological agents (Casey, 1998a). Casey, Montgomery and Scheltens (1997) showed that treatment with zilpaterol hydrochloride (0.2 mg/kg) in combination with an anabolic implant (24mg oestradiol + 120mg trenbolone acetate) proved to be agonistic, improving the biological efficiency of production but without the fat reducing properties of zilpaterol hydrochloride being affected. A unique characteristic of zilpaterol is that unlike other beta-agonists which are lipophillic, zilpaterol hydrochloride is not (Casey, 1998b).

2.2.2 Effects of beta-adrenergic agonists on skeletal muscle and adipose tissue

Treatment of mammals with a beta-agonist causes an increase in the amount of RNA transcript for many skeletal muscle proteins. The result being that the mRNA for myosin light chain (Smith, Garcia & Anderson, 1989), α-actin (Koohmaraie, Shackelford, Muggli-Cockett & Stone, 1991) and calpastatin (Killefer & Koohmaraie, 1994) are increased after beta-agonist treatment with the most obvious effect being an increase in muscle mass. The other obvious effect of dietary beta-agonist supplementation is a decrease in carcass fat due to the beta-agonist stimulating adipocyte triacylglycerol degradation and inhibiting fatty acid and triacylglycerol synthesis (Mersmann, 1998).

Maritz (1996) found that zilpaterol hydrochloride had a significant effect ($P < 0.05$) on growth performance with there being an increase in both average daily gain and feed efficiency. The most prominent improvement occurred during the first few weeks of treatment. Zilpaterol hydrochloride also significantly ($P < 0.05$) reduced the proportion of carcass fat (subcutaneous, intramuscular and total dissectible fat), with this shift in carcass composition giving rise to a corresponding increase in the muscle-to-bone and muscle-to-fat ratio. This was in agreement with Morris (1997) whose steers receiving zilpaterol hydrochloride during the growth phase had significantly increased ($P < 0.01$) mean daily body weight gain and were therefore significantly heavier ($P < 0.01$) and had better feed conversions ratios ($P < 0.001$). Steers receiving an additional low dose of zilpaterol hydrochloride, during a phase where other treatment groups were in a withdrawal period, showed higher protein and lower fat content in rib analysis samples compared to the groups that were no longer being supplemented. O'Neill (2001) also found zilpaterol hydrochloride improved both average daily gain and feed efficiency although the improvement was not significant. This trial however showed no changes for percentage lean or subcutaneous fat thickness in carcasses.

Results obtained by Webb and Casey (1994) suggest that a beta-agonist may influence the proportions of fatty acids synthesised in both the subcutaneous adipose tissue and *M. longissimus lumborum* of feedlot steers, with the beta-agonist resulting in a shift towards the deposition of saturated fatty acids in the *M. longissimus lumborum*. This shift is presumed to be related to the increased rate of lipolysis resulting in a subsequent release of free fatty acids in the subcutaneous fat and muscle. In the same trial, Webb (1994) found that the lipolytic effects of zilpaterol hydrochloride may elicit insulin secretion but also blunt insulin sensitivity up to 12 h post treatment with these changes ultimately influencing the synthesis or deposition of fatty acids in ruminants.

Beta-agonists however, tend to have a negative effect on tenderness and animals supplemented with beta-agonists seem to produce tougher meat. It has been found that a potential cause for the decrease in tenderness of beta-agonist supplemented meat is due to the effect it has on the activity levels of calpains and their inhibitor, calpastatin. Kretchmar, Hathaway, Epley and Dayton (1990) reported that in lambs there was a 15% decrease in µ-calpain activity in animals fed beta-agonists compared to the control group. Not only do beta-agonists decrease µ- calpain activity, they can also increase the level of calpastatin by up to 150% (Koohmaraie & Shakelford, 1991) as well as increasing the level of m- calpains which is the less active calpain out of the two (Dransfield, 1992). This is in agreement with Strydom, Frylinck, Montgomery and Smith (2009) who found that calpastatin activity was 2.4 and 3.2 units lower on *M. longissimus* muscles from the control group compared to the two beta-agonist groups (zilpaterol and ractopamine) and with Geesink, Smulders, Van Laack, Van der Kolk, Wensing and Breukink (1993) who also found a significant increase in calpastatin in clenbuterol supplemented animals. In both cases however, there was no difference between any of the groups for µ- and m-calpain activity.

Strydom, Frylinck, Montgomery and Smith (2009) found that beta-agonists increased the WBSF values of both the *M. longissimus* and semitendinosus muscles compared to a control group at 2, 7 and 14 days aging. Schroeder, Polser, Laudert and Vogel (2003a), reported a significant negative effect on shear force tenderness for ractopamine supplemented animals, while more recent studies by Rathmann et al. (2009), Hilton et al. (2009) and Kellermeier et al. (2009) found that zilpaterol increased WBSF at 7, 14 and 21 days aging. Monson, Sanudo, Bianchi, Alberti, Herrera and Arino (2007) found that beta-agonists only caused a small increase in shear force values. These animals were however also supplemented with dexamethasone which could have caused an increase in soluble collagen. O'Neill, Casey and Webb (2010) concluded however that with the implementation of electrical stimulation coupled with a 10 day aging period, that zilpaterol hydrochloride could be supplemented for 35 days without any detrimental effect on meat quality.

2.3 VITAMIN D_3

2.3.1 Function of Vitamin D_3

Vitamin D_3 is a fat soluble vitamin usually stored in the liver. Dietary vitamin D_3 is absorbed through the small intestine and transported in the blood to the liver, where it is converted into 25-hydroxycholecalciferol. 25-hydroxycholecalciferol is then transported to the kidney where it is converted into 1.25-dihydroxycholecalciferol, which is the most biologically active form of the vitamin (McDonald, Edwards, Greenhalgh & Morgan, 1995). From there the compound is transported in the blood to the various target tissues of the body. One of the most important functions of the compound 1.25-dihydroxycholecalciferol is the absorption of calcium from the intestinal lumen (McDonald, Edwards, Greenhalgh & Morgan, 1995).

The need for supplementing the diets of cattle with vitamin D_3 is generally not large, as adult ruminants can receive adequate amounts of the vitamin from irradiation (McDonald, Edwards, Greenhalgh & Morgan, 1995). The act of supplementing vitamin D_3 is therefore an attempt to increase the levels of calcium absorbed from the intestine and thereby increase calcium levels in the blood and possibly the muscle at slaughter.

2.3.2 Homeostasis of Vitamin D_3

The amount of 1.25-dihydroxycholecalciferol that the kidney produces is controlled by the parathyroid hormone. When the level of calcium in the blood is low, the parathyroid gland is stimulated to secrete more parathyroid hormone. Parathyroid hormone induces the kidney to produce more 1.25-dihydroxycholecalciferol which in turn enhances the intestinal absorption of calcium and phosphorus (since calcium is combined with phosphorus in the bone), as well as enhancing calcium and phosphorus resorption from the kidney and the bone (McDonald, Edwards, Greenhalgh & Morgan, 1995). However, when blood calcium

content increases, the hormone calcitonin is released from the thyroid gland. Once released into the blood, calcitonin has the opposite effect to that of parathyroid hormone and inhibits the resorption of bone and decreases the release of calcium from bone to the blood. High levels of calcium, as well as high levels of 1.25-dihydroxycholecalciferol, in the blood also inhibit the production of parathyroid hormone. Therefore the control system that keeps the blood's calcium supply at a stable level consists of two feedback loops. These two loops are parathyroid hormone operating to sustain the supply of calcium, and calcitonin operating to prevent calcium from rising above the desired level in the blood (Frandson & Spurgeon, 1992).

2.3.3 Hypervitaminosis D_3

The pathophysiology of vitamin D_3 toxicity is due partially to the severe hypercalcemia that is the result of the exaggerated response the body has to the vitamin. The symptoms of hypercalcemia are renal calculi (calcium phosphorus salts which form in the renal tubules eventually leading to kidney failure), joint and skeletal pain, weakness, decrease in feed intake (leading to anorexia), vomiting and polyuria (increased urine output). Toxicity can also lead to salt depositions in other soft tissues such as various organs as well as the inner walls of large blood vessels. In acute cases of vitamin D_3 toxicity death of bone cells can occur. Vitamin D_3 toxicity is a very serious disease and is difficult to treat as many of the pathological changes it causes are either difficult or impossible to reverse. Initial treatment is to alleviate the hypercalcemia to relieve clinical signs (Dukes, 1993).

2.3.4 Vitamin D_3 supplementation of feedlot cattle

A survey in Australia found that 77% of consumers would buy more beef if they knew it was always going to be more tender than previously purchased beef (Lawrence et al., 2006), whilst it was found in the USA that the top three quality concerns of consumers

included low overall consistency of beef products, inadequate tenderness and overall palatability (Vargas, Down, Webb, Han, Morgan & Dolezal, 1999). Post-mortem aging of carcasses at 0-2ºC for 7-21 days has been proven to increase tenderness in beef with proteolysis of key myofibrillar proteins by the calpains (especially µ–calpain) being implicated as the major cause of this process (Veiseth, Shackelford, Wheeler & Koohmaraie, 2001). Research has focused on increasing intracellular stores of calcium, thereby activating both µ–calpain and m-calpain to increase *post mortem* rates of proteolytic activity (Lawrence et al., 2006). Although the results vary as to the efficiency of dietary vitamin D_3 and beef tenderness, vitamin D_3 is in general a nutritional means of elevating muscle calcium concentration, with the ability to enhance the calcium dependant myofibrillar protein degradation *post mortem* to improve tenderness (Koohmaraie, 1996; Koohmaraie & Shakelford, 1991).So a potential means of improving tenderness in beef is to add supplemental vitamin D_3 to the diet shortly before cattle are slaughtered.

In a trial conducted by Karges et al. (2001), beef steers received supplemental vitamin D_3 of 6 x 10^6 IU for four or six days. Steaks were aged at 2 ºC for 7, 14 or 21 days. Feeding vitamin D_3 to feedlot steers for six days decreased (P = 0.04) WBSF values of *M. longissimus lumborum* steaks compared to control steers or steers fed vitamin D_3 for four days. Blood plasma calcium concentrations were significantly greater (P < 0.03) for all animals supplemented with vitamin D_3, and even more so for those supplemented for a longer period of time, compared to non-supplemented animals. Swanek et al. (1999) supplemented 7.5 x 10^6 IU vitamin D_3 for 10 days resulting in a significant (P < 0.05) increase in both plasma and muscle calcium concentrations. There was also a significant improvement in WBSF at 7 (P = 0.02) and 14 (P = 0.07) days aging. This is in agreement with previous findings of Karges, Morgan, Owens and Gill (1999) and Montgomery, Parrish, Beitz, Horst, Huff-Lonergan and Trenkle (2000). Tipton, King, Paschal, Hale and Savall (2007) supplemented 3 x 10^6 IU vitamin D_3 for 5 days immediately before slaughter and then a second group of 3 x 10^6 IU vitamin D_3 for 5 days followed by a 7 day withdrawal period

before slaughter. Serum calcium levels increased after supplement removal but not immediately following supplementation. There was no improvement in tenderness for the first group but tenderness did improve at day 7 of the withdrawal period. It was concluded that a withdrawal period made vitamin D_3 supplementation more effective as well as safer, as increased levels of vitamin D_3 that occurred during supplementation were back to normal levels at day 7 of the withdrawal period.

Montgomery, Parrish, Beitz, Horst, Huff-Lonergan and Trenkle (2000) found that all steaks from steers orally administered vitamin D_3 (5×10^6 IU or 7.5×10^6 IU for 9 days and slaughtered 1d later) had numerically lower WBSF values than control steaks at 3, 7 and 21 days aging. Oral supplementation of vitamin D_3 did however cause a significant difference ($P < 0.05$) in shear force in steaks aged for 14 days with shear force values being lower by about 0.5kg for steaks from supplemented steers compared to control steers. It was also found that the treatment groups had increased levels of vitamin D_3 in the muscle by approximately twenty four fold, and the levels were even higher in the liver and kidneys. Vargas, Down, Webb, Han, Morgan and Dolezal (1999) found similar results with steaks from control animals being tougher ($P < 0.05$) than steaks from treated groups (6×10^6 IU for 6.5 days prior to slaughter) up to 7 days *post mortem* storage. Shear force did not differ for steaks aged for more extended time periods, however, the steaks from supplemented animals did require fewer aging days to become more tender, which indicates that vitamin D_3 supplementation can be used to accelerate the aging process and improve the tenderness of beef products. Montgomery et al. (2002) achieved similar results when supplementing beef steers with various levels of vitamin D_3. It was found that plasma calcium increased linearly with vitamin D_3 treatment ($P < 0.01$) with there being a significant increase in muscle calcium ($P < 0.05$) as well. Calpastatin and calpain activity were however not influenced by treatment ($P < 0.05$) but there were differences in tenderness. Vitamin D_3 treatments of 0.5, 1.5 and 7.5×10^6 IU/d reduced strip loin steak WBSF values at 7 days aging but WBSF values did not decrease at any other time *post mortem*. Montgomery et al. (2004) also found that giving

beef steers vitamin D_3 supplementation increased total cytosolic calcium, phosphorus and magnesium concentrations in meat. Free cytosolic calcium could stimulate calcium-activated calpains and could be responsible for muscle structural alterations. It however remains unclear whether the activation of the calpain system and increased proteolysis are a result of increased cytosolic calcium or from *post mortem* changes in cytosolic calcium (Montgomery et al., 2004)

It must also be mentioned that in many of these experiments (Vargas, Down, Webb, Han, Morgan and Dolezal, 1999; Montgomery et al., 2004; Karges et al., 2001; Lawrence et al., 2006) it has been shown that steers receiving vitamin D_3 supplementation show a decrease in feed intake which can lead to a decrease in average daily gain. Factors such as this, as well as increased levels of vitamin D_3 in the muscle, liver and kidneys have to be taken into consideration regarding vitamin D_3 toxicity, although vitamin D_3 levels tend to drop in meat during the cooking process.

There have however also been many studies showing that supplementing vitamin D_3 has no effect on meat tenderness. In a study conducted by Foote, Horst, Huff-Lonergan, Trenkle, Parrish and Beitz (2004), results indicated that feeding supplemental 1.25 - $(OH)_2D_3$ and 25 – OHD_3 increased plasma calcium concentrations significantly ($P < 0.05$). All levels of treatment lead to an increase in plasma calcium concentration, with the highest concentrations of vitamin D_3 leading to the highest concentrations of calcium. However, even with elevated levels of plasma calcium concentration, total calcium concentration in the muscle was not affected ($P > 0.10$). Supplementation did however cause an increase in concentration of vitamin D_3 in the blood, liver, kidneys and muscles. There was also a trend for vitamin D_3 to decrease ($P < 0.01$) shear force values of *M. longissimus lumborum* steaks aged for 14 days, compared with those of controls aged for 14 days, but with further aging the control steaks became more tender. This interestingly showed that vitamin D_3 had the potential to improve tenderness at a faster aging rate but only until a point after which aging

alone is enough to produce the desired effect. These results were in agreement with an experiment conducted by Rider Sell, Mikel, Xiong and Behrends (2004) who found that vitamin D_3 supplementation did not statistically increase muscle calcium concentrations, but did show a tendency (P = 0.14) to increase numerically with increasing dietary vitamin D_3. As for WBSF values, supplementation had no effect on un-aged steaks, but did have lower values at 7 days of aging. However, at 14 days WBSF values were actually higher than for control steaks. Results from this study therefore indicate that vitamin D_3 supplementation provided little benefit to muscle tenderness (Rider Sell, Mikel, Xiong and Behrends, 2004). These animals were however cull cows and were therefore older and more likely to produce tough carcasses. Lawrence et al. (2006) showed that supplementation had no significant effect on pH, sarcomere length, muscle colour or cooking loss. There was also no increase in calcium and vitamin concentrations in the muscle or blood plasma. Supplementation also had no effect on WBSF values with there being no difference between treated and control groups after aging for 1, 7 and 14 days.

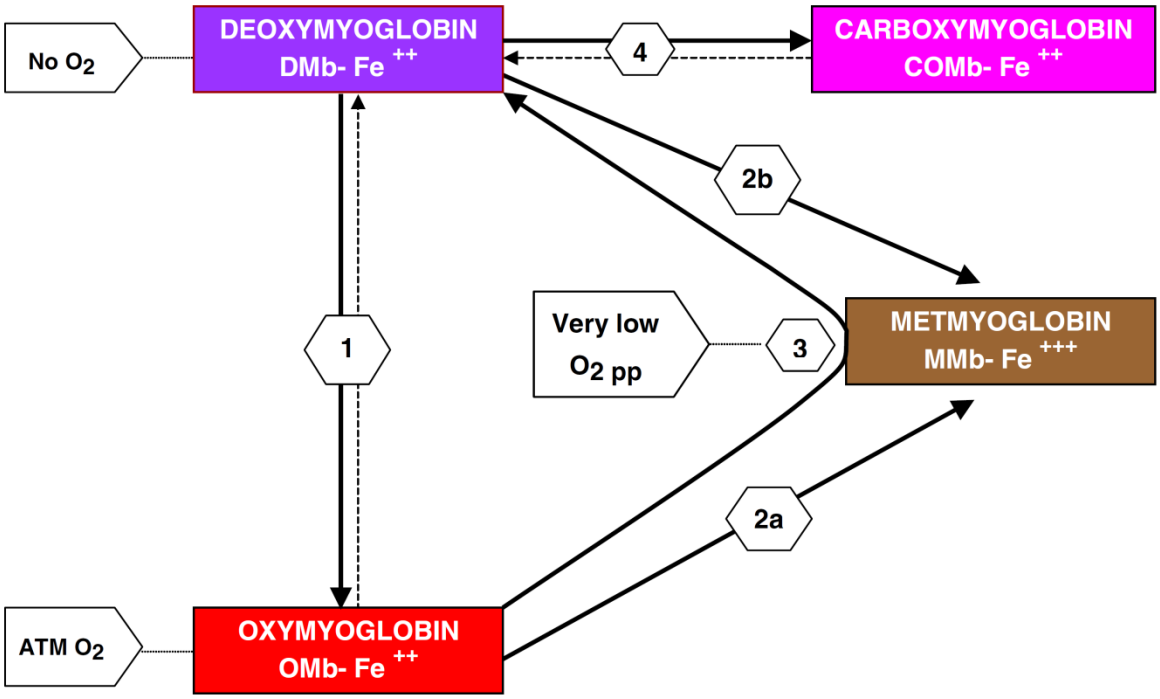
2.4 OTHER FACTORS AFFECTING MEAT QUALITY

There are many factors affecting meat quality. The most important quality attributes of beef include the tenderness, taste, juiciness (drip loss and water holding capacity), freshness, colour, lean content (and fatty acid composition), healthiness, nutrient content, safety and convenience (Webb, 2003).

2.4.1 Colour

The colour of meat is mainly determined by the amount of myoglobin in the muscle as well as the amount of oxygen available for it to react with. The amount of myoglobin in a muscle depends on many factors, namely species, breed, sex (more myoglobin in steers

and bulls than cows), age (more myoglobin in older muscles), and the type of muscle (more myoglobin in muscles that work more). The ligand present and the valence of iron present dictate muscle colour. There are three forms of myoglobin which can occur, namely, oymyoglobin, deoxymyoglobin and metmyoglobin formed by oxygenation, reduction and oxidation reactions respectively. Oxygenation occurs when myoglobin is exposed to oxygen forming oxymyoglobin. The formation of oxymyoglobin gives meat its bright cherry red colour. This is the colour that consumers associate with fresh meat. Oymyoglobin penetrates deeper into the meat's surface with increased exposure to oxygen (Mancini & Hunt, 2005). Oxygen consumption rate is associated with residual mitochondrial respiration in *post mortem* muscle and is related to the depth of oxygen penetration into the exposed surface of the muscle. Lower oxygen consumption rate allows for greater penetration of oxygen into the muscle and is associated with more colour stable muscles (McKenna, Mies, Baird, Pheiffer, Ellebracht & Saval, 2005) and oxygen diffusion into meat is also greater at lower temperatures (MacDougal, 1977). Deoxymyoglobin gives meat a purplish-red/grey colour. Very low oxygen tension is required to maintain myoglobin in a deoxygenated state, such as in vacuum packaged meat or meat just after cutting. When oxygen partial pressure is low, or there is oxygen consumption, metmyoglobin is formed giving the meat a brown colour. Discolouration results from oxidation of both ferrous myoglobin derivatives to ferric iron (Fe^{2+} → Fe^{3+}) and is defined as the amount of surface area covered by metmyoglobin (Fig. 1). Metmyoglobin beneath the surface of the meat, located between superficial oxymyoglobin and interior deoxymyoglobin, can gradually thicken and move towards the surface (Mancini & Hunt, 2005). Colour in meat can be measured as L* (lightness), a* (redness), b* (yellowness) and chroma (saturation index).



Rx 1 (Oxygenation): DMb + $O_2 \rightarrow$ OMb

Rx 2a (Oxidation): OMb + [oxygen consumption or low O_2 partial pressure] – $e^- \rightarrow$ MMb

Rx 2b (Oxidation): [DMb – hydroxyl ion – Hydrogen ion complex]+ $O_2 \rightarrow$ MMb + O_2^-

Rx 3 (Reduction): MMb + Oxygen consumption + metmyoglobin reducing activty $\rightarrow$ DMb

Rx 4 (CarboxyMb): DMb + carbon monoxide $\rightarrow$ COMb

Fig. 1. Visible myoglobin redox interconversions on the surface of meat (Mancini & Hunt, 2005).

Another factor which has a great impact on the colour of meat is the rate and extent that muscle pH declines *post mortem* and the temperature that this occurs at. An increasing paleness in meat is inversely proportional to pH meaning that a decrease in pH results in an increase in paleness. If the pH decline happens too rapidly, resulting in a very low pH at a high temperature, it will result in very pale meat. If the ultimate pH is high (where glycogen depletion occurs pre-slaughter resulting in little or no lactic acid production) the meat will be dark with a dry surface (DFD). DFD meat occurs when there is exercise or stress prior to slaughter resulting in the muscle being deficient in glycogen and therefore having a higher ultimate pH (5.7 and higher). DFD meat allows the growth of spoilage organisms which are inhibited at the usual ultimate pH of meat (Newton & Gill, 1981).

As electrical stimulation causes a more rapid decrease in pH it has to be taken into account when discussing colour. As mentioned previously, depth of oxygen penetration into meat depends on oxygen pressure, temperature and oxygen consumption rate by residual enzyme activity. The latter decreases with duration of aging after slaughter (MacDougall, 1977). Electrical stimulation will therefore speed up this process. Both Strydom, Frylinck & Smith (2005) and McKenna, Maddock & Savell (2003) found that electrical stimulation had no effect on L*, a* or b* values. Strydom, Frylinck & Smith (2005) however, concluded that chilling rates could make the effects of electrical stimulation negligible with regards to meat colour. Devine, Payne, Peachey, Lowe, Ingram and Cook (2002) found that the onset of rigor at a higher temperature usually results in a higher L* value which is a paler colour.

In this study we also have to consider the effects that zilpaterol and vitamin D_3 could have on colour. No differences in L*, a* or b* where recorded by Quin et al. (2008) in heifers fed a beta-agonist or by Avendaño-Reyes, Torres-Rodriques, Meraz-Murillo, Pérez-Linares, Figueroa-Saavedra and Robinson (2006), who observed no difference in meat colour during display from steers fed a beta-agonist. This does not agree with Geesink, Smulders, Van Laack, Van der Kolk, Wensing, & Breukink (1993) who found that beta-agonists significantly increased L* resulting in paler meat. This difference was attributed to L* being associated with water holding capacity in muscle. In this experiment electrical stimulation was applied resulting in a pH drop causing protein denaturation and therefore an increase in drip loss leading to increased L* values. In both Quin et al. (2008) and Avendaño-Reyes, Torres-Rodriques, Meraz-Murillo, Pérez-Linares, Figueroa-Saavedra and Robinson (2006), there were no differences in drip loss between beta-agonist supplemented and control groups. Hilton et al. (2009) obtained similar results regarding L* (no significant difference) but found that a*, b* and chroma were all significantly decreased by zilpaterol supplementation. Strydom, Buys & Strydom (2000) found that zilpaterol supplementation increased colour shelf-life by one day by improving colour stability by decreasing metmyoglobin development. Lawrence et al. (2006) found that vitamin D_3 supplementation had no effect on colour at all in

beef, while both Lahucky et al. (2007) and Wiegand et al. (2002) showed significantly higher a* values (and a significantly lower L* value in the case of Wiegand et al., 2002) in pork loin chops.

2.4.2 Water holding capacity/ drip loss

Water holding capacity is the ability of meat to bind its own water or, under the influence of external forces such as heat and pressure, to bind additional water. When meat loses water it is known as drip loss. There are three kinds of water found in muscle. The first is bound water. Bound water is found near non-aqueous constituents like proteins and does not easily move to other compartments. The second is immobilized water which is held either by steric effects or by attraction to the bound water. This water is held within the structure of the muscle but is not bound to the protein. This water does not flow freely from the tissue in early *post mortem* tissue. The third is free water whose flow from the tissue is unimpeded. This fraction of water is held to the meat by weak surface forces (Huff-Lonergan & Lonergan, 2005). Immobilized water is the most affected by the rigor process and the conversion of muscle to meat and can eventually escape as drip loss (Offer & Knight, 1988b).

pH has a large effect on water holding capacity. During the conversion of muscle to meat, water holding capacity will be reduced. The rate at which pH falls as well as the ultimate pH of the meat will have an effect on this. The higher the ultimate pH, the higher the water holding capacity will be. A fast rate in pH decline, as well as a fast rate of pH decline at high temperatures, will both result in a loss of water holding capacity. This can be attributed to the denaturation of muscle proteins, in particular myosin (Offer, 1991). The accelerated pH decline caused by electrical stimulation can contribute to reduced water holding capacity in beef. Strydom, Frylinck, & Smith (2005) found a small but significant increase in drip loss and attributed this to a rapid pH drop at a slightly slower chilling rate.

Both Strydom, Frylinck, Montgomery, & Smith (2009) and Kellermeier et al. (2009) found that beta-agonist supplementation led to a significant increase in drip loss. Kellermeier et al. (2009) suggested that this was due to zilpaterol supplementation causing an increase in carcass protein and moisture while Strydom, Frylinck, Montgomery, & Smith (2009) agreed with the increased moisture content as well as speculating that higher glycogen breakdown rates led to the increase in drip loss. Montgomery et al. (2002) found that supplementation with various levels of vitamin D_3 had no effect on the percent free, bound or immobilized water. This does not agree with Karges et al. (2001) who found that water holding capacity was increased with vitamin D_3 supplementation and increased with an increase in duration of supplementation.

2.5 CONCLUSION

Results regarding the effect of vitamin D_3 on meat tenderness are still varied. Vitamin D_3 has the potential to increase plasma calcium levels and therefore increase levels of calcium in the muscles, resulting in more calcium being available for the calcium dependant proteinases. This increase in calcium levels does not however always occur and could be due to the counteractive effect that the two feedback loops have, which are in place for calcium homeostasis. Even so, when calcium levels of the muscle are raised this does not seem to always result in increased calpain activity. Other negative effects of vitamin D_3 also have to be taken into account. These include vitamin D_3 toxicity in the animal or high levels of vitamin D_3 in the liver, kidneys and muscle leading to toxicity in humans after consumption. High levels of vitamin D_3 have also been shown to reduce feed intake and therefore average daily gain of supplemented animals. All these factors, as well as the high cost of vitamin D_3 and the possible positive effects on tenderness have to be weighed up. More research needs to be conducted on vitamin D_3 supplementation before it can be confirmed that it does indeed improve beef tenderness.

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CHAPTER 3: MATERIALS AND METHODS

3.1 EXPERIMENTAL DESIGN AND FEEDLOT PHASE

One hundred and twenty Bonsmara steers of approximately 9 months of age were raised on a commercial feedlot diet for 120 days. The animals were divided into six groups (n=20) so that the average weight and variation was the same for all groups. The groups represented 6 treatments, namely a control (C), which received the feedlot diet only, while the five remaining groups were supplemented with the beta-agonist, zilpaterol hydrochloride, (Intervet/Schering-Plough Animal Health, South Africa) at 0.15 mg/kg live weight for thirty days during the final weeks of finishing. One of the five groups only received zilpaterol (Z), while the other four groups received zilpaterol and vitamin D_3 (Vitamin D_3 500, Advit Animal Nutrition S.A. (PTY) LTD, Sebenza, South Africa) at the following levels and durations before slaughter: 7×10^6 IU/animal /day for 3 days prior to slaughter (3D7M); 7×10^6 IU/animal /day for 6 days prior to slaughter (6D7M); 7×10^6 IU/animal /day for 6 days followed by 7 days of no supplementation prior to slaughter (6D7M7N) and 1×10^6 IU/animal /day for 9 days prior to slaughter (9D1M). Zilpaterol was withdrawn from feed 4 days prior to slaughter. The vitamin D_3 supplement was mixed with maize to increase the volume and supplied as (1kg) top dressing on the daily supply of the feedlot diet (10 animals per pen). To ensure equal and complete consumption of the supplement, a clean bunk approach was followed, i.e. all feed was consumed before new feed was given each morning. All animals in each pen therefore approached the bunk at the same time and sufficient bunk space was provided so that all animals per pen could have access to feed at the same time.

3.2 SLAUGHTER AND SAMPLING PROCEDURES

The animals were slaughtered at the abattoir of the Animal Production Institute (Agricultural Research Council, Irene, Gauteng Province). Blood was collected from all

steers at exsanguination using sodium EDTA (parathyroid analysis) and lithium heparinised (calcium analysis) containers and stored on ice. After centrifugation, plasma and serum was stored at -18 °C until analysed. Carcasses were split and the left sides were electrically stimulated for 30 seconds (400V peak, 5ms pulses at 15 pulses per second) within 30 minutes of killing. Carcass sides were then chilled at 3 ± 2ºC (chiller temperature at loading). Temperature and pH measurements (Eutech Instruments, CyberScan pH II pH/mV/°C meter) of the *M. longissimus lumborum* (LL) (reading taken at the last lumbar vertebra) were taken every hour for 4 h and a final measurement was taken at 18 h *post mortem*. All samples were collected from the LL on the day of slaughter, or after rigor mortis, depending on the purpose of the sample. The following tests were conducted: a) Meat tenderness, measured by Warner Bratzler shear force (WBSF), and myofibril fragment length (MFL) on LL aged 3 and 14 days *post mortem* (2 ± 1°C). b) Sarcomere length measured at 1 day *post mortem*. c) Proteinase enzyme system measured as μ and m-calpain and calpastatin activities at 1 and 24 h *post mortem*. d) Instrumental colour and drip loss on fresh or vacuum-aged samples (14 days *post mortem*; 2 ± 1°C).

3.3 WARNER BRATZLER SHEAR FORCE MEASUREMENTS

Aged samples for Warner Bratzler shear force (WBSF) were frozen at -20°C and then processed into 30 mm steaks by means of a band saw. The frozen steaks were thawed at 2 ± 1°C for 24 h and cooked using an oven-broiling (Mielé, model H217, Mielé & Cie, Gütersloh, Germany) method with direct radiant heat (American Meat Science Association (AMSA, 1995). The steaks were broiled at 260°C (pre-set) to 70°C internal temperature and cooled down to 18 °C. Six round cores (12.7 mm diameter) were removed from the steaks parallel to the muscle fibres (American Meat Science Association (AMSA), 1995). Each core was sheared once through the centre, perpendicular to the fibre direction, by a Warner Bratzler shear device mounted on an Universal Instron apparatus (Model 4301,

Intsron Ltd, Buckinghamshire, England;.cross head speed = 200 mm/min) and the mean value of the 6 recordings used as a shear value.

3.4 HISTOLOGICAL MEASUREMENTS

Samples for sarcomere lengths of fresh LL samples (24 h *post mortem*), were prepared according to the method of Hegarty and Naudé (1970), by using distilled water instead of Ringer Locke solution (Dreyer, Van Rensburg, Naudé, Gouws & Stiemie, 1979). Fifty sarcomeres per sample were measured by means of a video image analyses (VIA) using an Olympus BX40 system microscope at a 1000X magnification equipped with CC12 video camera (Olympus, Tokyo, Japan). AnalySIS Life Science software package (Soft Imaging Systems Gmbh, Münster, Germany) was used to process and quantify measurements.

Myofibril fragment lengths (MFL) of LL aged for 3 and 14 days *post mortem* were measured by means of VIA. Myofibrils were extracted according to Culler, Parrish, Smith and Cross (1978) as modified by Heinze and Bruggemann (1994). One hundred myofibril fragments per sample were examined and measured with an Olympus BX40 system microscope at a 400X magnification.

3.5 MUSCLE BIOCHEMISTRY

Samples collected for enzyme studies (1 and 24 h *post mortem*) were snap-frozen in liquid nitrogen and preserved at –70ºC. Calpastatin, µ-calpain and m-calpain were extracted from 5g of the LL frozen samples as described by Dransfield (1996) and separated by means of the two-step gradient ion-exchange chromatography-method according to Geesink

and Koohmaraie (1999). Calpain assays were determined by using azo-casein as substrate according to Dransfield (1996). The use of azo-casein eliminates the problem of background absorbance of non-specific proteins in the extracts. One unit of calpain activity was defined as an increase in absorbance at 366nm of 1.0 per hour, at 25°C. One unit of calpastatin activity was defined as the amount that inhibited one unit of m-calpain activity. Data were expressed as units per gram of muscle.

3.6 SERUM CALCIUM AND PARATHYROID ANALYSES

Serum calcium concentrations were analyzed using a colorimetric assay kit (Roche, Mannheim, Germany). Plasma parathyroid hormone levels (PTH) were determined by electrochemiluminescence immunoassay employing a sandwich test principle on a Modular Analytics E 170 (Roche Diagnostic Systems, Nutley , NJ).

3.7 COLOUR AND DRIP LOSS OF FRESH MEAT

Instrumental meat colour was measured with a Minolta meter (Model CR200, Osaka, Japan; 8mm diameter measuring area, diffuse 228 illumination and 0° viewing angel) on fresh samples (24 h *post mortem*) and vacuum-packed aged samples (14 days). The sampled 30 mm LL steaks were divided in two steaks of 15 mm thickness and one steak was allowed to bloom for 60 minutes at chiller temperatures (2 ± 1 ºC) with its freshly cut surface facing upwards before colour recording. Recordings were done in triplicate at three positions on the steak surface. Colour measurements followed the CIE colour convention, where the three fundamental outputs are L*, a* and b*. L* is lightness on a scale of 0 (all light absorbed) to 100 (all light reflected); a* spans from +60 (red) to -60 (green) and b* spans from +60 (yellow) to -60 (blue). Saturation index, also known as chroma, was calculated as square root of $a^{*2} + b^{*2}$ and hue angle, defined as $\tan^{-1}$ (b/a) that describes the

fundamental colour of a substance (MacDougall, 1977). Mean values were used for statistical analysis.

Two cubes of 10 x 10 x 20mm were cut from the remaining fresh steak used for colour measurement to determine drip loss of fresh loin muscle. The cubes were suspended on a pin inside a sample bottle (200ml) taking care that the meat did not touch the sides of the bottle and stored for 3 days at 2 ± 1°C. The amount of drip measured as the difference between the sample mass before and after was expressed as a percentage of the starting mass. Drip loss or purge for aged samples were determined by measuring the amount of purge remaining in the bag after removing the 30 mm LL steak for colour measurement. The steak was removed and lightly dried with tissue paper. Drip was expressed as a percentage of the combined mass of the steak and drip.

3.8 STATISTICAL ANALYSES

Data of WBSF, MFL, the three enzyme activities and colour and drip loss were subjected to analysis of variance for a split-plot design (GenStat® VSN International, Hemel Hempstead, UK; Payne, Murray, Harding, Baird & Soutar, 2007) with the six treatment groups (C, Z, 3D1M, 9D1M, 6D7M7N, 6D7M) as whole plots and the two stimulation sub-treatments (ES and NES) and the two ageing periods (3 and 14 days *post mortem*) or time of analyses of enzyme activity (1 and 24 h *post mortem*) or two aging periods for colour (24 h and 14 days) as sub-plots. Data of sarcomere length was subjected to a similar analyses, six treatment groups (C, Z, 3D1M, 9D1M, 6D7M7N, 6D7M) as whole plots and only the two stimulation sub-treatments (ES and NES) as sub-plots. Means for the interactions between the whole plot and sub-plots were separated using Fisher's protected t-test least significant difference (LSD) at the 5% level (Snedecor & Cochran, 1980). The effect of pen allocation within each treatment was included in the statistical model but had no significant effect on any measurement and was therefore left out of the final model.

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CHAPTER 4: THE EFFICIENCY OF ELECTRICAL STIMULATION TO COUNTERACT THE NEGATIVE EFFECTS OF BETA-AGONISTS ON MEAT TENDERNESS OF FEEDLOT CATTLE

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4.1 ABSTRACT

Beta-agonists used as growth enhancers are known to affect the ageing potential of beef muscle negatively. On the other hand, procedures like electrical stimulation could accelerate rigor and the aging process. In this study, 20 out of 40 young steers received no beta-agonist (C), the remaining twenty steers received a beta-agonist (zilpaterol hydrochloride) (Z) for the 30 days prior to slaughter followed by four days withdrawal.

After slaughter carcasses were split, the left side electrically stimulated (ES) and the right side not stimulated (NES). Samples were aged for 3 or 14 days *post mortem*. Parameters included Warner Bratzler shear force (WBSF), myofibril filament length (MFL), sarcomere length and calpastatin and calpain enzyme activity.

Zilpaterol resulted in increased ($P < 0.001$) WBSF mainly due to an increased ($P < 0.001$) calpastatin activity. ES improved tenderness ($P < 0.001$) in general by early onset of rigor triggering the activity of calpains. ES also reduced the calpastatin activity ($P < 0.001$), which partially countered the effect of high calpastatin activity on the aging potential of Z loins. ES can therefore be implemented to improve meat tenderness in zilpaterol supplemented steers, although steers without zilpaterol will still have an advantage in final tenderness.

4.2 INTRODUCTION

It is the aim of any livestock industry to improve efficiency and economic return. Beta-agonists are compounds fed to animals to improve rate of gain, improve feed efficiency and to increase carcass meat yield efficiency (Dikeman, 2007). In this way both the producer and consumer could benefit as meat becomes less expensive to produce.

It is however common knowledge that beta-agonist supplemented animals produce tougher meat due to an increase in the activity of the inhibitor calpastatin and a reduction in calpain activity (Wheeler & Koohmaraie, 1997). These changes depend on species, type of muscle, the particular compound as well as the time and duration of supplementation (Dransfield, 1994). The beta-agonist zilpaterol was recently registered in Mexico, South Africa and the USA and, together with ractopamine, is probably the most commonly utilised beta-agonist in commercial beef production. Recent studies have found that tenderness problems generally related to beta-agonists also occur with this product (Strydom, Frylinck, Montgomery & Smith, 2009; Hilton et al., 2009; Kellermeier et al., 2009; Rathmann et al., 2009).

Variations in meat tenderness and consumer complaints regarding tough meat are general concerns to the meat industry worldwide. Rodas- González, Huerta-Leidenz, Jerez-Timaure and Miller (2009) showed that consumers are able to detect changes in tenderness of steaks with different WBSF. Furthermore, Miller, Carr, Ramsey, Crockett, and Hoover (2001) found that consumers can segregate differences in beef tenderness and that they are willing to pay more for more-tender beef. It would therefore be beneficial to improve meat tenderness of beta-agonist supplemented steers. A common way of doing this would be to age meat for longer although this would lead to extended storage costs. Electrical stimulation has been shown to hasten rigor and cause the tenderisation process to start earlier at a higher temperature (Dransfield, Etherington & Taylor, 1992) thereby reducing

aging time. In this trial we look at the efficiency of electrical stimulation with and without extended aging, to improve meat tenderness of beta-agonist treated meat and the mechanisms behind the process.

4.3 MATERIALS AND METHODS

4.3.1 Experimental design and feedlot phase

Forty Bonsmara steers of approximately 9 months of age were raised on a commercial feedlot diet for 120 days. The animals were divided in two (n=20) so that the average weight and variation was the same for both groups. The groups represented two treatments, namely a control (C), which received the feedlot diet only, and a zilpaterol group (Z), which received zilpaterol hydrochloride (Intervet/ Schering-Plough Animal Health, South Africa) at 0.15mg/kg live weight/day, for thirty days during the final weeks of finishing. The zilpaterol hydrochloride was withdrawn four days prior to slaughter.

4.3.2 Slaughter and sampling procedures

The animals were slaughtered at the abattoir of the Animal Production Institute (Agricultural Research Council, Irene, Gauteng Province). Carcasses were split and the left sides were electrically stimulated for 30 seconds (400V peak, 5ms pulses at 15 pulses per second) within 30 minutes of killing. Carcass sides were then chilled at 3 ± 2ºC (chiller temperature at loading). Temperature and pH measurements (Eutech Instruments, CyberScan pH II pH/mV/°C meter) of the *M. longissimus lumborum* (LL) (reading taken at the last lumbar vertebra) were taken every hour for 4 h and a final measurement was taken at 18 h *post mortem*.All samples were collected from the LL on the day of slaughter, or after rigor mortis, depending on the purpose of the sample. The following tests were conducted: a) Meat tenderness, measured by Warner Bratzler shear force (WBSF), and myofibril

fragment length (MFL) on LL aged 3 and 14 days *post mortem* (2 ± 1°C). b) Sarcomere length measured at 1 day *post mortem*. c) Proteinase enzyme system measured as µ-calpain, m-calpain and calpastatin activity at 1 and 24 h *post mortem*.

4.3.3 Warner Bratzler shear force measurements

Aged samples for Warner Bratzler shear force (WBSF) were frozen at -20°C and then processed into 30 mm steaks by means of a band saw. The frozen steaks were thawed at 2 ± 1°C for 24 h and cooked using an oven-broiling (Mielé, model H217, Mielé & Cie, Gütersloh, Germany) method with direct radiant heat (American Meat Science Association (AMSA), 1995). The steaks were broiled at 260°C (pre-set) to 70°C internal temperature and cooled down to 18 °C. Six round cores (12.7 mm diameter) were removed from the steaks parallel to the muscle fibres (American Meat Science Association (AMSA), 1995). Each core was sheared once through the centre, perpendicular to the fibre direction, by a Warner Bratzler shear device mounted on an Universal Instron apparatus (Model 4301, Intsron Ltd, Buckinghamshire, England;.cross head speed = 200 mm/min) and the mean value of the six recordings used as a shear value.

4.3.4 Histological measurements

Samples for sarcomere lengths of fresh LL samples (24 h *post mortem*), were prepared according to the method of Hegarty and Naudé (1970), by using distilled water instead of Ringer Locke solution (Dreyer, Van Rensburg, Naudé, Gouws & Stiemie, 1979). Fifty sarcomeres per sample were measured by means of a video image analyses (VIA) using an Olympus BX40 system microscope at a 1000X magnification equipped with CC12 video camera (Olympus, Tokyo, Japan). AnalySIS Life Science software package (Soft

Imaging Systems Gmbh, Münster, Germany) was used to process and quantify measurements.

Myofibril fragment lengths (MFL) of LL aged for three and 14 days *post mortem* were measured by means of VIA. Myofibrils were extracted according to Culler, Parrish, Smith and Cross (1978) as modified by Heinze and Bruggemann (1994). One hundred myofibril fragments per sample were examined and measured with an Olympus BX40 system microscope at a 400X magnification.

4.3.5 Muscle biochemistry

Samples collected for enzyme studies (1 and 24 h *post mortem*) were snap-frozen in liquid nitrogen and preserved at –70ºC. Calpastatin, µ-calpain and m-calpain were extracted from 5g of the LL frozen samples as described by Dransfield (1996) and separated by means of the two-step gradient ion-exchange chromatography-method according to Geesink and Koohmaraie (1999). Calpain assays were determined by using azo-casein as substrate according to Dransfield (1996). The use of azo-casein eliminates the problem of background absorbance of non-specific proteins in the extracts. One unit of calpain activity was defined as an increase in absorbance at 366nm of 1.0 per hour, at 25°C. One unit of calpastatin activity was defined as the amount that inhibited one unit of m-calpain activity. Data were expressed as units per gram of muscle.

4.3.6 Statistical analyses

Data of WBSF, MFL and the three enzyme activities were subjected to analysis of variance for a split-plot design (GenStat® VSN International, Hemel Hempstead, UK; Payne, Murray, Harding, Baird & Soutar, 2007) with the two treatment groups (C and Z) as whole plots and the two stimulation sub-treatments (ES and NES) and the two ageing periods (3 and 14 days *post mortem*) or time of analyses of enzyme activity (1 and 24 h *post mortem*)

as sub-plots. Data of sarcomere length was subjected to a similar analyses, two treatment groups (C and Z) as whole plots and only the two stimulation sub-treatments (ES and NES) as sub-plots. Means for the interactions between the whole plot and sub-plots were separated using Fisher's protected t-test least significant difference (LSD) at the 5% level (Snedecor & Cochran, 1980). The effect of pen allocation within each treatment was included in the statistical model but had no significant effect on any measurement and was therefore left out of the final model.

4.4 RESULTS

4.4.1 Main effects of treatment, stimulation and aging

The differences in WBSF between the treatment (C and Z), stimulation and aging groups are presented in Table 1, Table 2 and Table 3, respectively. There was a significant difference ($P < 0.001$) between the C and Z groups, with the C group producing more tender meat than the beta-agonist supplemented group. Both electrical stimulation (ES) and prolonged aging reduced WBSF significantly ($P < 0.001$) relative to no stimulation (NES) and aging for three days, respectively. Higher WBSF for Z was accompanied by significantly ($P < 0.001$) longer MFL (less myofibrillar degradation), although the significantly ($P = 0.011$) shorter MFL of non- stimulated (NES) compared to ES samples contrasted the positive effect of ES on WBSF. Aging had a positive effect on meat tenderness with filament lengths (MFL) being significantly ($P < 0.001$) shorter at 14 days aging when compared to 3 days aging. Neither treatment ($P = 0.581$) nor stimulation ($P = 0.276$) had a significant effect on sarcomere length.

Table 1

Effect of zilpaterol on Warner Bratzler shear force (WBSF), histological and biochemical characteristics of *M. longissimus lumborum* (data pooled for stimulation and *post mortem* aging/or time of measurement)

Treatment	Control	Zilpaterol	SEM[a]	*P* value
WBSF (kg)	4.1	5.8	0.1787	<0.001
MFL (µm)[b]	30.1	36.7	0.968	<0.001
Sarcomere length (µm)	1.8	1.8	0.0084	0.581
Calpastatin activity[c]	2.2	2.6	0.0431	<0.001
µ-calpain activity[d]	0.91	0.96	0.0241	0.124
m-calpain activity[d]	0.94	0.99	0.0156	0.051

[a] Standard error of means

[b] MFL: Myofibril filament length

[c] Calpastatin activity: One unit of calpastatin activity is defined as the amount that inhibited one unit of m-calpain activity.

[d] One unit of calpain activity is defined as an increase in absorbance at 366 nm of 1.0 absorbance unit per g of muscle per hour, at 25 °C.

Table 2

Effect of electrical stimulation on Warner Bratzler shear force (WBSF), histological and biochemical characteristics of *M. longissimus lumborum* (data pooled for treatment and *post mortem* aging/or time of measurement).

	Stimulation[a]			
	ES	NES	SEM[b]	*P* value
WBSF (kg)	4.6	5.3	0.0975	<0.001
MFL (µm)[c]	34.3	32.5	0.454	0.011
Sarcomere length (µm)	1.8	1.8	0.0054	0.276
Calpastatin activity[d]	2.3	2.5	0.0226	<0.001
µ-calpain activity[e]	0.85	1.0	0.0183	<0.001
m-calpain activity[e]	0.95	0.98	0.0062	0.001

[a] NES: Non-stimulated; ES: Electrically stimulated

[b] Standard error of means

[c] MFL: Myofibril filament length

[d] Calpastatin activity: One unit of calpastatin activity is defined as the amount that inhibited one unit of m-calpain activity.

[e] One unit of calpain activity is defined as an increase in absorbance at 366 nm of 1.0 absorbance unit per g of muscle per hour, at 25 °C.

Table 3

Effect of *post mortem* aging (3 days and 14 days) on Warner Bratzler shear force (WBSF) and myofibril length (MFL) of *M. longissimus lumborum* (Data pooled for treatment and electrical stimulation)

	Aging			
	3 days	14 days	SEM [a]	*P* value
WBSF (kg)	5.7	4.1	0.0708	<0.001
MFL (µm)	37.8	29.1	0.429	<0.001

[a] Standard error of means

4.4.2 Interactions between treatment, stimulation and aging

A significant interaction (P = 0.003) occurred between treatment and stimulation regarding WBSF. Fig. 1 shows that ES had very little effect on the tenderness of C but did have a significant effect on Z, although ES did not improve shear force of Z to the level of C. In agreement with the effect of ES on tenderness of Z, there was a significant interaction between treatment and stimulation for calpastatin activity (P = 0.015) (Fig. 2). Overall ES decreased calpastatin activity although the effect was larger for Z than for C. In both cases however, calpastatin activity was still higher for Z than for C after 24 h. Fig. 3 shows the interaction between treatment and aging for WBSF ($P < 0.001$) where Z aged at a faster rate than C between 3 and 14 days, although the final WBSF was still in favour of C.

Treatment did not affect µ-calpain activity at 1 h *post mortem*, but a significantly higher activity was found for Z samples at 24 h compared to C samples for µ-calpain (P = 0.002; Fig. 4). Stimulation and time of measurement showed significant interactions for calpastatin, µ and m-calpain. ES significantly decreased calpastatin activity (P = 0.014) at 1 and 24 h *post mortem* (Fig. 5), although the difference was much higher at 24 h. No difference in µ-calpain activity occurred at 1 h, while activity was significantly lower for ES at 24 h compared with NES (Fig. 6) ($P < 0.001$). M-calpain activity increased significantly between 1 and 24 h for both ES and NES, although the difference was much larger at 24 h than at 1 h *post mortem* (Fig. 7, Table 4).

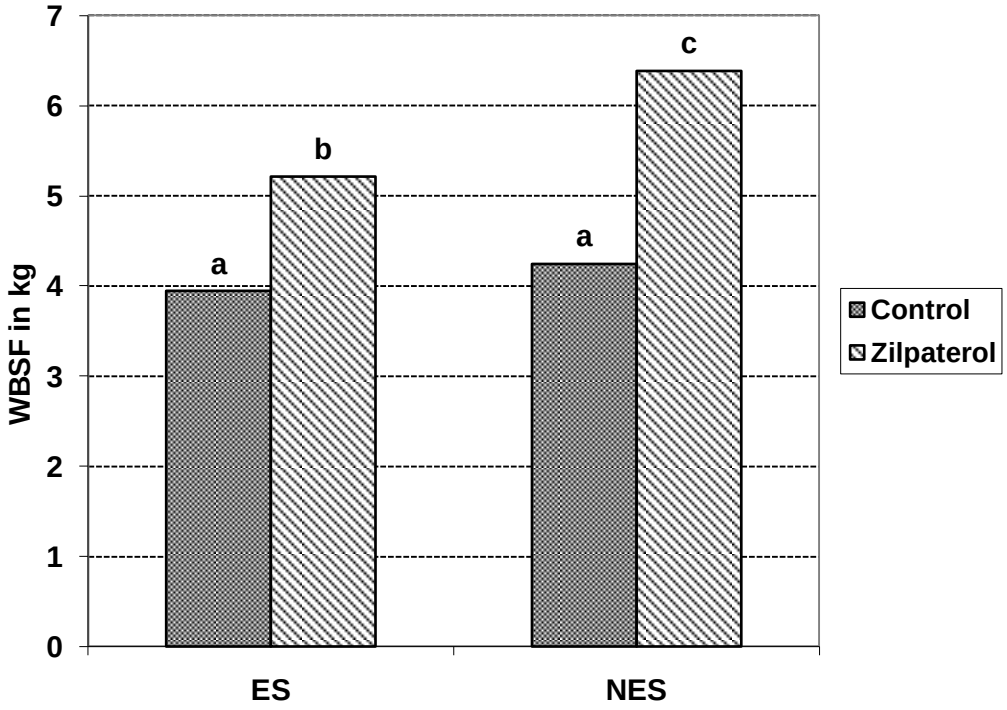

Fig. 1. Interaction between treatment (control and zilpaterol) and electrical stimulation in relation to Warner Bratzler shear force (WBSF; *P* = 0.003). (Bars with different superscripts differ significantly, *P* < 0.05; ES and NES = stimulated and non-stimulated)

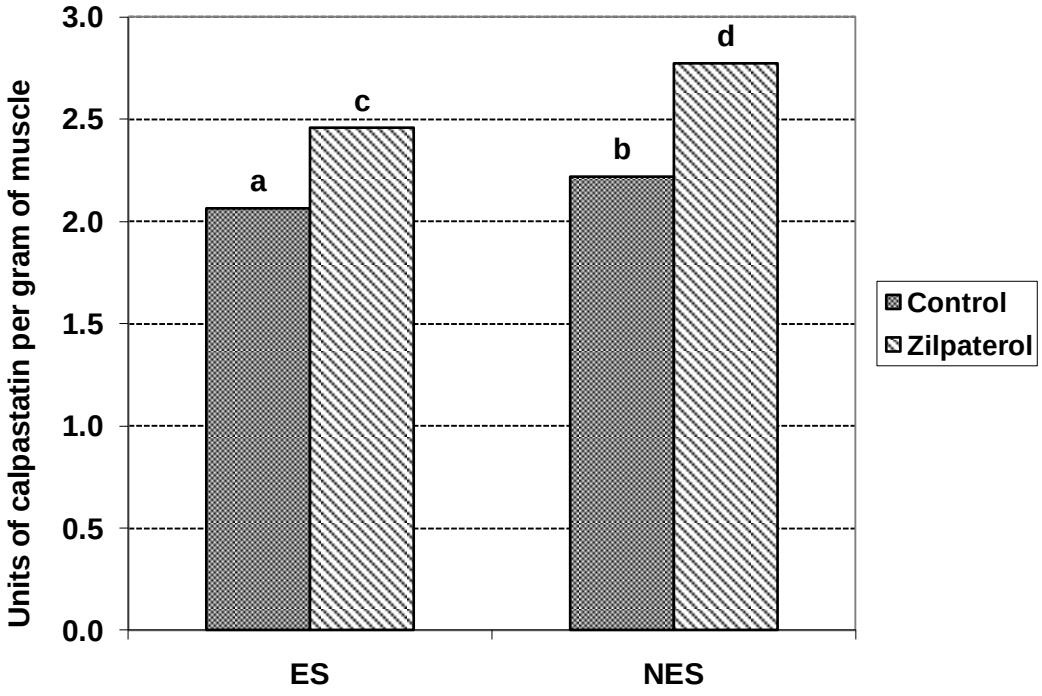

Fig. 2. Interaction between treatment (control and zilpaterol) and electrical stimulation in relation to calpastatin activity (*P* = 0.015). (Bars with different superscripts differ significantly, *P* < 0.05; ES and NES = stimulated and non-stimulated)

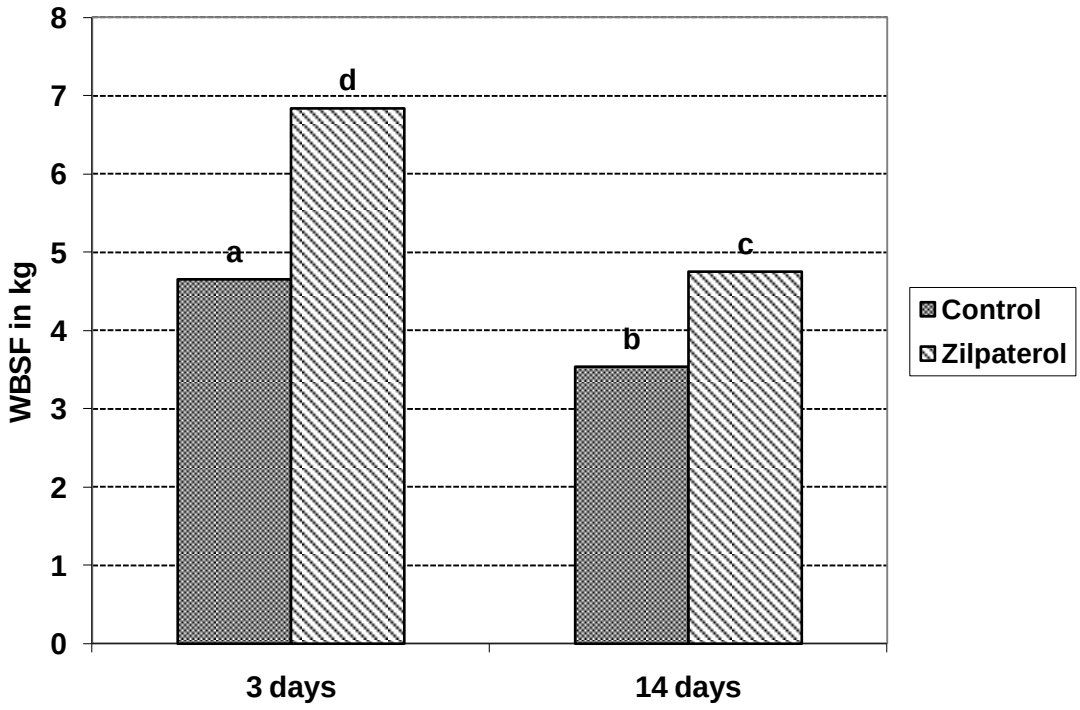

Fig. 3. Interaction between treatment (control and zilpaterol) and *post mortem* aging (3 days and 14 days) in relation to Warner Bratzler shear force (WBSF; $P < 0.001$). (Bars with different superscripts differ significantly, $P < 0.05$)

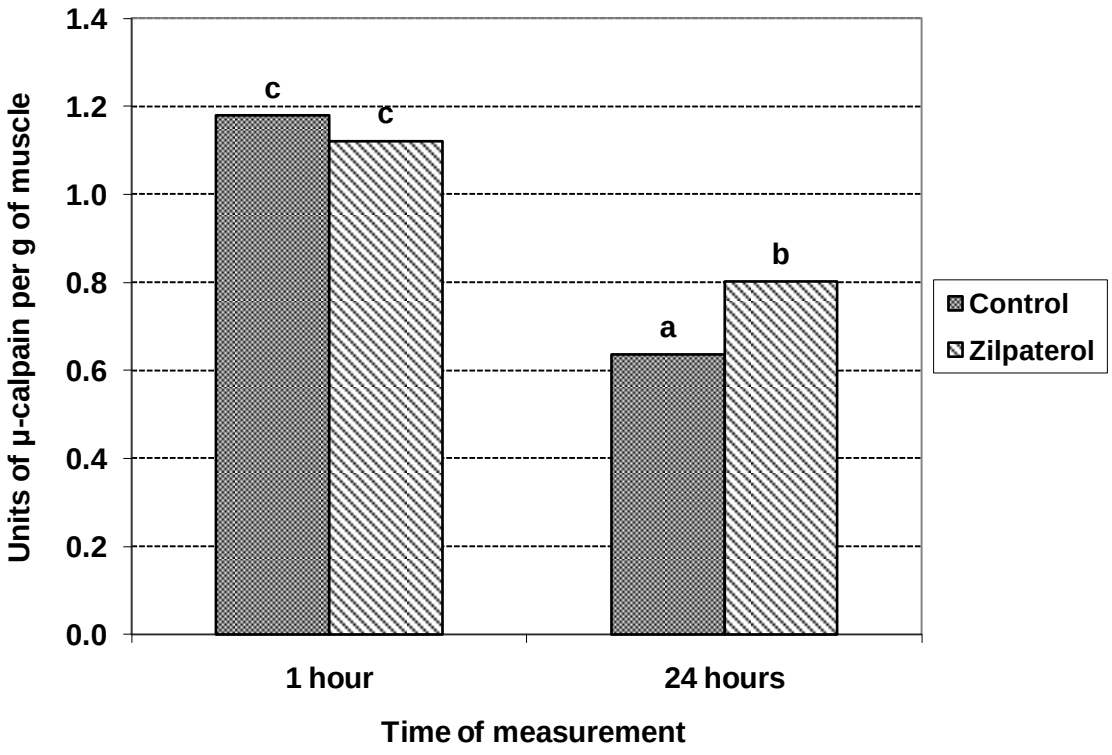

Fig. 4. Interaction between treatment (control and zilpaterol) and time of measurement (1 and 24 hours *post mortem*) in relation to µ-calpain activity ($P < 0.002$). (Bars with different superscripts differ significantly, $P < 0.05$)

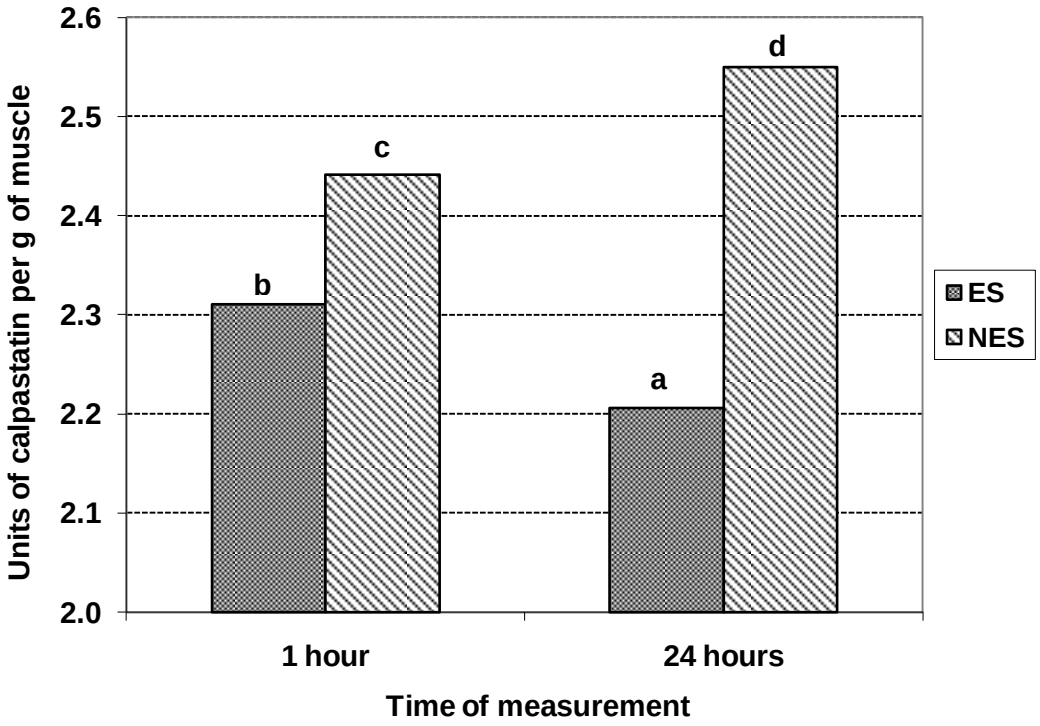

Fig. 5. Interaction between stimulation and time of measurement (1 and 24 hours *post mortem*) in relation to calpastatin activity (P = 0.014). (Bars with different superscripts differ significantly, P < 0.05; ES and NES = stimulated and non-stimulated)

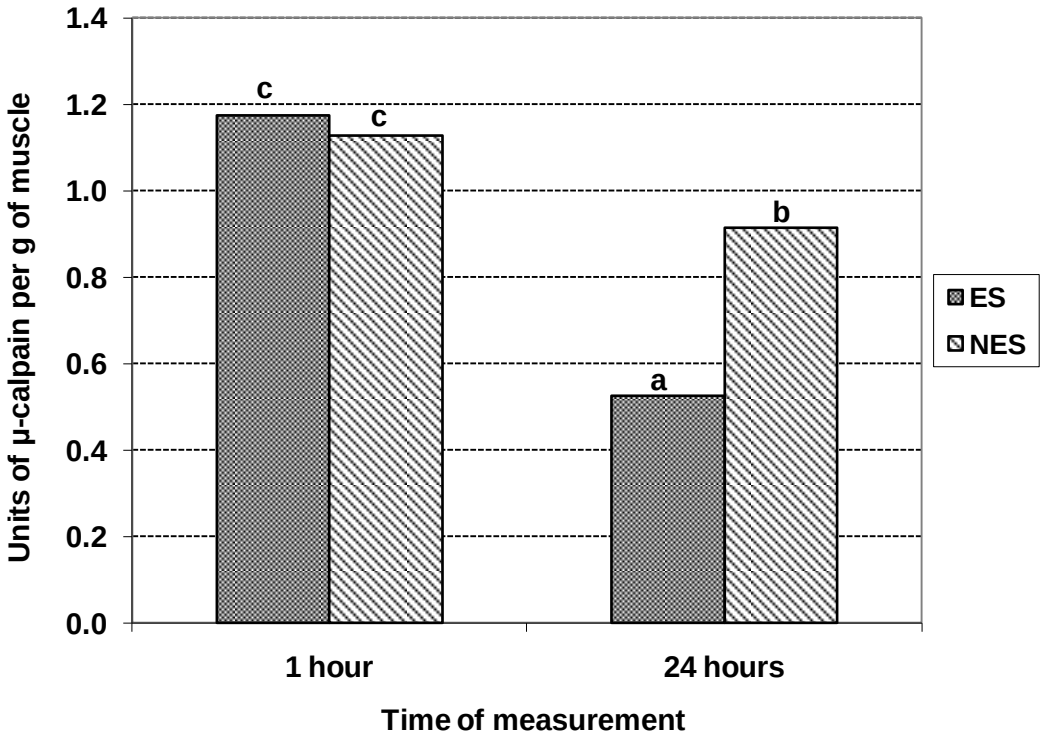

Fig. 6. Interaction between stimulation and time of measurement (1 and 24 hours *post mortem*) in relation to µ-calpain activity (P < 0.001). (Bars with different superscripts differ significantly, P < 0.05; ES and NES = stimulated and non-stimulated)

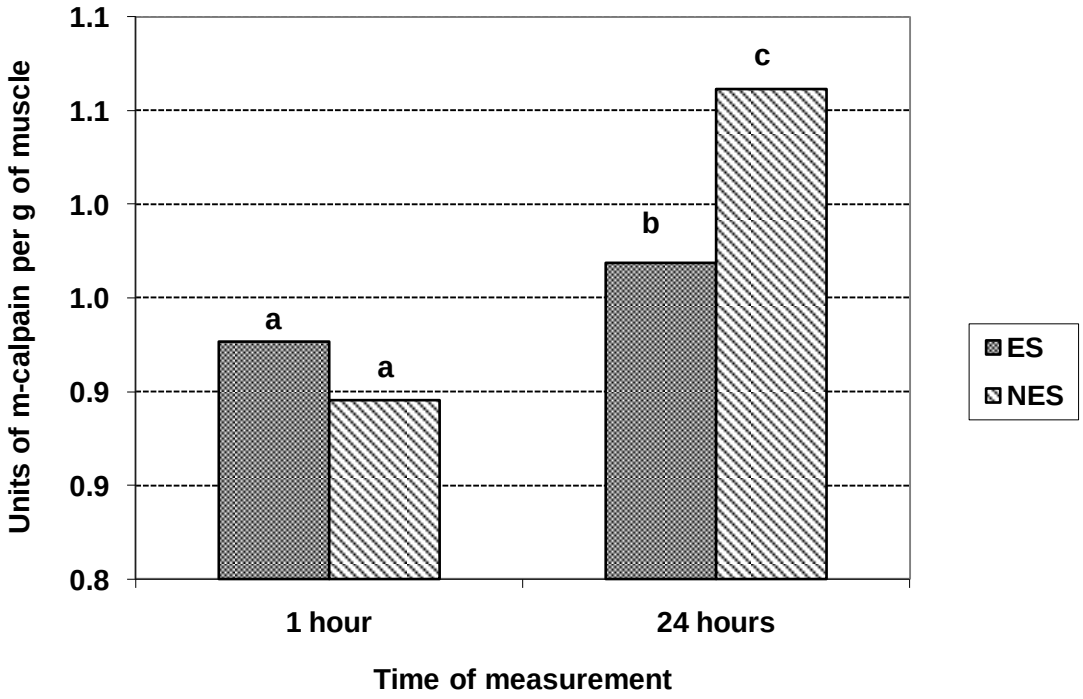

Fig. 7. Interaction between stimulation and time of measurement (1 and 24 hours *post mortem*) in relation to m-calpain activity ($P < 0.001$). (Bars with different superscripts differ significantly, $P < 0.05$; ES and NES = stimulated and non-stimulated)

Table 4

Effect of time of measurement (*post mortem*) on calcium dependent protease activity of *M. longissimus lumborum* (Data pooled for treatment and stimulation)

	Time of measurement			
	1 hour	24 hours	SEM[a]	*P* value
Calpastatin activity[b]	2.4	2.4	0.0301	0.967
µ-calpain activity[c]	1.2	0.72	0.0246	<0.001
m-calpain activity[c]	0.91	1.0	0.0101	<0.001

[a] Standard error of means

[b] Calpastatin activity: One unit of calpastatin activity is defined as the amount that inhibited one unit of m-calpain activity.

[c] One unit of calpain activity is defined as an increase in absorbance at 366 nm of 1.0 absorbance unit per g of muscle per hour, at 25 °C.

4.5 DISCUSSION

WBSF was affected negatively (Table 1) by Z which agrees with various other studies regarding the effect of beta-agonists on beef tenderness (for a review: Dunshea, D'Souza, Pethick, Harper & Warner, 2005; Dikeman, 2007). Prolonged aging did improve WBSF of Z in our study, even showing a greater aging rate than C samples (Interaction: $P < 0.05$), although the ultimate tenderness was still significantly in favour of C (Fig. 3). Recent studies on zilpaterol by Hilton et al. (2009), Kellermeier et al. (2009), Leheska et al. (2009) and Rathmann et al. (2009) agree with our results showing differences in favour of C for WBSF even after 28 days *post mortem*, emphasizing how the aging ability of treated samples is impaired. The negative effect of beta-agonists on tenderness has mainly been attributed to increased calpastatin activity which retards *post mortem* aging (Geesink, Smulders, Van Laack, Van der Kolk, Wensing & Breukink, 1993; Koohmaraie & Shackelford, 1991; Wang & Beerman 1988; Simmons, Young, Dobbie, Singh, Thompson & Speck, 1997), while reports on calpain activity are less consistent (Geesink, Smulders, Van Laack, Van der Kolk, Wensing & Breukink, 1993; Simmons, Young, Dobbie, Singh, Thompson & Speck, 1997) and dependant on the time of analysis *post mortem* and the product in question. In our study calcium dependant proteinase (CDP) activities were measured at 1 and 24 h *post mortem*. In agreement with Geesink, Smulders, Van Laack, Van der Kolk, Wensing and Breukink (1993), Koohmaraie and Shackelford (1991), Wang and Beerman (1988) and Simmons, Young, Dobbie, Singh, Thompson and Speck (1997) calpastatin activity was higher for Z than for C in (Table 1) at 1 h *post mortem* and remained higher at 24 h *post mortem* (data of interaction not shown). Initial µ-calpain activity at 1 h was similar for the Z and C, with µ-calpain activity of C declining more over the following 23 hours compared with Z (Fig. 4). This probably reflects the inhibiting action of higher calpastatin on µ-calpain in Z (Simmons, Young, Dobbie, Singh, Thompson & Speck 1997; Geesink & Koohmaraie, 1999) and at the same time the high rate of action and autolysis of µ-calpain in C (Veiseth, Shackelford, Wheeler & Koohmaraie, 2004). The fact that shorter MFL of C coincided with

lower WBSF even at 3 days combined with a sharper decrease in µ-calpain activity agree with the findings of Veiseth, Shackelford, Wheeler and Koohmaraie (2004) who reported changes in MFI (a similar measurement of fragmentation as MFL) during the first 12 h and accompanied effects of µ-calpain on substrates in the first 9 hours *post mortem* in ovine muscle. The continued shorter MFL of C at 14 days mirrored differences in WBSF at 14 days *post mortem* and the limiting effect of Z on aging due to initial and probably continued effects on the CDP system. It is perhaps relevant to note at this point that both Hilton et al. (2009) and Rathmann et al. (2009) found no effect of Z on CDP even though their reports showed that the aging ability of meat in treated samples was impaired.

The main objective of this experiment was to investigate the ability of ES to overcome the negative effect of beta-agonists on meat tenderness. It was previously reported by Strydom, Osler, Leeuw & Nel (1999) that ES on its own could reduce aging time to reach a specific level of tenderness or reduce the ultimate difference in tenderness even further between C and Z after extended aging. Similar results in WBSF were recorded in the present trial (Fig. 8), showing improvements between C and Z from differences of 2.7 kg (NES aged 3 days) to 0.8 kg (ES aged 14 days) (although this interaction was not significant). The similar initial µ-calpain activity and subsequent sharper decline in activity of ES samples (compared with NES; Fig. 6) at 24 h *post mortem* in the present study corroborates with previous reports by Ducastaing, Valin, Schollmeyer and Cross (1985), Dransfield, Etherington and Taylor (1992) and Hwang and Thompson (2001a) that ES advances the onset of rigor (pH = 6.1; Dransfield, Etherington & Taylor, 1992) whereby activation of µ-calpain is initiated due to the release of Ca+ ions and which causes proteolyses and tenderisation. This effect is enhanced by high temperatures (Dransfield, Etherington & Taylor, 1992; Hwang & Thompson, 2001a). While Ducastaing, Valin, Schollmeyer and Cross (1985) also recorded an overall decline in calpastatin activity for ES samples similar to our study (Table 2) the increase in calpastatin activity of NES samples

from 1 h to 24 h (Fig. 5) is difficult to explain. Nevertheless, the decrease in calpastatin activity of ES samples over time could imply a lower inhibitory effect for this treatment in general. Therefore, the changes in µ-calpain and calpastatin activity support the positive effect of ES on WBSF in general (Table 2), independent of treatment (C or Z) or duration of aging. When both treatment (Z and C) and stimulation are considered, ES decreased the activity of calpastatin more in Z samples than in C samples (Fig. 2) and this effect was larger at 24 h *post mortem* (data not shown). Collectively, these events explain the greater advantage of ES for Z at both 3 and 14 days *post mortem* if relative changes and actual values occurring in the CDP system in the first 24 h *post mortem* are accurate predictions for tenderness development later on. This line of thinking is widely supported (Koohmaraie & Geesink, 2006; Koohmaraie, 1996; Koohmaraie, 1994; Dransfield, Etherington & Taylor, 1992; Veiseth, Shackelford, Wheeler & Koohmaraie, 2004; Veiseth, Shackelford, Wheeler & Koohmaraie, 2001) although some grey areas still exist on the specific action and relationship between CDP and prolonged aging (Veiseth, Shackelford, Wheeler & Koohmaraie, 2004). In support of the distinctive effect of ES on Z other examples of similar actions when tenderness potential was compromised by the CDP system are demonstrated by Ferguson, Jiang, Hearnshaw, Rymill and Thompson (2000) and Wheeler, Savell, Cross, Lunt & Smith (1990) where breed (*Bos indicus*) effect on tenderness was substantially reduced by ES.

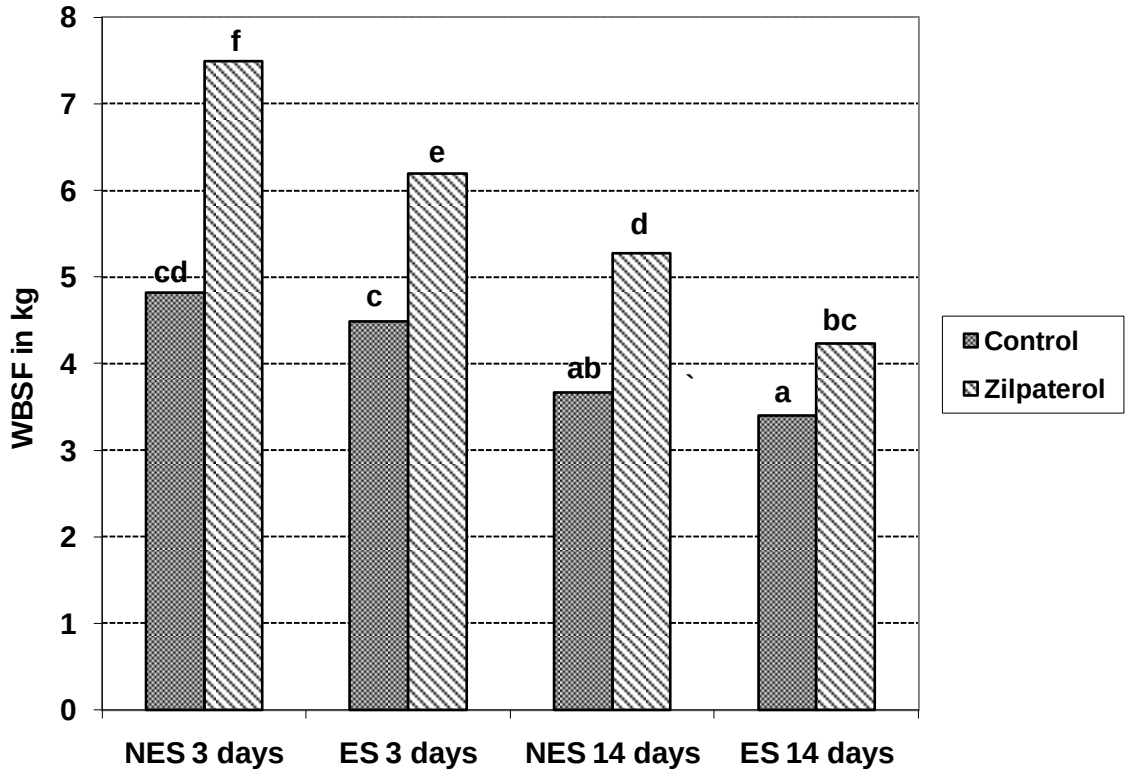

Fig. 8. Interaction between treatment (control and zilpaterol) and electrical stimulation and *post mortem* aging (3 and 14 days) in relation to Warner Bratzler shear force (WBSF; $P = 0.610$). (Bars with different superscripts differ significantly, $P < 0.05$; ES and NES = stimulated and non-stimulated)

The question may be raised about the almost lack of effect of ES on C WBSF and also the proportionally small effect of aging on C. Besides the fact that C loins could have been considered inherently tender (no beta-agonist disposition), WBSF was only measured on day three *post mortem* samples meaning that substantial aging for both ES and NES of C could have taken place in the first 3 days (Veiseth, Shackelford, Wheeler & Koohmaraie, 2004) due to lower calpastatin activity, and that the advantage of ES was already cancelled out by day 3. It could also be reasoned that high rigor temperatures lead to higher rates of autolysis of calpains leading to fast but ineffective aging (Hwang & Thompson, 2001a, 2001b). According to Fig. 9, neither NES nor ES carcasses were close to the optimum 29-30°C at pH 6 recommended by these authors for optimum tenderness at 14 days. Considering final WBSF values of ES and NES, however, this was not detrimental in terms of benchmarked WBSF values for consumer satisfaction (Miller, Carr, Ramsey, Crockett &

Hoover, 2001) which indicated values of < 3.0, 3.4 and 4.0 for 100, 99 and 94 % satisfaction respectively. On the other hand, it may also mean that ES could have been even more beneficial for Z if stimulation was optimised in terms of temperature and pH decline ratios. Williams, Pagliani, Innes, Pennie, Harris, & Garthwaite (1987) also suggested that the leaner carcasses of beta-agonist treated animals may allow rapid *post mortem* cooling inducing cold shortening which could result in tougher meat although the lack of any effect on sarcomere length due to stimulation (Table 2) rules out this possibility.

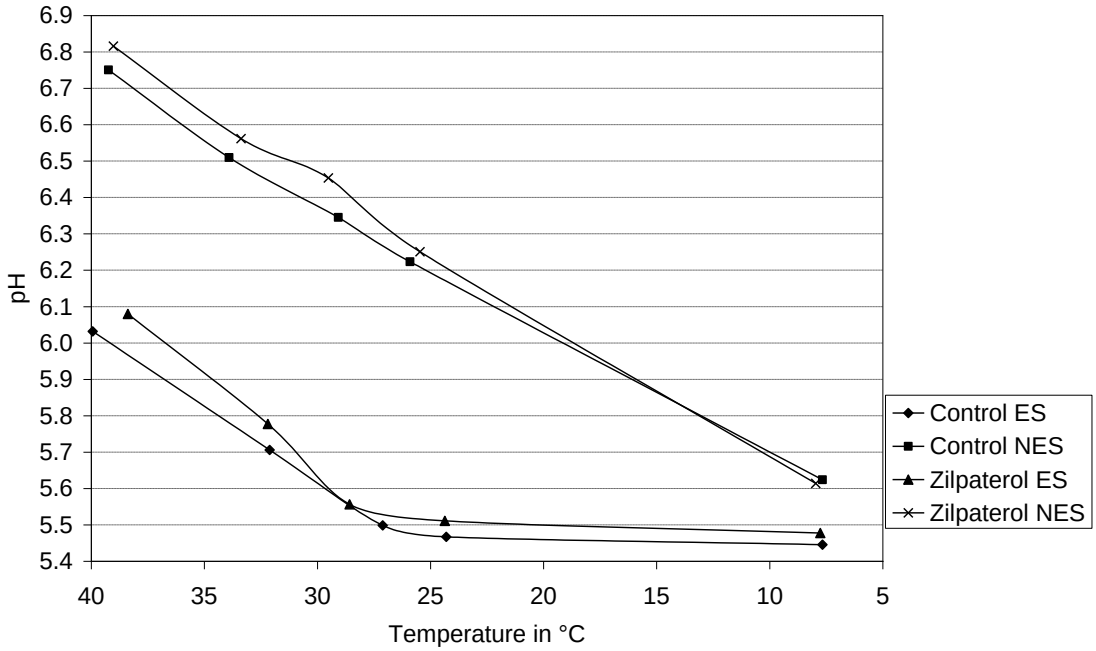

Fig. 9. pH/temperature dynamics of the *M. longissimus lumborum* for different combinations of treatment and stimulation (ES and NES = stimulated and non-stimulated; data points represent recordings at 1, 2, 3, 4 and 18 hours *post mortem*)

M-calpain is generally regarded as having a minor or no part in proteolysis due to the unavailability of sufficient free calcium in *post mortem* muscle to activate the enzyme and a lack of evidence of autolyzation or decline in *post mortem* activity and therefore proteolyses (Boehm, Kendall, Thompson & Goll, 1998; Veiseth, Shackelford, Wheeler & Koohmaraie, 2001; 2004). In our study, Z increased m-calpain activity, which is difficult to explain, but probably had no effect on tenderness. M-calpain activity increased between 1 h and 24 h which is strange but not uncommon (Veiseth, Shackelford, Wheeler & Koohmaraie, 2001; 2004). ES reduced m-calpain activity at 24 h probably suggesting autolysis of the enzyme. Considering all this and variations in tenderness, not much can be concluded from the results on m-calpain and its effect on tenderness when considering other results in this regard.

4.6 CONCLUSION

The study confirmed that the negative effect on meat tenderness by the beta-agonist, zilpaterol, was mainly caused by increased calpastatin activity. Electrical stimulation improved loin tenderness of both beta-agonist supplemented and non-supplemented animals. This effect was mainly mediated through early onset of rigor, triggering the CDP system and advancing tenderisation in general. In addition, ES advanced the tenderisation process of Z by reducing the activity of calpastatin, but could not completely cancel out the effect of Z on the aging process.

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CHAPTER 5: THE EFFECTS OF A BETA-AGONIST TREATMENT, VITAMIN D_3 SUPPLEMENTATION AND ELECTRICAL STIMULATION ON MEAT QUALITY OF FEEDLOT STEERS.

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5.1 ABSTRACT

In this study, 20 young steers received no beta-agonist (C), 100 animals all received zilpaterol hydrochloride (Z), with 1 group only receiving Z while the other 4 groups received zilpaterol and vitamin D_3 at the following levels (IU/animal/day) and durations before slaughter: 7 million for 3 days (3D7M); 7 million for 6 days (6D7M); 7 million for six days with 7 days no supplementation (6D7M7N) and 1 million for 9 days (9D1M). Left carcass sides were electrically stimulated (ES) and the right side not stimulated (NES). Samples were aged for 3 or 14 days *post mortem*. Parameters included Warner Bratzler shear force (WBSF), myofibril filament length, sarcomere length and calpastatin and calpain enzyme activity.

Both ES and prolonged aging reduced WBSF ($P < 0.001$). 6D7M, 6D7M7N and Z remained significantly tougher than C ($P < 0.001$), while 3D7M and 9D1M improved WBSF under NES conditions. ES is more effective to alleviate beta-agonist induced toughness than high vitamin D_3 supplements.

5.2 INTRODUCTION

A large portion of South African feedlot cattle are supplemented with a beta-agonist to improve feed efficiency and yield. Beta-agonists are known to affect meat tenderness (and other quality traits) negatively due to an increase in calpastatin activity (Koohmaraie, Shackelford, Muggli-Cockett, & Stone, 1991). Electrical stimulation (ES) could improve meat

tenderness by early activation of the calpain system (calcium-dependent proteases) (Hwang & Thompson 2001a). Hope-Jones, Strydom, Frylinck, and Webb (2010) showed that electrical stimulation combined with *post mortem* aging could improve, but not completely overcome, the negative effect of a beta-agonist on beef loin tenderness.

Various other attempts have been made to activate the calcium-dependent protease system and overcome meat tenderness problems. Wheeler, Koohmaraie, Lansdell, Siragusa, & Miller, (1993), Kerth, Miller, and Ramsey (1995) and others have used $CaCl_2$ injections in pre- and post-rigor cuts to activate intra-cellular µ- and m-calpain (Goll, Thompson, Taylor, & Zalewska., 1992; Koohmaraie & Shackelford, 1991). Koohmaraie and Shackelford (1991) also showed that $CaCl_2$ infusion was effective in overcoming beta-agonist induced toughness. In more recent years, supplementation of very high levels of vitamin D_3 over the final days before slaughter has been investigated by Montgomery, Carr, Kerth, Hilton, Price and Galyean (2002), Montgomery et al. (2004b) and others to increase plasma calcium levels by stimulating intestinal calcium absorption, mobilizing calcium from bone mineral and increasing renal re-absorption of calcium (as reviewed by Swanek et al., 1999). Higher blood and muscle calcium would lead to increased activation of the calpains and improved tenderness (Karges, Brooks, Morgan, Gill, Breazile, & Owens, 2001; Montgomery, Carr, Kerth, Hilton, Price & Galyean, 2002; Montgomery, King, Gentry, Barham, Barham & Hilton, 2004a). Various studies achieved success with the latter method but reports are not consistent with regards to the level and duration of supplementation as well as the magnitude of the effects. In addition, no studies have reported on vitamin D_3 supplementation to overcome the negative effects of beta-agonists. It is also yet to be verified if the effect of electrical stimulation and vitamin D_3 are additive.

In this trial we investigated various levels and durations of vitamin D_3 supplementation in an attempt to establish the best scenario in terms of cost, safety and efficacy. Our choices were based on previous studies with variable outcomes. Karges, Brooks, Morgan,

Gill, Breazile, & Owens (2001) used 6 million IU/animal per day for 4 days and 6 million IU/animal for 6 days resulting in increased blood plasma concentrations and improved beef tenderness. Tipton, King, Paschal, Hale & Savell (2007), on the other hand, used 3 million IU/animal per day for 5 days and found an increase in calcium levels but no improvement in WBSF. Tenderness did however improve when vitamin D_3 was withdrawn for 7 days after 5 days supplementation. The withdrawal also resulted in lower levels of vitamin D_3 in the liver, but higher levels of calcium were maintained improving tenderness. Montgomery, Carr, Kerth, Hilton, Price and Galyean (2002) reported positive effects for tenderness at a relatively low dose (0.5 IU/animal per day) of vitamin D_3 for 9 days without negatively affecting feedlot performance (feed intake) or tissue residues. We have used these scenario's to design our study and also added a short duration of 3 days at 7 million IU/animal per day in an attempt to take advantage of increased calcium levels before homeostasis could restore blood calcium to normal levels.

5.3 MATERIALS AND METHODS

5.3.1 Experimental design and feedlot phase

One hundred and twenty Bonsmara steers of approximately 9 months of age were raised on a commercial feedlot diet for 120 days. The animals were divided into six groups (n = 20) so that the average weight and variation was the same for all groups. The groups represented 6 treatments, namely a control (C), which received the feedlot diet only, while the five remaining groups were supplemented with the beta-agonist, zilpaterol hydrochloride, (Intervet/Schering-Plough Animal Health, South Africa) at 0.15 mg/kg live weight for 30 days during the final weeks of finishing. One of the five groups only received zilpaterol (Z), while the other four groups received zilpaterol and vitamin D_3 (vitamin D_3 500, Advit Animal Nutrition S.A. (Pty) Ltd, Sebenza, South Africa) at the following levels and durations before

slaughter (D = days supplemented; M = million IU; N = days withdrawn): 7 x 10^6 IU/animal /day for 3 days prior to slaughter (3D7M); 7 x 10^6 IU/animal /day for 6 days prior to slaughter (6D7M); 7 x 10^6 IU/animal /day for 6 days followed by 7 days of no supplementation prior to slaughter (6D7M7N) and 1 x 10^6 IU/animal /day for 9 days prior to slaughter (9D1M). Zilpaterol was withdrawn from feed 4 days prior to slaughter. The vitamin D_3 supplement was mixed with maize to increase the volume and supplied as (1 kg) top dressing on the daily supply of the feedlot diet (10 animals per pen). To ensure equal and complete consumption of the supplement, a clean bunk approach was followed, i.e. all feed was consumed before new feed was given each morning. All animals in each pen therefore approached the bunk at the same time and sufficient bunk space was provided so that all animals per pen could have access to feed at the same time.

5.3.2 Slaughter and sampling procedures

The animals were slaughtered at the abattoir of the Animal Production Institute (Agricultural Research Council, Irene, Gauteng Province). Blood was collected from all steers at exsanguination using sodium EDTA (parathyroid analysis) and lithium heparinised (calcium analysis) containers and stored on ice. After centrifugation, plasma and serum was stored at -18 °C until analysed. Carcasses were split and the left sides were electrically stimulated for 30 seconds (400 V peak, 5 ms pulses at 15 pulses per second) within 30 minutes of killing. Carcass sides were then chilled at 3 ± 2 ºC (chiller temperature at loading). Temperature and pH measurements (Eutech Instruments, CyberScan pH II pH/mV/°C meter) of the *M. longissimus lumborum* (LL) (reading taken at the last lumbar vertebra) were taken every hour for 4 h and a final measurement was taken at 18 h *post mortem*. All samples were collected from the LL on the day of slaughter, or after rigor mortis, depending on the purpose of the sample. The following tests were conducted: a) Meat tenderness, measured by Warner Bratzler shear force (WBSF), and myofibril fragment length (MFL) on LL aged 3 and 14 days *post mortem* at 2 ± 1 °C). b) Sarcomere length

measured at 1 day *post mortem*. c) Proteinase enzyme system measured as μ and m-calpain and calpastatin activity at 1 and 24 h *post mortem*.

5.3.3 Warner Bratzler shear force measurements

Aged samples for Warner Bratzler shear force (WBSF) were frozen at -20 °C and then processed into 30 mm steaks by means of a band saw. The frozen steaks were thawed at 2 ± 1 °C for 24 h and cooked using an oven-broiling (Mielé, model H217, Mielé & Cie, Gütersloh, Germany) method with direct radiant heat (American Meat Science Association (AMSA), 1995). The steaks were broiled at 260 °C (pre-set) to 70 °C internal temperature and cooled down to 18 °C. Six round cores (12.7 mm diameter) were removed from the steaks parallel to the muscle fibres (American Meat Science Association (AMSA), 1995). Each core was sheared once through the centre, perpendicular to the fibre direction, by a Warner Bratzler shear device mounted on an Universal Instron apparatus (Model 4301, Intsron Ltd, Buckinghamshire, England;.cross head speed = 200 mm/min) and the mean value of the 6 recordings were used as a shear value.

5.3.4 Histological measurements

Samples for sarcomere lengths of fresh LL samples (24 h *post mortem*), were prepared according to the method of Hegarty and Naudé (1970), by using distilled water instead of Ringer Locke solution (Dreyer, Van Rensburg, Naudé, Gouws, & Stiemie, 1979). Fifty sarcomeres per sample were measured by means of a video image analyses (VIA) using an Olympus B x 40 system microscope at a 1 000 x magnification equipped with CC12 video camera (Olympus, Tokyo, Japan). AnalySIS Life Science software package (Soft Imaging Systems Gmbh, Münster, Germany) was used to process and quantify measurements.

Myofibril fragment lengths (MFL) of LL aged for 3 and 14 days *post mortem* were measured by means of VIA. Myofibrils were extracted according to Culler, Parrish, Smith and Cross (1978) as modified by Heinze and Bruggemann (1994). One hundred myofibril fragments per sample were examined and measured with an Olympus B x 40 system microscope at a 400 x magnification.

5.3.5 Muscle biochemistry

Samples collected for enzyme studies (1 and 24 h *post mortem*) were snap-frozen in liquid nitrogen and preserved at -70 ºC. Calpastatin, µ-calpain and m-calpain were extracted from 5g of the LL frozen samples as described by Dransfield (1996) and separated by means of the two-step gradient ion-exchange chromatography-method according to Geesink and Koohmaraie (1999a). Calpain assays were determined by using azo-casein as substrate according to Dransfield (1996). The use of azo-casein eliminates the problem of background absorbance of non-specific proteins in the extracts. One unit of calpain activity was defined as an increase in absorbance at 366 nm of 1.0 per h, at 25 °C. One unit of calpastatin activity was defined as the amount that inhibited 1 unit of m-calpain activity. Data were expressed as units per gram of muscle.

5.3.6 Serum calcium and parathyroid analyses

Serum calcium concentrations were analyzed using a colorimetric assay kit (Roche, Mannheim, Germany). Plasma parathyroid hormone levels (PTH) were determined by electrochemiluminescence immunoassay employing a sandwich test principle on a Modular Analytics E 170 (Roche Diagnostic Systems, Nutley, NJ).

5.3.7 Statistical analyses

Data of WBSF, MFL, calpastatin, µ-calpain and m-calpain activities were subjected to analysis of variance for a split-plot design (GenStat® VSN International, Hemel Hempstead, UK; Payne, Murray, Harding, Baird, & Soutar, 2007) with the six treatment groups (C, Z, 3D1M, 9D1M, 6D7M7N, 6D7M) as whole plots and the two stimulation sub-treatments (ES and NES) and the two ageing periods (3 and 14 days *post mortem*) or time of analyses of enzyme activity (1 h and 24 h) as sub-plots. Data of sarcomere length was subjected to a similar analyses, six treatment groups (C, Z, 3D1M, 9D1M, 6D7M7N, 6D7M) as whole plots and only the two stimulation sub-treatments (ES and NES) as sub-plots. Means for the interactions between the whole plot and sub-plots were separated using Fisher's protected t-test least significant difference (LSD) at the 5% level (Snedecor & Cochran, 1980). The effect of pen allocation within each treatment was included in the statistical model but had no significant effect on any measurement and was therefore left out of the final model.

5.4 RESULTS

5.4.1 Effect of treatment, stimulation and aging on physical and histological characteristics

General statistics for the effects of treatment (C, 3D7M, 6D7M, 6D7M7N, 9D1M, Z), stimulation and aging on WBSF, MFL and sarcomere length are presented in Table 1. Treatment had a significant effect ($P < 0.001$) on WBSF. Both electrical stimulation (ES) and prolonged aging reduced WBSF significantly ($P < 0.001$) relative to no stimulation (NES) and aging for 3 days, respectively. A significant interaction ($P < 0.001$) occurred between treatment and stimulation ($P < 0.001$), treatment and aging ($P < 0.001$) and stimulation and

aging (P = 0.053) for WBSF. Stimulation had very little effect on the tenderness of the control, but did have a significant effect on all other treatments in particular the zilpaterol group, 6D7M7N and 6D7M (Fig. 1a). However, the variation (standard deviation) within non-stimulated and 3 days aged treatment groups were higher than within stimulated and 14 days aged groups (data not shown). Furthermore, stimulation also reduced the variation between mean values of all zilpaterol treated groups (zilpaterol and vitamin D_3 treatments). Nevertheless, the control still had a significant (P < 0.05) advantage over other treatments even after 14 days aging (Fig. 1a).

The control recorded shorter (P < 0.001) MFL's (more myofibrillar degradation) than the other treatment groups under any aging and stimulation scenario (Fig. 1b). Aging reduced MFL (P < 0.001) indicating myofibrillar breakdown through proteolyses. The longer MFL's (or no change) of stimulated samples (Fig. 1b), indicating less myofibrillar breakdown, were not expected as it contrasts the lower WBSF (more tender meat) of stimulated samples (Fig. 1a). This suggests the involvement of other tenderizing mechanisms. Neither treatment (P = 0.732) nor stimulation (P = 0.626) had a significant effect on sarcomere length.

Table 1

General statistics for the effects of treatment, stimulation and aging and their first order interactions on Warner Bratzler shear force (WBSF) and histological characteristics of *M. longissimus lumborum* (Data pooled for treatment, stimulation and *post mortem* aging; degrees of freedom parenthesized)

Effect	WBSF		Myofibrillar fragment length		Sarcomere length	
	Significance	*F* ratio	Significance	*F* ratio	Significance	*F* ratio
Main effect						
Treatment	*P* < 0.001	15.53 (5,54)	*P* < 0.001	6.81 (5,54)	*P* = 0.732	0.56 (5,114)
Stimulation	*P* < 0.001	118.37 (1,54)	*P* < 0.001	34.84 (1,54)	*P* = 0.626	0.24 (1,113)
Aging	*P* < 0.001	897.3 (1,108)	*P* < 0.001	933.9 (1,108)		
Interactions						
T x S	*P* < 0.001	6.89 (5,54)	*P* = 0.394	1.06 (5,54)		
T x A	*P* < 0.001	7.08 (5,108)	*P* = 0.071	2.10 (5,108)		
S x A	*P* = 0.056	3.74 (1,108)	*P* = 0.518	0.42 (1,108)		
T x S x A	*P* = 0.703	0.60 (5,108)	*P* = 0.375	1.08 (5,108)		

T: Treatment (control, zilpaterol and 4 vitamin D_3 supplemented groups)

S: Stimulation (stimulated and non-stimulated)

A: Aging (3 and 14 days *post mortem*)

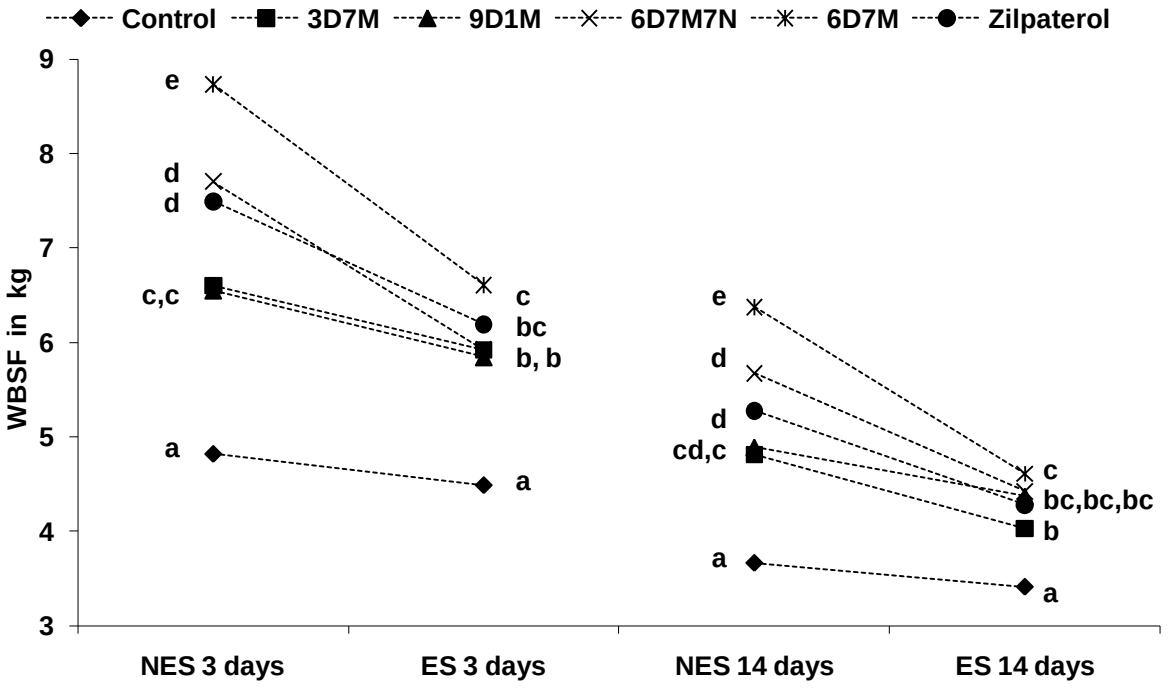

Fig. 1a. Interaction between treatment (Control, 3D7M, 9D1M, 6D7M7N, 6D7M, Zilpaterol), electrical stimulation (NES and ES) and *post mortem* aging (3 and 14 days) for Warner Bratzler shear force.

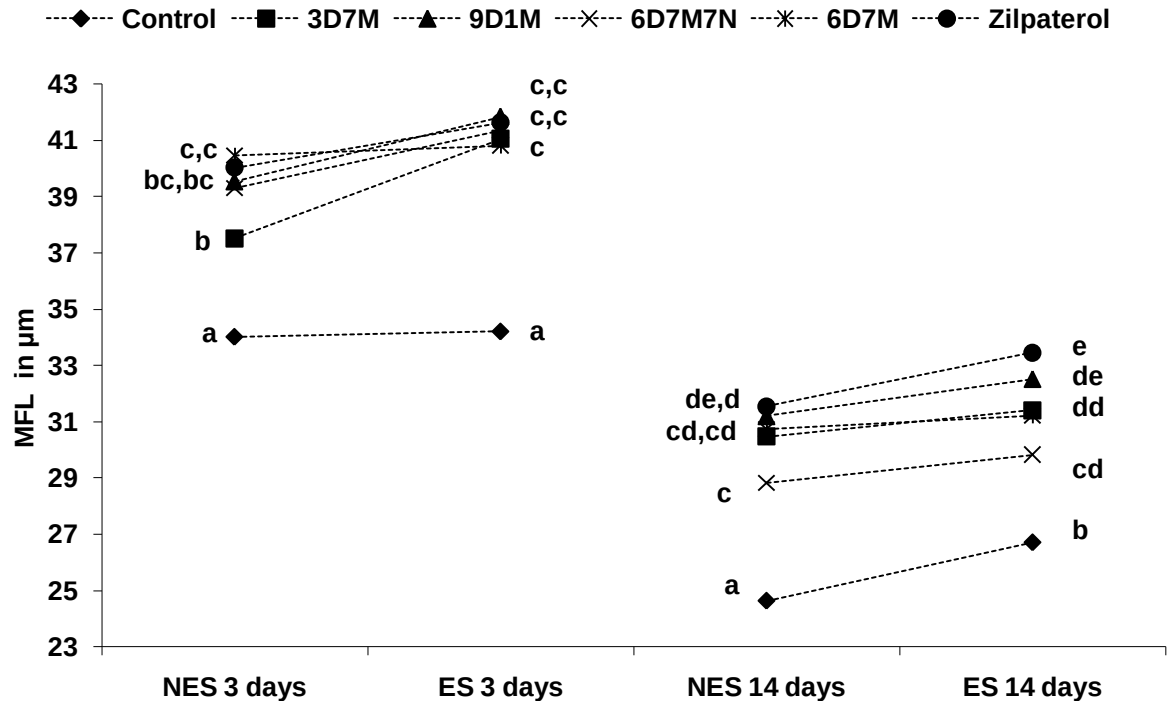

Fig. 1b. Interaction between treatment (Control, 3D7M, 9D1M, 6D7M7N, 6D7M, Zilpaterol), electrical stimulation (NES and ES) and *post mortem* aging (3 and 14 days) for Myofibril fragment length.

5.4.2 Effect of treatment, stimulation and aging on the calcium dependant proteinase system.

General statistics for the effects of treatment (C, 3D7M, 6D7M, 6D7M7N, 9D1M, Z), stimulation and time of measurement on the calcium dependent proteinase system (CDP) are presented in Table 2. Control samples had lower calpastatin values than zilpaterol and zilpaterol combined with vitamin D_3 samples independent of time of measurement (Fig. 1c). Stimulation reduced µ-calpain and calpastatin activity measured at 24 h in contrast to 1 h measurements (Fig. 1c and 1d) (Interaction: $P < 0.001$). Available µ-calpain activity was also lower at 24 h compared with 1 h recording, although the effect was much more pronounced for the control and 3D7M than for the other treatments (interaction: $P < 0.001$; Fig. 1d). Stimulation tended to increase m-calpain activity at 1 h and reduce activity at 24 h (Fig. 1e).

Table 2

General statistics for the effects of treatment, stimulation, time of measurement and their first order interactions on calpastatin and calpain activity of *M. longissimus lumborum* (degrees of freedom parenthesized)

Effect	Calpastatin activity[a]		μ-calpain activity[b]		m-calpain activity[b]	
	Significance	*F* ratio	Significance	*F* ratio	Significance	*F* ratio
Main effect						
Treatment	$P < 0.001$	13.09 (5,54)	$P = 0.005$	3.80 (5,54)	$P < 0.001$	4.94 (5,54)
Stimulation	$P < 0.001$	75.33 (1,54)	$P < 0.001$	89.32 (1,54)	$P = 0.018$	5094 (1,54)
Time of measurement	$P = 0.608$	0.26 (1,108)	$P < 0.001$	346.0 (1,108)	$P < 0.001$	328.1 (1,108)
Interactions						
T x S	$P = 0.489$	0.90 (5,54)	$P = 0.102$	1.94 (5,54)	$P = 0.346$	1.15 (5,54)
T x M	$P = 0.412$	1.02 (1,108)	$P < 0.001$	7.42 (5,108)	$P = 0.006$	3.49 (5,108)
S x M	$P < 0.001$	34.1 (1,108)	$P < 0.001$	83.86 (1,108)	$P < 0.001$	92.43 (1,108)
T x S x M	$P = 0.697$	0.60 (5,108)	$P = 0.977$	83.86 (1,108)	$P = 0.261$	92.43 (1,108)

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

[b] One unit of calpain activity is defined as an increase in absorbance at 366 nm of 1.0 absorbance unit per g of muscle per h, at 25 °C.

T: Treatment (control, zilpaterol and 4 vitamin D_3 supplemented groups)

S: Stimulation (stimulated and non-stimulated)

M: Time of measurement (1 and 24 h *post mortem*)

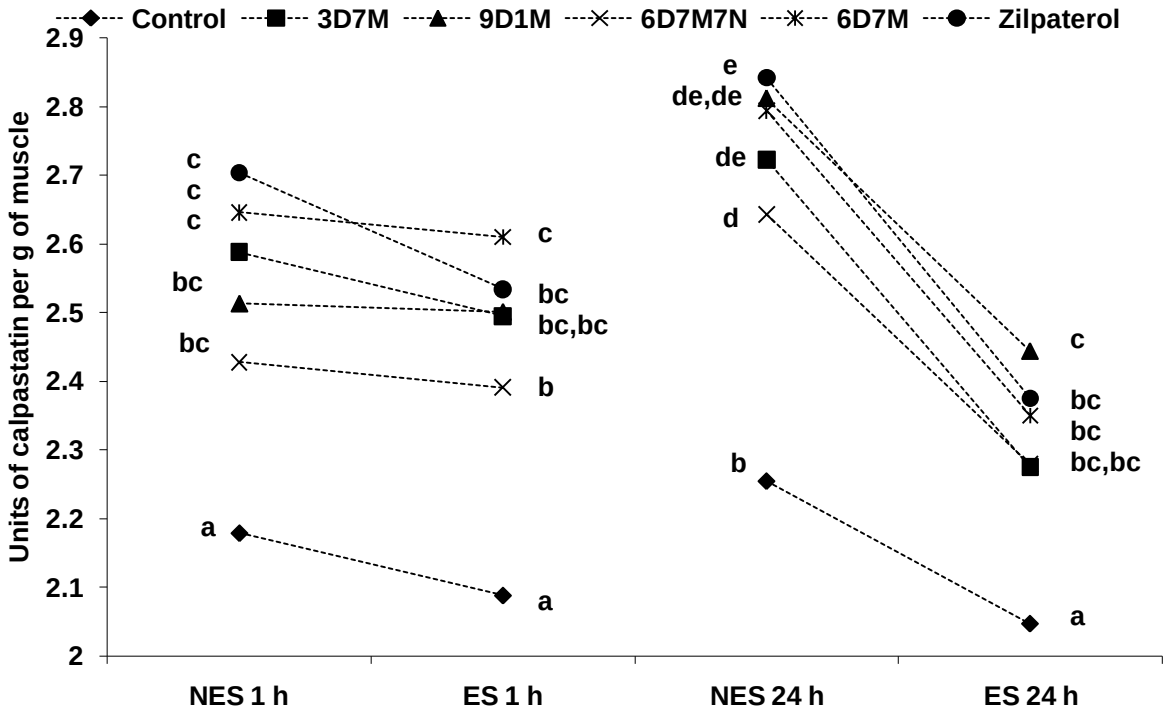

Fig. 1c. Interaction between treatment (Control, 3D7M, 9D1M, 6D7M7N, 6D7M, Zilpaterol), electrical stimulation (NES and ES) and time of measurement *post mortem* (1 h and 24 h) for Calpastatin activity.

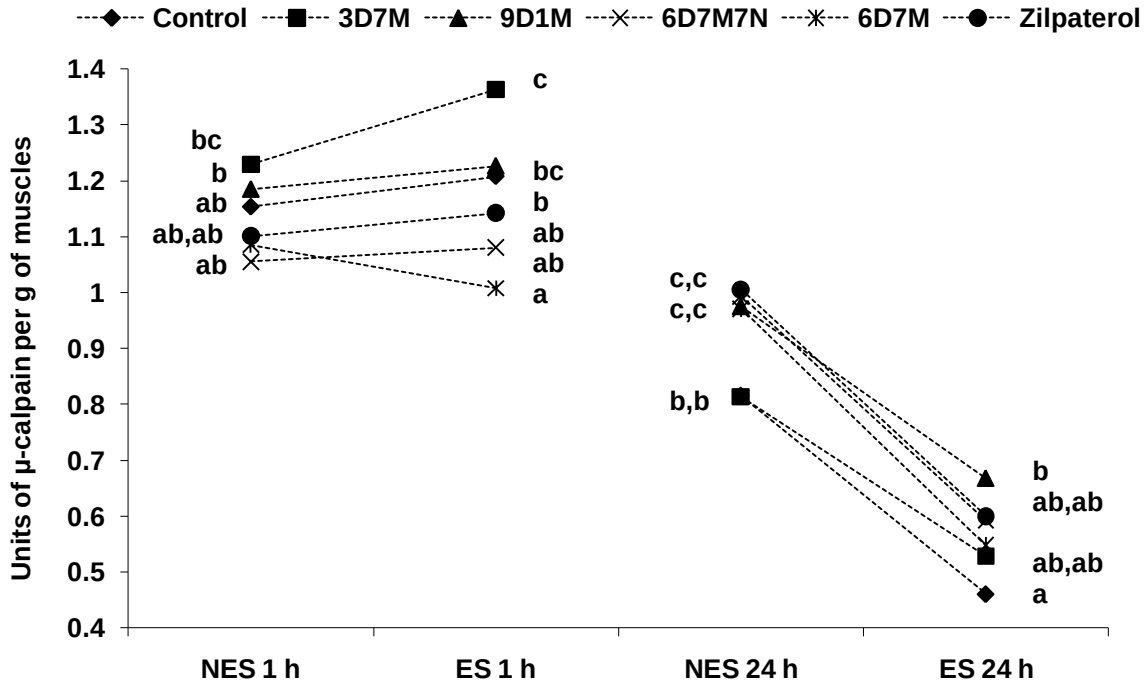

Fig. 1d. Interaction between treatment (Control, 3D7M, 9D1M, 6D7M7N, 6D7M, Zilpaterol), electrical stimulation (NES and ES) and time of measurement *post mortem* (1 h and 24 h) for µ-calpain activity.

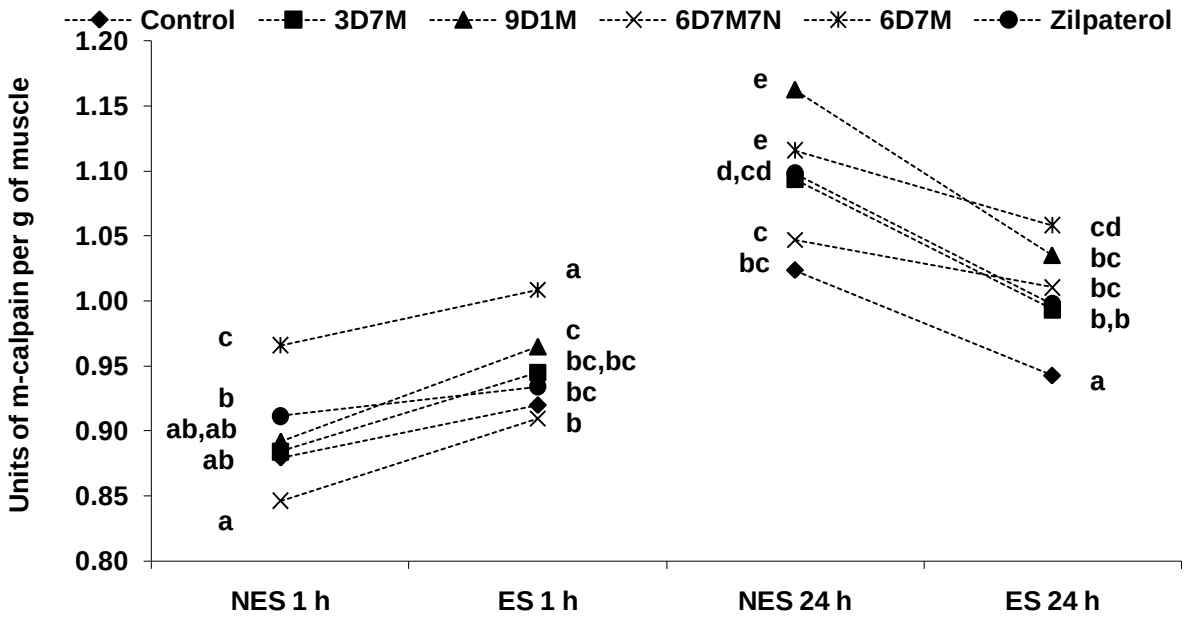

Fig. 1e. Interaction between treatment (Control, 3D7M, 9D1M, 6D7M7N, 6D7M, Zilpaterol), electrical stimulation (NES and ES) and time of measurement *post mortem* (1 h and 24 h) for m-calpain activity.

5.4.3 Effect of treatment on blood parameters.

Table 3 shows the effect of treatment on calcium (Ca) and parathyroid hormone (PTH) levels in the blood. All the vitamin D_3 supplemented groups showed higher blood Ca levels ($P < 0.001$) than the control and zilpaterol groups with the exception of 6D7M that did not differ from zilpaterol. PTH levels were higher for the control than for all vitamin D_3 treatments and zilpaterol, and zilpaterol alone had higher levels than all vitamin D_3 treatments.

Table 3:

Effect of treatment on serum calcium and parathyroid hormone (PTH).

Treatment	Control	Zilpaterol	Vitamin D_3 treatments				SEM[e]	*P* value
			3D7M	9D1M	6D7M7N	6D7M		
Calcium (mg/100 ml)	9.39[a]	9.57[ab]	10.85[c]	10.43[c]	10.76[c]	10.17[bc]	0.255	<0.001
PTH (pg/mL)	70.0[d]	49.4[c]	24.9[b]	13.2[ab]	5.9[a]	10.7[a]	4.36	<0.001

[a,b,c,d] Different superscripts in a row shows significant difference ($P < 0.05$)

[e] Standard error of means.

Control: no zilpaterol and no vitamin D_3 supplement.

Zilpaterol: 0.15 mg zilpaterol hydrochloride per kg live weight for 30 days during the final weeks of finishing.

Vitamin D_3 treatments: D = number of days supplemented; M = x million IU vitamin D_3/animal/day; N = number of days vitamin D_3 was withdrawn; all vitamin D_3 received 0.15 mg zilpaterol hydrochloride per kg live weight for 30 days during the final weeks of finishing.

5.5 DISCUSSION

The expectation that control samples would be more tender than zilpaterol samples was confirmed and this is in agreement with other studies showing the negative effects of beta-agonists on tenderness (Hilton et al., 2009; Rathmann et al., 2009; Strydom, Frylinck, Montgomery, & Smith, 2009). However considering the reported improvement in tenderness with high vitamin D_3 supplements in other studies (Swanek et al., 1999; Varges, Down, Webb, Han, Morgan, & Dolezal, 1999; Montgomery, Parrish, Beitz, Horst, Huff-Lonergan, & Trenkle, 2000; Montgomery, King, Gentry, Barham, Barham & Hilton, 2004a) the poor response of improvement in tenderness by vitamin D_3 in our study was unexpected. Closer examination of the various studies show an inconsistent response to vitamin D_3 supplement in any event related to dose or withdrawal times (Foote, Horst, Huff-Lonergan, Trenkle, Parrish, & Beitz, 2004; Lawrence et al., 2006, Wertz et al., 2004). In some studies even negative results were reported (sheep: Boleman, McKenna, Ramsey, Peel, & Savell, 2004;

Berry, Gill & Ball, 2000). The main difference between the present study and others is that it had a beta-agonist in the protocol that is known to affect tenderness and it is clear that vitamin D_3 supplement has very little effect on increasing tenderness under these conditions.

The argument behind supplementing super levels of vitamin D_3 would be to raise Ca levels above the blood Ca homeostasis level of 8 to 12 mg/dL (in cattle) but more particularly to increase free cytosolic Ca during early rigor and binding of this excess Ca at strategic places in the muscle cell that would increase calpain activity and increase myofibrillar proteolysis (Boehm, Kendall, Thompson, & Goll 1998; Montgomery et al., 2004b). Swanek et al. (1999) and Montgomery, Carr, Kerth, Hilton, Price and Galyean (2002) speculated that increased Ca recorded with high vitamin D_3 supplements may even be sufficient to activate m-calpain. Although the activities of Ca dependent proteases (CDP) and their relation to tenderness measurements have been inconsistent among studies it is suggested that the activation and subsequent proteolytic action of µ-calpain starts soon after killing (within the first 3 hours *post mortem*) and become undetectable after 3 days (Veiseth, Shackelford, Wheeler, & Koohmaraie, 2004). They also found that m-calpain remained constant throughout aging and calpastatin activity remained stable over the first 12h and then declined. Dransfield (1996) regarded the reduced recovered activity of calpains during rigor mortis and subsequent storage as unknown but important in relation to tenderization, while he accounted the reduction to either autolysis of activated calpain, a low extractability due to *post mortem* protein aggregation, or post rigor degradation. Several studies have demonstrated a negative relationship between calpastatin activity and proteolysis in *post mortem* muscle (Geesink & Koohmaraie,1999a; Geesink & Koohmaraie, 1999b; Koohmaraie, Shackelford, Muggli-Cockett & Stone, 1991) which corresponds with the increased calpastatin activities and reduced tenderness of zilpaterol treated steers in our study and our previous study involving zilpaterol and other beta-agonists (Strydom, Frylinck, Montgomery, & Smith, 2009). Following this line of reasoning, any CDP mediated effect of supplemented vitamin D_3 on tenderness of zilpaterol treated samples would therefore show

through increased action of calpains (possibly lower 24 h activities) and a consequent reduced activity of calpastatin (Doumit & Koohmaraie, 1999) at 24 h as was reported by Swanek et al. (1999; 7.5 million IU per day for 10 days). In our study, the best positive effect on calpastatin was recorded for 6D7M7N at 1 h, yet in all scenarios the calpastatin activities for vitamin D_3 treatments were still significantly higher than for the control (Fig. 1c). µ-calpain showed some variation at 1 h for stimulated samples but apart from a higher activity recorded for 3D7M, the variation in enzyme activity among all treatments did not agree with the response to vitamin D_3 reported by Swanek et al. (1999) nor did they relate to variation in tenderness among treatments as presented in Fig. 1a. Only the study of Tipton, King, Paschal, Hale, & Savell (2007) investigated the effect of vitamin D_3 under conditions tenderness could have been compromised (*Bos indicus*) and reported positive results on tenderness when vitamin D_3 was supplemented for 5 days and then withdrawn for 7 days. No effect was recorded when cattle were slaughtered directly after the 5 days supplement period. No CDP related tests were performed but serum and tissue Ca was significantly elevated when vitamin D_3 was withdrawn for 7, but not directly after the 5 day supplement. Despite elevated levels in serum Ca for 3D7M, 6D7M7N and 9D1M, it probably had little effect on free cytosolic Ca to activate sufficient µ-calpain to neutralize the effect of raised calpastatin activities caused by beta-agonists. M-calpain is generally regarded as having a minor or no part in proteolysis due to the unavailability of sufficient free Ca in *post mortem* muscle to activate the enzyme, and a lack of evidence of autolyzation (measured as decline in *post mortem* activity) and therefore proteolyses (Boehm, Kendal, Thompson, & Goll, 1998; Veiseth, Shackelford, Wheeler, & Koohmaraie, 2001; 2004). M-calpain activity increased between 1 h and 24 h in our study which is strange but not uncommon (Veiseth, Shackelford, Wheeler & Koohmaraie, 2001; 2004). Swanek et al. (1999) reported lower 24 h for µ- and m-calpain activities with associated improvement in tenderness for vitamin D_3 treated loins. They also argued that the levels of water-extractable muscle Ca recorded in their treated samples were sufficient to activate both µ- and m-calpain activities. We could

show no evidence that vitamin D_3 supplements increased Ca levels to affect m-calpain activity and subsequently WBSF tenderness.

In relation to increased Ca levels, 3D7M and 9D1M showed the best response in tenderness during early stages of aging and when samples were not stimulated, while 6D7M7N and 6D7M recorded tenderness values similar and higher than to zilpaterol samples, respectively. A plausible explanation for the extreme effect of 6D7M (and to a lesser extent 6D7M7N) on WBSF could be that the high levels of vitamin D_3 maintained for relatively long periods could have caused an over compensation and therefore a relative shortage of available Ca due to the reaction of regulating hormones. Decreased PTH levels in all vitamin D_3 groups, but particularly 6D7M and 6D7M7N, support this argument as this hormone decreases in response to increased serum Ca levels (Aranda-Osorio, 2002; Littledike & Goff, 1987).

In general, other reports showed very little consistent results with regard to dosages and duration of vitamin D_3 in relation to Ca levels and tenderness. Karges et al. (2001) tended to find better WBSF results with longer (6 vs. 4 days) supplement periods, while Scanga, Belk, Tatum & Smith (2001) reported elevated Ca levels with no response in tenderness improvement similar to our study. Montgomery, Carr, Kerth, Hilton, Price and Galyean (2002) showed a positive dose response to levels of vitamin D_3 for plasma Ca but trends for improved WBSF in the opposite direction, i.e. lower supplement levels gave numerically better WBSF results than increasing dosages.

Electrical stimulation (ES) had an overall positive effect on WBSF (lowered values) which coincided with lower 24 h calpastatin activity in general (Fig. 1c) and a larger reduction in available µ-calpain activity in ES samples between 1 and 24 h (Fig. 1d). This is in agreement with previous reports by Rosenvold et al. (2008), Dransfield, Etherington, & Taylor (1992) and Hwang & Thompson (2001a) who found that ES advances the onset of

rigor (pH = 6.1, Dransfield et al.,1992) where the activation of µ-calpain is initiated due to the release of Ca^{+} ions which causes proteolysis and tenderization (Ducastaing, Valin, Schollmeyer, & Cross, 1985). Changes in calpastatin and µ-calpain activities due to ES was accompanied by reduced variation in WBSF among all treatments groups and had the greatest effect on those treatments that were most compromised under NES conditions, such as 6D7M, 6D7M7N and zilpaterol (Fig. 1a). Similar results were reported by Ferguson, Jiang, Hearnshaw, Rymill and Thompson (2000) and Wheeler, Savell, Cross, Lunt and Smith (1990) when tenderness potential facilitated by the CDP system was compromised by breed (*Bos indicus*) effect. The negative breed effect was substantially reduced by ES on *Bos indicus* samples and the total effect was higher than for the non-*indicus* group. The combined effect of ES and aging in our study reduced the difference between the control and the other treatments to between 1.3 kg (6D7M) and 0.6 kg (3D7M) (Fig. 1a) but could not completely overcome the effect of zilpaterol on WBSF toughness. Furthermore none of the vitamin D_3 treatments differed from zilpaterol, although the 3D7M group recorded lower WBSF values than 6D7M ($P < 0.05$). Only two other studies used electrical inputs in combination with either vitamin D_3 or their metabolites (Lawrence et al., 2006; electrical inputs such as rigidity probe and immobiliser; Tipton, King, Paschal, Hale & Savall, 2007; dual system, 150 and 300 V, 28 s) but only Tipton, King, Paschal, Hale and Savall (2007) recorded a slight advantage recorded an advantage for vitamin D_3 treatments of 3 to 4 N (0.3 to 0.4 kg) above control samples. This agrees with our results that stimulation generally reduces variation in tenderness.

5.6 CONCLUSION

High vitamin D_3 supplement levels does not seem to be a viable option for improving meat tenderness in beta-agonist treated beef. Only a shorter but higher dose (3D7M) and a longer but lower dose (9D1M) of vitamin D_3 showed small but significant improvements in tenderness, under conditions of no electrical stimulation. The benefit of using electrical stimulation on its own should be less costly and show better results on improving beta-agonist treated beef compared to any vitamin D_3 treatment with no stimulation. Furthermore, with electrical stimulation, no added advantage of feeding vitamin D_3 is achieved. Even though stimulation is effective in reducing the effect of a beta-agonist on tenderness, the beta-agonist treated meat remains tougher than non-treated meat and should be avoided if meat quality is the primary objective.

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CHAPTER 6: EFFECT OF DIETARY BETA-AGONIST TREATMENT, VITAMIN D_3 SUPPLEMENTATION AND ELECTRICAL STIMULATION OF CARCASSES ON COLOUR AND DRIP LOSS OF STEAKS FROM FEEDLOT STEERS

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6.1 ABSTRACT

In this study, 20 young steers received no beta-agonist (C), 100 animals all received zilpaterol hydrochloride (Z), with 1 group only receiving Z while the other 4 groups received zilpaterol and vitamin D_3 at the following levels (IU/animal/day) and durations before slaughter: 7 million for 3 days (3D7M); 7 million for 6 days (6D7M); 7 million for six days with 7 days no supplementation (6D7M7N) and 1 million for 9 days (9D1M). Left carcass sides were electrically stimulated (ES) and right sides not (NES). Samples were analysed fresh or vacuum-aged for 14 days *post mortem*. Parameters included drip loss and instrumental colour measurements. In general, zilpaterol showed increased drip loss, lighter meat, and reduced redness. Vitamin D_3 supplementation could not consistently overcome these negative effects. All vitamin D_3 treatments reduced drip loss of stimulated aged steaks.

6.2 INTRODUCTION

Physical appearance of retail meat cuts during display is a critical link in the first step of the consumer satisfaction process. The bright red colour of red meat is the most important quality attribute to a consumer at the point of purchase (Jeremiah, Carpenter & Smith, 1972; Killinger, Calkins, Umberger, Feuz, & Eskridge, 2004; Kropf, 1980). In addition, so is unacceptable water holding capacity which results in increased drip loss in packaging that is unattractive to consumers, more susceptible to bacterial growth and that leads to deterioration of eating quality (Lagerstedt, Ahnström, & Lundström, 2011; Vázquez, Carriera,

Franco, Fente, Cepeda, & Barros-Velázquez, 2004). Various pre-harvest, harvest and post-harvest processes could affect the colour and water holding qualities of meat.

Beta-agonists are supplemented to feedlot cattle to improve weight gain and feed efficiency and increase carcass yield (Dunshea, D'Souza, Pethick, Harper, & Warner, 2005; Dikeman, 2007; Kellermeier et al., 2009; Shook et al., 2009). However, beta-agonists also induce muscle toughness in supplemented animals (Brooks et al., 2009; Holmer et al., 2009) especially in ruminants, mainly due to an increase in the activity of the inhibitor calpastatin and a reduction in calpain activity (Koohmaraie & Shackelford, 1991; Wheeler & Koohmaraie, 1997; Strydom, Frylinck, Montgomery & Smith, 2009). In addition, various studies have reported effects on other quality attributes like colour and water holding capacity (Avendaño-Reyes, Torres-Rodríguez, Meraz-Murillo, Pérez-Linares, Figueroa-Saavedra, & Robinson, 2006; Geesink, Smulders, Van Laack, Van der Kolk, Wensing, & Breukink, 1993; Gonzalez Johnson, Thrift, Savell, Ouellette, Johnson, 2009; Hilton, et al., 2009; Rogers et al., 2010; Vestergaard, Sejrsen, & Klastrup, 1994), which could probably be associated with the shift in fibre type composition (more glycolytic or white)(Geesink et al., 1993; Gonzalez et al., 2009; Strydom, Frylinck, Montgomery & Smith, 2009; Vestergaard, Henckel, Oksbjerg, & Sejren, 1994) and muscle hypertrophy (Kellermeier et al., 2009) caused by beta-agonists. It is also well-known that processes like electrical stimulation and *post mortem* aging may affect colour (Devine, Payne, Peachey, Lowe, Ingram & Cook, 2002; Ledward, 1985; Ledward, Dickinson, Powell & Shorthose, 1968; MacDougall, 1977; Renerre, 1990) and water holding capacity (Den Hertog-Meischke, Smulders, Van Logtestijn & van Knapen, 1997; Devine, 2009; Strydom, Frylinck, & Smith, 2005) and that this procedure combined with beta-agonists may have additive effects on these parameters (Geesink et al., 1993).

Supplementation with ultra-high levels of vitamin D_3 over the final days before slaughter has been used to improve meat tenderness in steers (Montgomery et al., 2002).

This method is motivated by the suggestion that an increased calcium ion level, stimulated by high vitamin D_3 levels (Swanek et al., 1999), contributes to meat tenderization directly by weakening of myofibrillar structures (Takahashi, 1992) as well as indirectly through activation of µ-calpain (Koohmaraie, 1992). Lahucky et al. (2007) showed that supplementation of vitamin D_3 could improve antioxidative capacity of pork loin muscle, thereby maintaining the cell structure, which could affect colour and water binding qualities of muscle.

The beta-agonist zilpaterol is approved for use in Mexico, South Africa and USA and, together with ractopamine, is probably the most commonly utilised beta-agonist in commercial beef production. In a recent study Strydom, Hope-Jones, Frylinck and Webb (2011) compared the effect of supplementation of ultra-high levels of vitamin D_3 on the tenderness (Warner-Bratzler shear force) of shorter (3 days) and longer vacuum-aged (14 days) beef loins from carcasses that were either not stimulated or subjected to electrical stimulation within 30 minutes of killing. As subpart of this study, we investigated the effect of various combinations of vitamin D_3 supplement on the instrumental colour and drip loss of fresh and vacuum-aged beef loin steaks. Our hypothesis is that the anti-oxidative protection of vitamin D_3 observed in pork could play a role in alleviating the negative effects of zilpaterol combined with other processes on moisture and colour quality parameters of beef loin.

6.3 MATERIALS AND METHODS

6.3.1 Experimental design and feedlot phase

One hundred and twenty Bonsmara steers (the same animals used for the experiment described in Chapter 5)of approximately 9 months of age were raised on a commercial feedlot diet for 120 days. The animals were divided into six groups (n=20) so that the average weight and variation was the same for all groups. The groups represented 6

treatments, namely a control (C), which received the feedlot diet only, while the five remaining groups were supplemented with the beta-agonist, zilpaterol hydrochloride, (Intervet/Schering-Plough Animal Health, South Africa) at 0.15 mg/kg live weight for thirty days during the final weeks of finishing. One of the five groups only received zilpaterol (Z), while the other four groups received zilpaterol and vitamin D_3 (Vitamin D_3 500, Advit Animal Nutrition S.A. (PTY) LTD, Sebenza, South Africa) at the following levels and durations before slaugher: 7 x 10^6 IU/animal /day for 3 days prior to slaughter (3D7M); 7 x 10^6 IU/animal /day for 6 days prior to slaughter (6D7M); 7 x 10^6 IU/animal /day for 6 days followed by 7 days of no supplementation prior to slaughter (6D7M7N) and 1 x10^6 IU/animal /day for 9 days prior to slaughter (9D1M). Zilpaterol was withdrawn from feed 4 days prior to slaughter. The vitamin D_3 supplement was mixed with maize meal to increase the volume and supplied as (1kg) top dressing on the daily supply of the feedlot diet (10 animals per pen). To ensure equal and complete consumption of the supplement, a clean bunk approach was followed, i.e. all feed was consumed before new feed was given each morning. All animals in each pen therefore approached the bunk at the same time and sufficient bunk space was provided so that all animals per pen could have access to feed at the same time.

6.3.2 Slaughter and sampling procedure

The animals were slaughtered at the abattoir of the Animal Production Institute (Agricultural Research Council, Irene, Gauteng Province). Carcasses were split and the left sides were electrically stimulated for 30 seconds (400V peak, 5ms pulses at 15 pulses per second) within 30 minutes of slaughter (ES) and the left sides were not stimulated (NES). Carcass sides were then chilled at 2 ± 2 ºC (chiller temperature at loading). Temperature and pH measurements (Eutech Instruments, CyberScan pH II pH/mV/°C meter) of the *M. longissimus lumborum* (LL) (reading taken at the last lumbar vertebra) were taken every hour for 4 h and a final measurement was taken at 18 h *post mortem*. All samples were collected from the LL on the day after slaughter. Measurements were performed on fresh

(24 h *post mortem*) or vacuum-aged samples (14 days *post mortem*; 2 ± 1°C). For both fresh and aged samples, a steak of 30 mm was sampled from the LL at the position of the last rib.

6.3.3 Colour and drip loss of fresh meat

Instrumental meat colour was measured with a Minolta meter (Model CR200, Osaka, Japan; 8mm diameter measuring area, diffuse 228 illumination and 0° viewing angel) on fresh samples (24 h *post mortem*) and vacuum-packed aged samples (14 days). The sampled 30 mm LL steaks were divided in two steaks of 15 mm thickness and one steak was allowed to bloom for 60 minutes at chiller temperatures (2 ± 1 °C) with its freshly cut surface facing upwards before colour recordings. Recordings were done in triplicate at three positions on the steak surface. Colour measurements followed the CIE colour convention, where the three fundamental outputs are L*, a* and b*. L* is lightness on a scale of 0 (all light absorbed) to 100 (all light reflected); a* spans from +60 (red) to -60 (green) and b* spans from +60 (yellow) to -60 (blue). Saturation index, also known as chroma, was calculated as square root of $a^{*2} + b^{*2}$ and hue angle, defined as $\tan^{-1}$ (b/a) that describes the fundamental colour of a substance (MacDougall, 1977). Mean values were used for statistical analysis.

Two cubes of 10 x 10 x 20mm were cut from the remaining fresh steak used for colour measurement to determine drip loss of fresh loin muscle. The cubes were suspended on a pin inside a sample bottle (200ml) taking care that the meat did not touch the sides of the bottle and stored for 3 days at 2 ± 1 °C. The amount of drip measured between 24 h and 96 h *post mortem*, as the difference between the sample mass before and after, was expressed as a percentage of the starting mass. Drip loss or purge for aged samples were determined by measuring the amount of purge remaining in the vacuum bag after removing the 30 mm LL steak for colour measurement. The steak was removed and lightly dried with tissue

paper. Drip was expressed as a percentage of the combined mass of the aged steak and the drip.

6.3.4 Statistical analyses

Data of colour and drip loss were subjected to analysis of variance for a split-plot design (GenStat® VSN International, Hemel Hempstead, UK; Payne, Murray, Harding, Baird & Soutar, 2007) with the six treatment groups (C, Z, 3D1M, 9D1M, 6D7M7N, 6D7M) as whole plots and the two stimulation sub-treatments (ES and NES) as a sub-plots and the two aging periods (24 h and 14 days). Means for the interactions between the whole plot and sub-plots were separated using Fisher's protected t-test least significant difference (LSD) at the 5% level (Snedecor & Cochran, 1980). The effect of pen allocation within each treatment was included in the statistical model but had no significant effect on any measurement and was therefore left out of the final model.

6.4 RESULTS

6.4.1 Effect of treatment, stimulation and vacuum-packed aging on drip loss

Treatment (P = 0.024), electrical stimulation (P < 0.001) and aging (P < 0.001) had significant effects on drip loss (Table 1). Furthermore, significant interactions between treatment and electrical stimulation (P < 0.001), treatment and aging (P = 0.053) and between electrical stimulation and aging (P = 0.014) were recorded. According to Table 4 and 5, drip loss was generally higher for stimulated samples than for non-stimulated samples and vacuum-aging increased drip loss compared to fresh samples.

Fresh control samples recorded lower drip loss than zilpaterol samples (Table 4; $P < 0.05$). Drip values of vitamin D_3 samples were closer to those of zilpaterol samples, except for non-stimulated 6D7M7N that was the same as the non-stimulated control. Zilpaterol samples showed the largest increase in drip when carcass sides were stimulated and 9D1M showed no difference between the two applications (ES and NES). Aged non-stimulated samples of all treatments recorded similar drip loss values irrespective of treatment (Table 5). However for aged stimulated samples, zilpaterol showed a significant increase ($P < 0.05$) in drip loss, while the control and vitamin D_3 samples recorded smaller increases ($P < 0.05$ for 6D7M7N only).

Table 1

General statistics for the effects of treatment, electrical stimulation and aging and their first order interactions for drip loss of the *M. longissimus lumborum* (Data pooled for treatment, electrical stimulation and *post mortem* aging; degrees of freedom for main effects and error in parenthesis)

Effect	Drip loss		
	Significance	*F* ratio	SEM[a]
Main effect			
Treatment	$P = 0.024$	2.70 (5, 114)	0.1386
Stimulation	$P < 0.001$	69.08 (1, 114)	0.0516
Aging	$P < 0.001$	76.19 (1, 218)	0.0467
Interactions			
T x S	$P < 0.001$	4.69 (5, 114)	0.1650
T x A	$P = 0.053$	2.23 (5, 218)	0.1605
S x A	$P = 0.014$	6.13 (1, 218)	0.0696
T x S x A	$P = 0.442$	0.96 (5, 218)	0.2007

[a] Standard error of means

T: Treatment (control, zilpaterol and vitamin D_3 supplemented groups).

S: Stimulation (electrically stimulated and not electrically stimulated).

A: Aging (1 and 14 days *post mortem;* "1 day" represents the amount of drip measured between 24 h and 96 h *post mortem*).

Table 4

Mean values for the effects of treatment and electrical stimulation on drip loss and colour properties of fresh *M. longissimus lumborum* samples (24 h *post mortem*).

	Treatment					
	Control	9D1M	3D7M	6D7M	6D7M7N	Zilpaterol
Drip loss[e]						
NES	1.30[a]	1.98[b]	1.78[ab]	1.86[b]	1.34[a]	1.84[b]
ES	1.45[a]	2.07[bc]	2.18[bc]	2.35[c]	2.03[bc]	2.68[d]
Lightness						
NES	39.2[a]	41.2[c]	40.6[bc]	41.3[c]	40.2[b]	41.3[c]
ES	38.8[a]	41.3[c]	40.7[bc]	41.0[c]	40.1[b]	41.5[c]
Redness						
NES	14.8[b]	14.9[b]	14.7[b]	13.9[ab]	14.0[ab]	13.6[a]
ES	14.9[b]	15.0[b]	14.8[b]	14.1[ab]	14.6[b]	13.8[a]
Yellowness						
NES	6.46[ab]	6.88[b]	6.80[b]	6.56[ab]	6.30[a]	6.66[ab]
ES	6.22[a]	6.90[b]	6.78[b]	6.39[ab]	6.27[a]	6.45[ab]
Chroma						
NES	16.2[b]	16.4[b]	16.2[b]	15.4[ab]	15.4[ab]	15.2[a]
ES	16.1[b]	16.5[b]	16.3[b]	15.5[ab]	15.9[ab]	15.2[a]
Hue angle						
NES	23.5[b]	24.8[c]	24.8[c]	25.3[c]	24.2[b]	26.1[d]
ES	22.7[a]	24.7[c]	24.6[c]	24.3[b]	23.2[ab]	25.1[c]

ES and NES = electrically stimulated and not electrically stimulated.

Control: no zilpaterol and no vitamin D_3 supplement.

Zilpaterol: 0.15 mg zilpaterol hydrochloride per kg live weight for 30 days during the final weeks of finishing.

Vitamin D_3 treatments: 3D7M, 9D1M, 6D7M, 6D7M7N : D = number of days supplemented; N = number of days vitamin D_3 was withdrawn; M = millions IU vitamin D_3/animal/day, all vitamin D_3 received 0.15 mg zilpaterol hydrochloride per kg live weight for 30 days during the final weeks of finishing.

[a,b,c,d] Means in the 2 rows of the same property and with different superscripts, differ significantly ($P < 0.05$).

[e] Measured as the amount of drip between 24 h and 96 h post mortem.

Table 5

Mean values for the effects of treatment and electrical stimulation on drip loss and colour properties of vacuum-aged (14 days *post mortem*) *M. longissimus lumborum* samples.

	Treatment					
	Control	9D1M	3D7M	6D7M	6D7M7N	Zilpaterol
Drip loss						
NES	2.20^{ab}	2.14^{ab}	2.12^{ab}	2.17^{ab}	2.01^{ab}	1.94^{a}
ES	2.49^{b}	2.74^{b}	2.52^{b}	2.92^{b}	2.88^{b}	3.66^{c}
Lightness						
NES	41.2^{bc}	42.0^{d}	41.3^{c}	42.5^{de}	39.8^{a}	42.9^{e}
ES	40.5^{b}	42.1^{d}	40.4^{ab}	42.4^{de}	40.5^{b}	42.4^{de}
Redness						
NES	18.7^{c}	17.5^{bc}	17.1^{b}	16.8^{b}	16.8^{b}	17.1^{b}
ES	18.2^{c}	16.8^{b}	15.8^{a}	16.9^{b}	16.1^{ab}	16.0^{a}
Yellowness						
NES	10.80^{c}	10.58^{bc}	10.55^{bc}	9.83^{ab}	9.84^{ab}	10.59^{bc}
ES	10.51^{bc}	10.27^{b}	9.70^{a}	9.79^{ab}	9.85^{ab}	9.99^{ab}
Chroma						
NES	21.6^{d}	20.4^{c}	20.1^{bc}	19.4^{b}	19.5^{b}	20.1^{bc}
ES	21.0^{cd}	19.7^{bc}	18.5^{a}	19.5^{b}	18.9^{ab}	18.9^{ab}
Hue angle						
NES	30.0^{a}	31.2^{b}	31.7^{b}	30.4^{ab}	30.4^{ab}	31.9^{b}
ES	30.0^{a}	31.4^{b}	31.4^{b}	30.2^{a}	31.1^{b}	31.8^{b}

ES and NES = electrically stimulated and not electrically stimulated.

Control: no zilpaterol and no vitamin D_3 supplement.

Zilpaterol: 0.15 mg zilpaterol hydrochloride per kg live weight for 30 days during the final weeks of finishing.

Vitamin D_3 treatments: 3D7M, 9D1M, 6D7M, 6D7M7N : D = number of days supplemented; N = number of days vitamin D_3 was withdrawn; M = millions IU vitamin D_3/animal/day, all vitamin D_3 received 0.15 mg zilpaterol hydrochloride per kg live weight for 30 days during the final weeks of finishing.

^{a,b,c,d,e} Means in the 2 rows of the same property and with different superscripts, differ significantly ($P < 0.05$).

6.4.2 Effect of treatment, stimulation and vacuum-packed aging on instrumental colour

Treatment, electrical stimulation and *post mortem* aging had significant effects on all aspects of instrumental colour measurements (Table 2 and 3). Furthermore, various interactions between the main effects were recorded.

The three way interaction between treatment, electrical stimulation and aging for lightness (L*) approached significance ($P = 0.088$; Table 2). In general, L* increased with aging for both stimulated and non-stimulated samples ($P < 0.001$) (Table 4 and 5). In fresh and aged samples electrical stimulation had no significant effect on L* except for 3D7M ($P < 0.05$) and C ($P < 0.10$) where electrical stimulation reduced the L* for aged samples compared to fresh samples. In fresh samples the control had lower ($P < 0.05$) L* values than all zilpaterol treatments (vitamin D_3 included) and 6D7M7N had lower values ($P < 0.05$) than Z, 9D1M and 6D7M (Table 4). Similar patterns among treatments were found for aged samples, except that the control, 3D7M and 6D7M7N were grouped together and non-stimulated 6D7M7N samples recorded uncharacteristically low L* values for non-stimulated samples ($P < 0.05$)(Table 5).

Vacuum-aged samples recorded higher ($P < 0.001$) values for redness, yellowness, chroma and hue angle than fresh samples. Aging interacted significantly with electrical stimulation and treatment for redness, yellowness, chroma and hue angle ($P < 0.001$; Table 2 and 3).

In fresh samples electrical stimulation had no effect on redness, yellowness or chroma. Zilpaterol recorded the lowest and the C, 9D1M and 3D7M the highest ($P < 0.05$) values for redness (Table 4). Redness values for 6D7M and 6D7M7N were closer to zilpaterol, except

for electrically stimulated 6D7M7N samples that showed slightly higher values than non-stimulated samples.

In aged samples electrical stimulation tended to decrease redness values ($P < 0.05$ for zilpaterol and 3D7M), while 6D7M was not affected by electrical stimulation (Table 5). The control samples recorded higher redness values ($P < 0.05$) than all other treatments. The range in yellowness values were less than 1 unit in aged and fresh samples (Table 4 and 5). In aged samples only 3D7M showed lower ($P < 0.05$) values for stimulated samples. Chroma values mirrored redness values for the different treatment combinations. Hue values for zilpaterol samples indicated a greater shift (higher hue angle) from red to yellow than control samples in fresh and aged samples (Table 5). In fresh samples electrical stimulation tended to reduce hue angle (closer to red) ($P < 0.05$; for control and zilpaterol) but this effect was not evident in aged samples (Table 4).

Table 2

General statistics for the effects of treatment, electrical stimulation and aging and their first order interactions on instrumental colour attributes of *M. longissimus lumborum* (Data pooled for treatment, electrical stimulation and *post mortem* aging; degrees of freedom for main effects and error in parenthesis)

Effect	Light reflection			Redness (a*)			Yellowness (b*)		
	Significance	*F* ratio	SEM[a]	Significance	*F* ratio	SEM[a]	Significance	*F* ratio	SEM[a]
Main effect									
Treatment	*P*<0.001	6.51 (5, 114)	0.3543	*P*<0.001	4.99 (5, 114)	0.2505	*P*<0.002	4.03 (5, 114)	0.1127
Stimulation	*P*=0.128	2.35 (1, 113)	0.0709	*P*<0.026	5.07 (1, 114)	0.0800	*P*<0.002	10.16 (1, 114)	0.0505
Aging	*P*<0.001	90.44 (1, 224)	0.0676	*P*<0.001	526.69 (1, 228)	0.0782	*P*<0.001	3711.2 (1, 228)	0.0422
Interactions									
T x S	*P*=0.147	1.67 (5, 113)	0.3749	*P*=0.398	1.04 (5, 114)	0.2862	*P*=0.459	0.94 (5, 114)	0.1426
T x A	*P*<0.001	9.23 (5, 224)	0.3731	*P*<0.001	6.12 (5, 228)	0.2848	*P*<0.001	6.31 (5, 228)	0.1343
S x A	*P*=0.501	0.45 (1, 224)	0.0980	*P*<0.001	16.45 (1, 228)	0.1118	*P*=0.044	4.11 (1, 228)	0.0658
T x S x A	*P*<0.088	1.94 (5, 224)	0.4099	*P*=0.497	0.88 (5, 228)	0.3444	*P*=0.204	1.46 (5, 228)	0.1762

[a] Standard error of means

T: Treatment (control, zilpaterol and vitamin D_3 supplemented groups)

S: Stimulation (electrically stimulated and not electrically stimulated)

A: Aging (1 and 14 days *post mortem*)

Table 3

General statistics for the effects of treatment, electrical stimulation and aging and their first order interactions on chroma and hue angle of *M. longissimus lumborum* (Data pooled for treatment, electrical stimulation and *post mortem* aging; degrees of freedom for main effects and error in parenthesis)

Effect	Chroma			Hue angle		
	Significance	*F* ratio	SEM[a]	Significance	*F* ratio	SEM[a]
Main effect						
Treatment	*P*<0.001	4.72 (5, 114)	0.2578	*P*<0.001	6.52 (5, 114)	0.2945
Stimulation	*P*=0.013	6.34 (1, 114)	0.0902	*P*<0.011	6.76 (1, 111)	0.0837
Aging	*P*<0.001	1093.2 (1, 228)	0.0844	*P*<0.001	3570.9 (1, 225)	0.0771
Interactions						
T x S	*P*=0.378	1.07 (5, 114)	0.3014	*P*=0.605	0.73 (5, 111)	0.3283
T x A	*P*<0.001	6.05 (5, 228)	0.2964	*P*<0.001	4.79 (5, 225)	0.3234
S x A	*P*<0.001	14.43 (1, 228)	0.1235	*P*<0.001	11.73 (1, 225)	0.1138
T x S x A	*P*=0.454	0.94 (5, 228)	0.3655	*P*=0.217	1.42 (5, 225)	0.3787

[a] Standard error of means

T: Treatment (control, zilpaterol and vitamin D_3 supplemented groups)

S: Stimulation (electrically stimulated and not electrically stimulated)

A: Aging (1 and 14 days *post mortem*)

6.5 DISCUSSION

In this study Strydom, Hope-Jones, Frylinck, and Webb (2011) showed that ultra-high dosages of vitamin D_3 could not overcome beta-agonist induced toughness (according to Warner-Bratzler shear force; WBSF), while electrical stimulation (ES) and/or *post mortem* aging reduced the difference in WBSF between zilpaterol and control samples significantly. In agreement with Avendaño-Reyes et al. (2006), Kellermeier et al. (2009) and Strydom et al. (2009), our study also showed that zilpaterol increased drip loss compared to control samples which was exacerbated by electrical stimulation especially in aged samples (Table 4). This combination of factors could be regarded as a worst case scenario for drip due to proliferation and/or hypertrophy of predominantly white fibres (fast glycolytic; FG) (Gonzalez et al., 2009; Strydom et al., 2009; Vestergaard et al., 1994; Wheeler & Koohmaraie, 1997) in beta-agonist treated animals that are more susceptible to protein denaturation (Maltin, Warkup, Matthews, Grant, Porter & Delday, 1997) especially when electrical stimulation is applied and glycolysis is accelerated. Our study also showed that the effect of electrical stimulation was enhanced with longer aging which was supported by the study of Simmons, Daly, Cummings, Morgan, Johnson and Lombard (2008). This effect was also more pronounced in steaks from zilpaterol-treated steers.

According to Table 4 vitamin D_3 (in particular 3D7M) reduced drip loss in aged stimulated steaks from zilpaterol-supplemented steers, while no other study on beef supported these results. Montgomery et al. (2002) found no effect with various supplementation levels of vitamin D_3 on free water content of beef loin. In addition, Montgomery et al. (2004) reported higher drip loss at 5 million IU vitamin D_3 supplemented for 8 days, which they attributed to higher proteolyses and instability of the muscle structure. Several studies on pork reported lower drip loss when high supplement levels of vitamin D_3 were used (Enright, Anderson, Ellis, McKeith, Berger, & Baker, 1998; Wilborn, Kerth, Owsley, Jones, & Frobish, 2004; Lahucky et al., 2007). The study of Lahucky et al. (2007)

associated the reduced drip loss with an increased anti-oxidative capacity in steaks from vitamin D_3-supplemented steers after incubation of muscle homogenates with Fe^{2+}/ascorbate and suggested that a higher level of Ca^{2+} (bivalent ion) due to vitamin D_3 in muscles were causing the positive influence on the lipid oxidation. Under similar increased anti-oxidative conditions obtained by vitamin E supplementation, Pettigrew and Esnaola (2001) suggested that the reduction in the oxidation of membrane lipids may improve the integrity of those membranes, and thus reduce fluid leakage. That would translate into reduced drip loss (increased water-binding capacity) and collectively these effects could explain the positive effects of vitamin D_3 seen in our study.

Paler meat, i.e. higher L* values, in steaks from zilpaterol-treated steers was expected. Firstly higher drip loss could increase surface moisture resulting in higher reflectance by the aqueous layer as reported by (Geesink et al., 1993). In addition, the shift towards whiter glycolytic fibre types and hypertrophy of these fibres caused by beta-agonist treatment (Gonzalez et al., 2009; Strydom et al., 2009; Vestergaard et al., 1994; Wheeler & Koohmaraie, 1997) would have reduced the heme iron pigment concentration and hence result in paler meat (Geesink et al., 1993). Certain vitamin D_3 treatments reduced the effect of the beta-agonist on lightness for both fresh and aged meat. In fresh samples, 6D7M7N, and to some extent 3D7M were closer to those of the control but still significantly higher ($P < 0.05$), but in aged meat the effects of both treatments were more pronounced. No effect on lightness was reported for vitamin D_3 supplemented beef in the literature (Montgomery et al., 2002; Montgomery et al., 2004; Reiling & Johnson, 2003), while Enright et al. (1998); Lahucky et al. (2007) (tendency; $P < 0.05$), Wiegand et al. (2002) and Wilborn et al. (2004). Wilborn et al. (2004) reported lower L* values for vitamin D_3 supplemented pigs. Wiegand et al. (2002) found no effect on day 1 *post mortem* but a significant effect on day 14 which agrees with our results for beef, in particular for 3D7m and 6D7M7N. None of the studies cited suggested a mechanism involved in the effect of vitamin D_3 but considering the relationship between drip loss, surface moisture and light reflection, it is reasonable to

believe that lower L* values in certain vitamin D_3 treatments was the result of lower drip loss which was more evident in aged samples. However, the reason for the lack of effect in other treatment combinations (9D1M and 6D7M) could not be explained.

Existing evidence for the effect of zilpaterol and other beta-agonists on the typical colour of meat (bright redness) is contradictive probably due to specific trial conditions in different studies. For zilpaterol in particular, Avendaño-Reyes et al. (2006) recorded lower values for redness (a*) and chroma and higher hue angle values for zilpaterol samples, in agreement with our results, although their colour recordings were done on thawed steaks that were previously frozen at -20°C. In addition, Hilton et al. (2009) recorded poorer visual colour scores and lower redness, yellowness and chroma values on freshly cut loin (probably not bloomed properly) during chiller assessment. However, better colour scores (brighter red) were recorded for steaks from zilpaterol-treated steers displayed under simulated retail conditions after vacuum-aging for 14 days. Rogers et al. (2010) also recorded more desirable visual colour scores and instrumental colour values for loin steaks from zilpaterol treated steers in a colour shelf life study, while VanOverbeke et al. (2009) found no effect of zilpaterol on visual colour and discolouration of displayed top sirloin steaks. Variable results were also recorded for other beta-agonists. Despite reduced heme iron concentrations in meat from clenbuterol-treated veal calves, Geesink et al. (1993) found no clear differences in a*- and b*-values between treated and non-treated animals. In contrast, Garssen, Geesink, Hoving-Bolink & Verplanke (1995) reported lower values for redness for steaks from clenbutarol and salbutamol-treated steers. The lower redness correlated with lower heme pigment and myoglobin which they attributed to a general shift in muscle fibre composition towards white muscle type accompanied by muscle fibre hypertrophy in steaks from beta-agonist-treated steers. In a colour shelf-life study, Gonzalez et al. (2009) reported poorer subjective scores for several muscles from ractopamine-treated steers. However, no differences in objective colour measurements, such as ratios of oxy- and metmyoglobin, percentage reduced nitric oxide metmyoglobin and Hunterlab a*- and b*-

values were reported despite an expected decrease in the metmyoglobin reducing ability (MRA) due to increased glycolytic fibre types with fewer mitochondria and less intracellular NADH in most of the studied muscles types of treated animals. NADH is key to colour development and stability in meat (Howlett & Willis, 1998). Since previous studies on the effect recorded a shift towards whiter glycolytic muscle fibre types (Strydom et al., 2009) in zilpaterol-treated steers, it is reasonable to believe that poorer instrumental colour values were due to lower MRA in the loin muscles of treated steers as discussed by Gonzalez et al. (2009).

The significant effect of aging on overall colour values (a*, b* and therefore chroma) in our study was unexpected as Renerre (1990) speculated that low oxygen partial pressure may exist in vacuum packs that will cause brown metmyoglobin (MetMb) due to higher oxidation rates. However, Hood (1980) and Ledward (1985) suggested that in fresh meat a high oxygen consumption rate (OCR) will favour the reduced form of myoglobin causing the formation of MetMb (Atkinson & Follet, 1973). Then, as the OCR decreases, so does MetMb formation during storage (Bendall & Taylor, 1972) and will become more dependent on the relative effectiveness of the enzymatic reducing system that is still active after several weeks *post mortem* and could therefore maintain colour. We speculate that it further explains the increased variation between the control and zilpaterol in aged samples since muscle with different fibre compositions would respond differently to these effects (Ledward, 1985). In the same line of reasoning, results from Ledward (1985) could explain why aged ES meat in our study tended to have lower values for redness (and chroma). High temperatures and low pH values found with electrical stimulation advance the depletion of OCR and MRA and hence the buffering effect of the latter will be less at the same stage of aging as non-stimulated meat.

Under these combined conditions of zilpaterol treatment, ES and aging, variable effects of vitamin D_3 supplementation on colour were recorded. In aged samples, only

9D1M and 6D7M in ES samples showed increased redness of 1 unit ($P < 0.05$), while in fresh ES and non-electrically stimulated (NES) samples, 3D7M and 9D1M (and 9D7M7N in ES) redness increased to similar levels as those of the control. Lahucky et al. (2007) (5 days PVC overwrapped display) and Wiegand et al. (2002) (vacuum-aged for 14 days) reported improved redness (CIE a*) values for vitamin D_3 supplemented pork loins (500,000 IU/day) and Wilborn et al. (2004) reported no effect on instrumental colour readings (a*), but recorded better visible colour scores in vitamin D_3 supplemented pigs. No effect on any colour attribute was recorded in the literature for beef (Montgomery et al. 2002; Montgomery et al. 2004, Reiling & Johnson, 2003). The anti-oxidative effect of vitamin D_3 discussed earlier for drip loss and lightness, was probably also involved here (Lahucky et al., 2007) with the small but positive response for selected treatment combinations. The specific conditions (aging and electrical stimulation) and variation in muscle composition as discussed by Ledward (1985) between the control and other treatments (zilpaterol with and without vitamin D_3) probably reduced the effect of certain vitamin D_3 treatments relative to the control. Since yellowness varied within a small range, chroma followed more or less the pattern of redness under the different scenarios. For hue angle, indicating the shift from redness to yellowness, 6D7M7N and 6D7M treatments showed some success in buffering the colour shift of zilpaterol treatments in aged and fresh meat.

6.6 CONCLUSION

In this study we confirmed the negative effects of the beta-agonist, zilpaterol, on drip loss and instrumental colour of loin steaks and that electrical stimulation and aging enhanced these negative effects on important meat quality traits. We hypothesized that very high levels of vitamin D_3 would exhibit anti-oxidative behaviour and alleviate negative effects of zilpaterol combined with other processes on moisture and colour quality of beef. This hypothesis was found to be true for drip loss under certain conditions but in particular when samples were stimulated and aged. For instrumental colour quality certain vitamin D_3 treatments gave

significant positive results but the effects were often small in magnitude and were also affected by aging and electrical stimulation. Considering the lack of positive effects on meat tenderness, ultra high levels of vitamin D_3 is probably not a viable option to improve the quality of meat that was compromised by feeding beta-agonists.

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CHAPTER 7: CONCLUSION

Vitamin D_3 has the potential to increase plasma calcium levels and therefore increase levels of calcium in the muscles, resulting in more calcium being available to activate the calcium dependant proteinases. Results regarding this effect of vitamin D_3 are however varied. One reason for this is the homeostasis mechanism in place to regulate plasma calcium levels which would have to be overcome by the correct dose and duration of vitamin D_3 supplementation. Even so, previous studies have shown that increased plasma calcium does not always lead to an increase in muscle calcium or any effect on the calcium dependent proteinase system. In addition, studies on vitamin D's ability to counteract the negative effects of beta-agonist supplementation are sparse.

In general electrical stimulation has its advantages in that it can counteract cold shortening where carcasses are chilled quickly. It can also result in more tender meat at an early stage without the prolonged aging. In this study we looked at the effect that both these methods had on the tenderness of meat from beta-agonist treated steers which would be tougher (when compared to control animals) due to an increased level of calpastatin activity, inhibiting the calcium dependent proteinase system. Another aim was to study the combined effects of these two treatments together (e.g. vitamin D supplementation in combination with electrical stimulation of carcasses) on meat tenderness.

In this study we have shown that the main reason for the decrease in tenderness of meat from beta-agonist supplemented animlas is due to an increase in the levels of calpastatin, which is an inhibitor of the calpains. Vitamin D_3 proved not to be the most effective method of counteracting this negative effect on tenderness. Only the short, high dose (3D7M) and the low dose over a long period of time (9D1M) showed significant but small improvements in tenderness, but only under conditions where no electrical stimulation was applied. The best positive effect on calpastatin was recorded for 6D7M7N at 1h *post*

mortem, yet in all the scenarios the calpastatin activities for vitamin D_3 treatments were still significantly higher than for the control group. Despite elevated levels in serum calcium for 3D7M, 6D7M7N and 9D1M, it probably had little effect on the free cytosolic calcium to activate sufficient µ-calpain to neutralize the effect of raised calpastatin activities caused by the beta-agonists. Vitamin D_3 also had only a small effect on the other quality traits (drip loss and colour) which were negatively affected by beta-agonist supplementation.

Electrical stimulation improved loin tenderness of both beta-agonist supplemented and non-supplemented animals and improved loin tenderness in general by the early onset of rigor by triggering the activity of the calpains. In addition electrical stimulation advanced the tenderisation process of steaks from the zilpaterol supplemented group of steers by reducing the activity of calpastatin and can therefore be implemented to improve meat tenderness of meat from zilpaterol supplemented steers. Electrical stimulation did however increase drip loss which also resulted in paler meat.

A significant interaction occurred between treatment and electrical stimulation. Electrical stimulation had very little effect on the tenderness of the control group, but had a significant effect on all the other treatment groups in particular on Z, 6D7M7N and 6D7M. Electrical stimulation reduced the variation between mean values for tenderness of all groups supplemented with zilpaterol. Furthemore, with electrical stimulation, no added advantage of feeding vitamin D_3 was achieved.

Electrical stimulation proved to be far more effective in improving meat tenderness compared to vitamin D_3 suplementation. The benefit of using electrical stimulation on its own is that it is less costly and easier to implement than vitamin D_3 supplementation. It also avoids the possible negative effects that vitamin D_3 can have on production due to a decrease in feed intake. However neither vitamin D_3 supplementation nor electrical

stimulation could completely counteract the effects of the beta-agonists and it is therefore advisable not to use zilpaterol if tenderness is the main focus of production.

CHAPTER 8: CRITICAL EVALUATION

The aim of this research project was to study the effects of dietary beta-agonist treatment of feedlot cattle in combination with vitamin D_3 supplementation and electrical stimulation of the carcasses on meat quality parameters. Although these effects have all been studied separately before, there are no studies on the combined effects of these treatment combinations on meat quality of South African feedlot cattle. This experiment also explored various doses and durations of vitamin D_3 supplementation and in addition was a large trial allowing us to properly study the interactions between treatments, therefore allowing us to conclude which treatments or combination of treatments could be best utilized to improve meat quality. The experiment also focussed on the calcium dependant proteinase system giving a better picture of the effect of each treatment (and their combinations) on meat tenderness.

Regarding cattle breed type, the Bonsmara breed was chosen as a model because it is a typical medium frame animal and the most common breed found in feedlots throughout the country. This breed is also representative of a large portion of South Africa's feedlot stock. There would however be some breed differences regarding zilpaterol supplementation in that its use would not be recommended for later maturing breeds as their carcasses would become too heavy before fattening occurs. Beta-agonist treatment is also not recommended in breeds such as the Brahman, which already produce tougher meat due to inherently higher levels of calpastatin activity. The results of the present study with Bonsmara type feedlot cattle, provides a good indication of what can be expected in the South African beef industry.

As far as the diet fed is concerned, the cattle in our trial were fed a commercial feedlot diet typical of the diet fed in many feedlots. This was to try and eliminate diet as a variable as much as possible. There were however differences between the site of the

experiment when compared with conditions at a commercial feedlot in that a commercial feedlot would have less space available per animal, less bunk space for feeding, and therefore there is a chance that shy feeders would have less intake of a particular supplement (in this case vitamin D_3). This is however an indication that the lack of response to vitamin D_3 supplementation in our trial, under controlled conditions, provides even stronger evidence that the current use of vitamin D_3 in commercial feedlots is a huge expense failure.

There are some changes or improvements that could have been made to the project. Firstly the trial could have included another treatment group of vitamin D_3 supplementation only. This could have shown if vitamin D_3 had any effect on improving meat tenderness when not under the challenge of zilpaterol supplementation. Muscle calcium could also have been measured in addition to the plasma calcium levels that were measured. This would possibly have given a better indication of whether increased plasma calcium levels led to an increase in calcium in the muscle and therefore to more calcium being available for the calcium dependent proteinase system.

It can once again be mentioned that so far no feed additive or pre- or post-slaughter practice has been able to improve tenderness of meat from steers supplemented with zilpaterol hydrochloride to the level of that of meat from steers not treated with the beta-agonist. A number of producers have already stopped the practice of supplementing with a beta-agonist which has resulted in the production of more tender meat being sold at a premium price. In South Africa however, the bulk of producers will continue to supplement with a beta-agonist to produce more meat per animal and therefore sell meat at a cheaper price to a population where red meat is often a luxury. It would therefore be beneficial to not only look at other methods of improving tenderness of meat from beta-agonist supplemented animals, but also to study the actual molecule more closely and gain a better knowledge of its mechanisms of action, thereby potentially being able to modify the molecule so that it

achieves the same results as a repartitioning agent but without the negative effects on meat tenderness.

This study ultimately showed that supplementation of vitamin D_3 had very little or no effect on meat quality. Consumers have shown that consistency in the quality of a product is important and with many feedlots across South Africa already supplementing vitamin D_3, in an attempt to improve meat tenderness, the results from this study are beneficial in proving that this expensive practice is doing little to improve meat quality and more importantly has shown that supplementation with vitamin D_3 leads to inconsistencies in meat quality. We have also shown that further research needs to be conducted to find other methods (other than or which could be additive to electrical stimulation) to improve tenderness of meat from zilpaterol supplemented steers in our country.