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 Zilpaterol was withdrawn from feed 4 days prior to slaughter.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.

3.9 REFERENCES

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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).

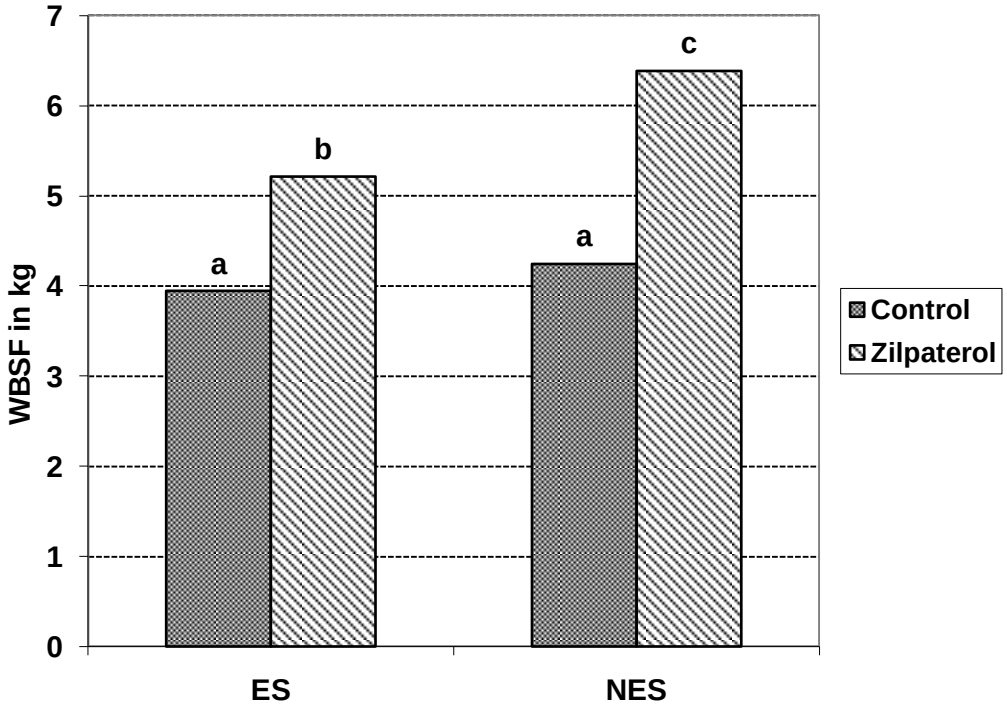

Fig. 1. Interaction between treatment (control and zilpaterol) and electrical stimulation in relation to Warner Bratzler shear force (WBSF; *P* = 0.003). (Bars with different superscripts differ significantly, *P* < 0.05; ES and NES = stimulated and non-stimulated)

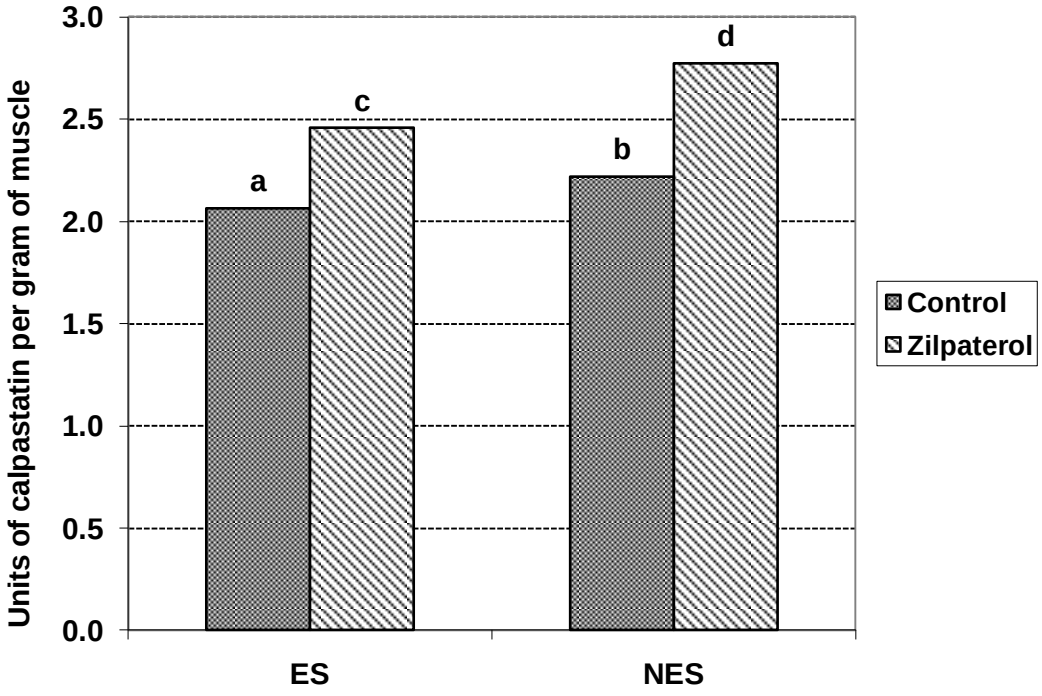

Fig. 2. Interaction between treatment (control and zilpaterol) and electrical stimulation in relation to calpastatin activity (*P* = 0.015). (Bars with different superscripts differ significantly, *P* < 0.05; ES and NES = stimulated and non-stimulated)

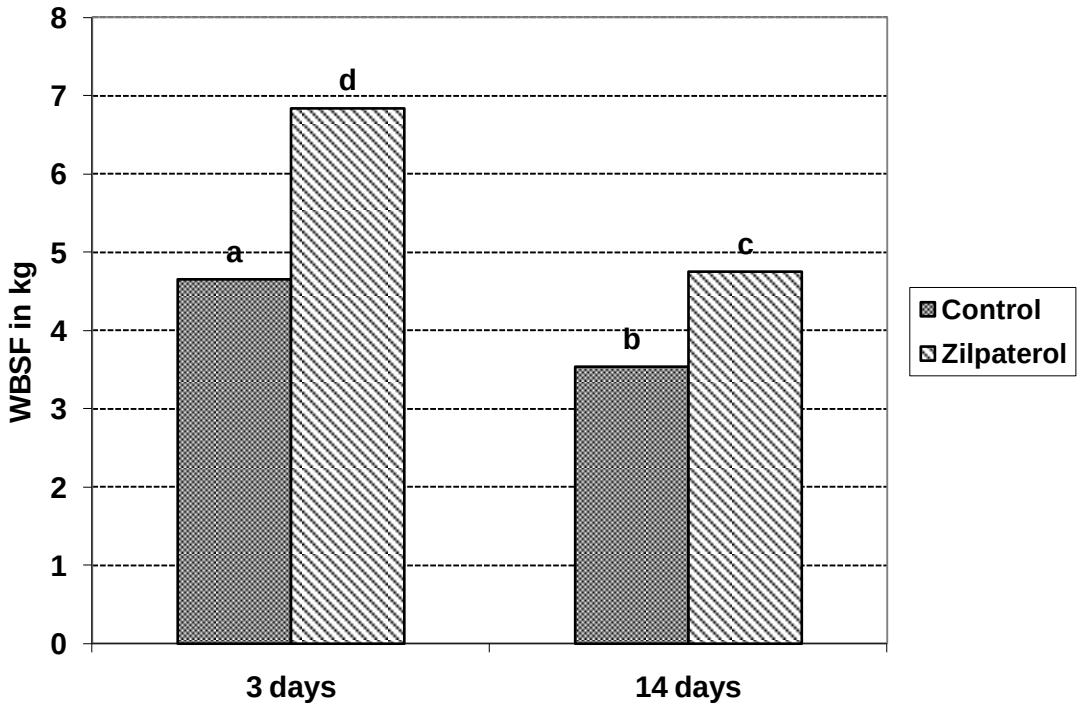

Fig. 3. Interaction between treatment (control and zilpaterol) and *post mortem* aging (3 days and 14 days) in relation to Warner Bratzler shear force (WBSF; $P < 0.001$). (Bars with different superscripts differ significantly, $P < 0.05$)

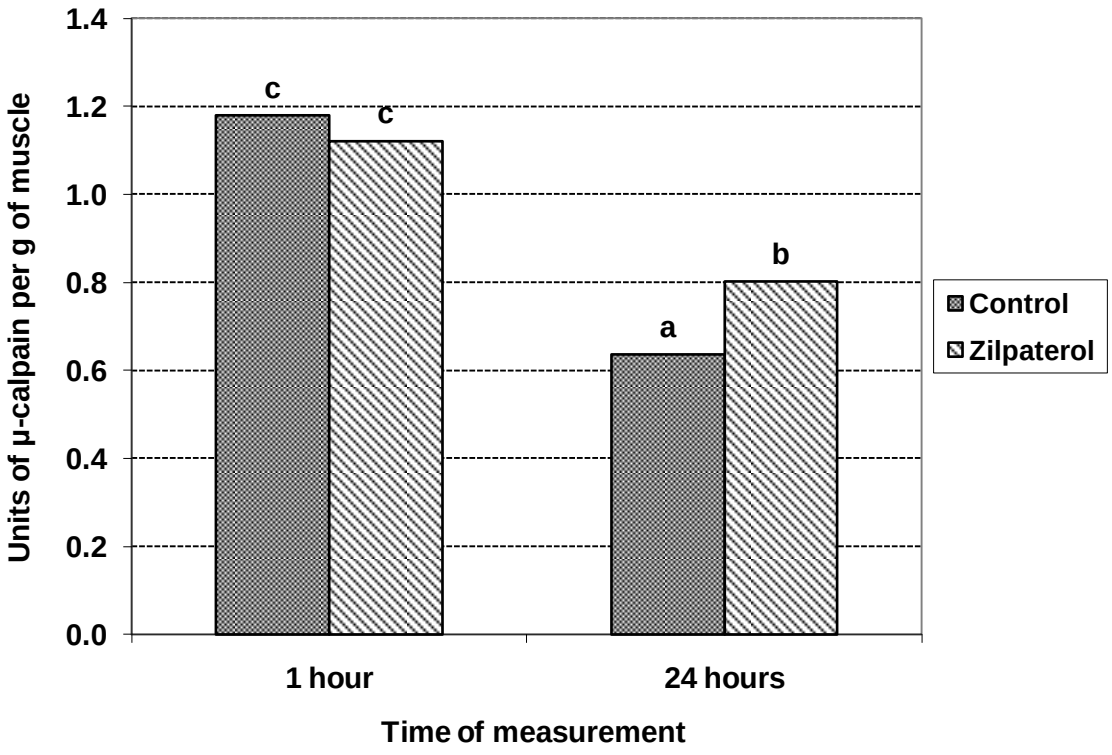

Fig. 4. Interaction between treatment (control and zilpaterol) and time of measurement (1 and 24 hours *post mortem*) in relation to µ-calpain activity ($P < 0.002$). (Bars with different superscripts differ significantly, $P < 0.05$)

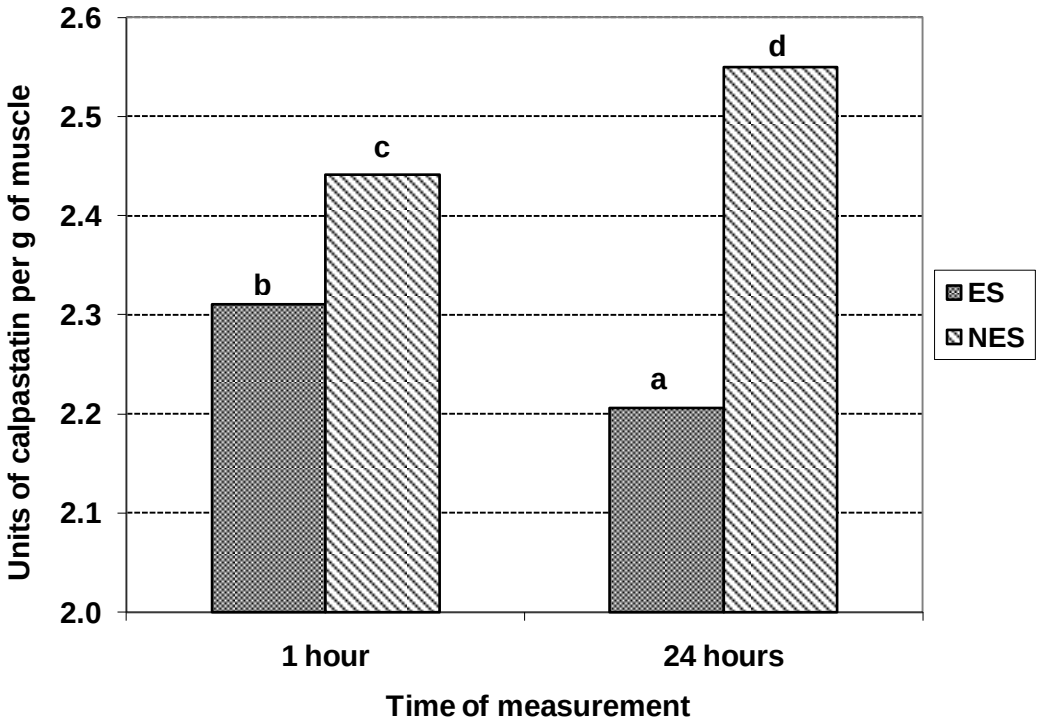

Fig. 5. Interaction between stimulation and time of measurement (1 and 24 hours *post mortem*) in relation to calpastatin activity (P = 0.014). (Bars with different superscripts differ significantly, P < 0.05; ES and NES = stimulated and non-stimulated)

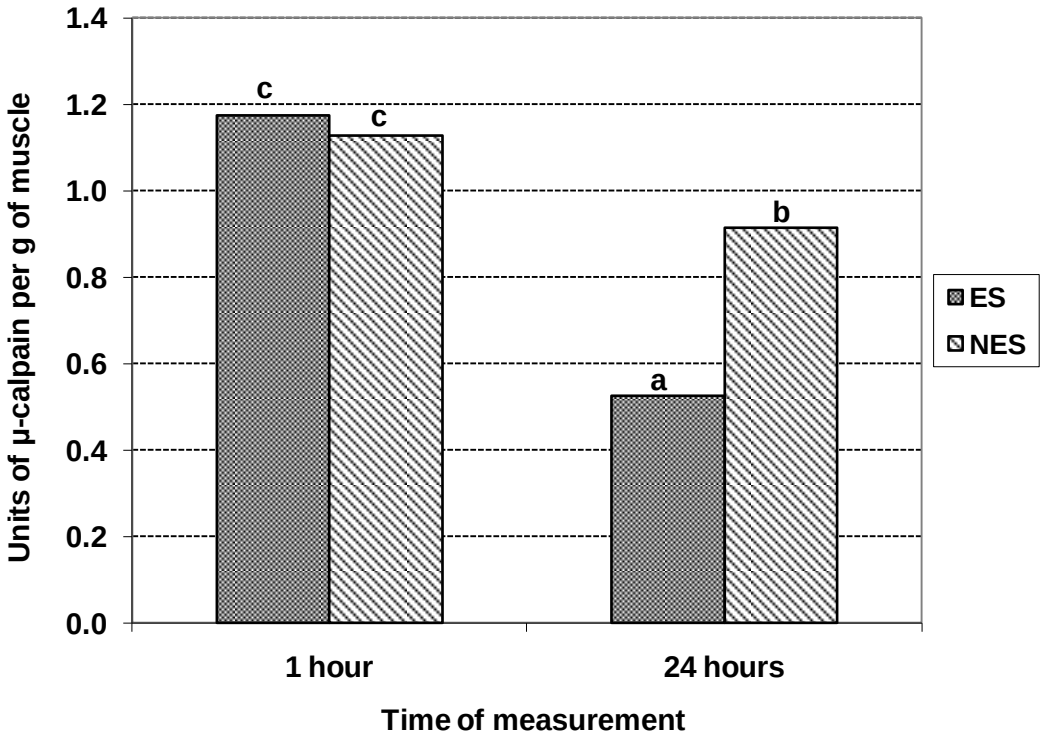

Fig. 6. Interaction between stimulation and time of measurement (1 and 24 hours *post mortem*) in relation to µ-calpain activity (P < 0.001). (Bars with different superscripts differ significantly, P < 0.05; ES and NES = stimulated and non-stimulated)

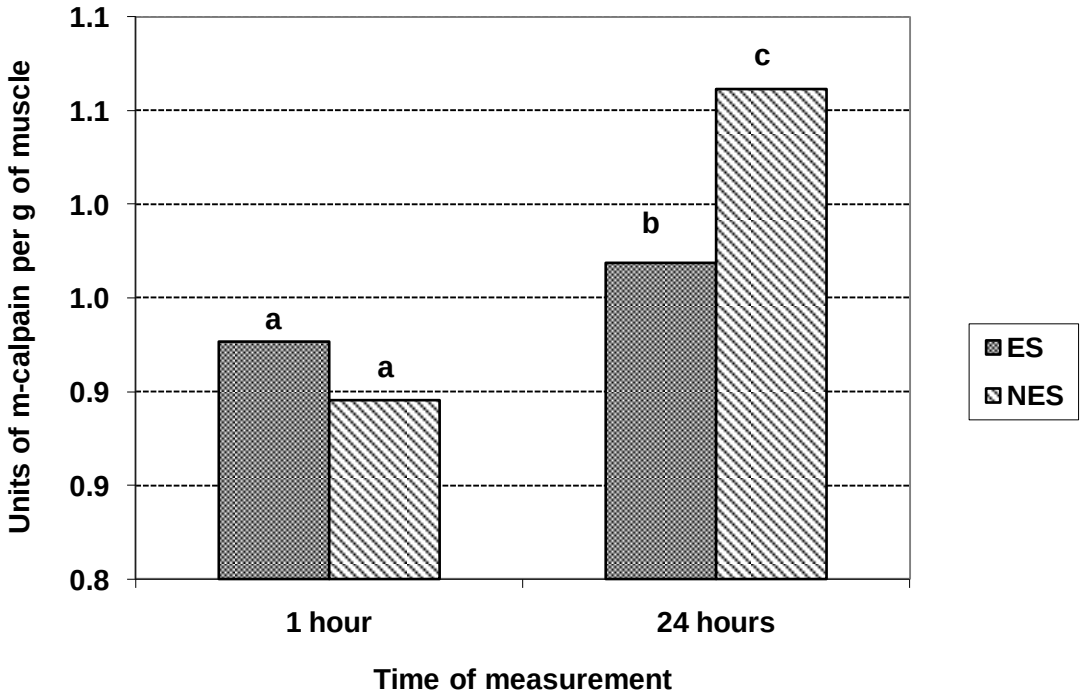

Fig. 7. Interaction between stimulation and time of measurement (1 and 24 hours *post mortem*) in relation to m-calpain activity ($P < 0.001$). (Bars with different superscripts differ significantly, $P < 0.05$; ES and NES = stimulated and non-stimulated)

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.

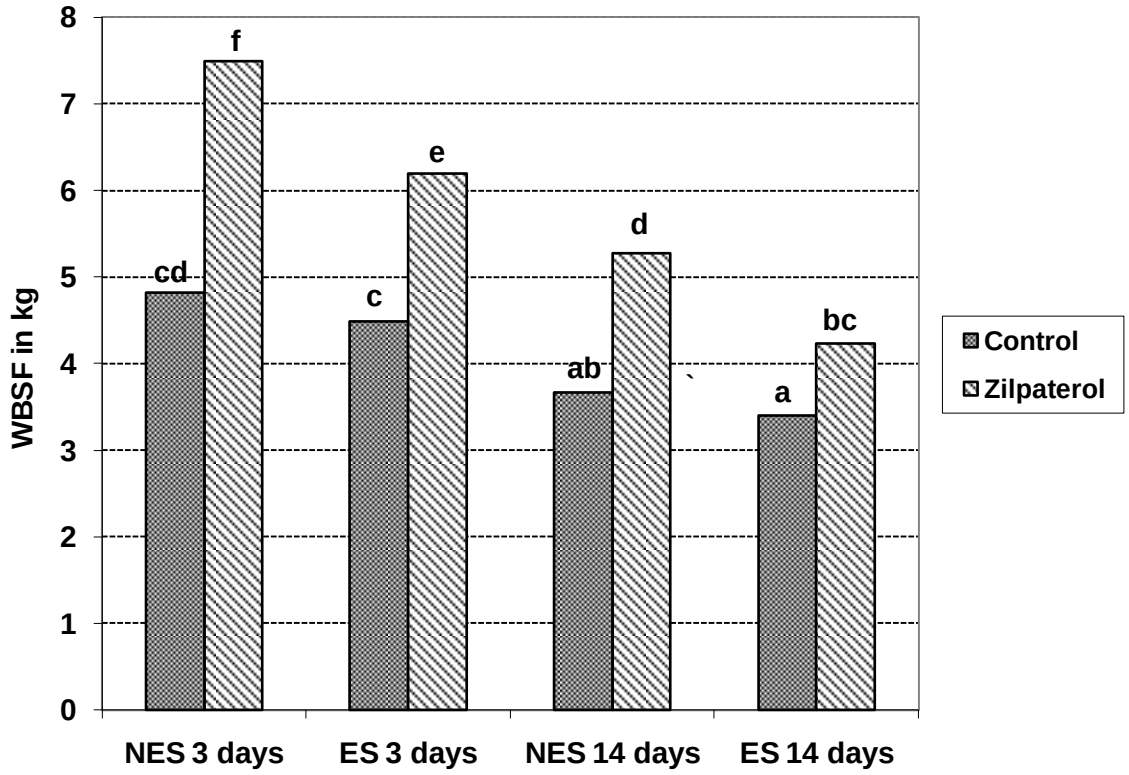

Fig. 8. Interaction between treatment (control and zilpaterol) and electrical stimulation and *post mortem* aging (3 and 14 days) in relation to Warner Bratzler shear force (WBSF; $P = 0.610$). (Bars with different superscripts differ significantly, $P < 0.05$; ES and NES = stimulated and non-stimulated)

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.

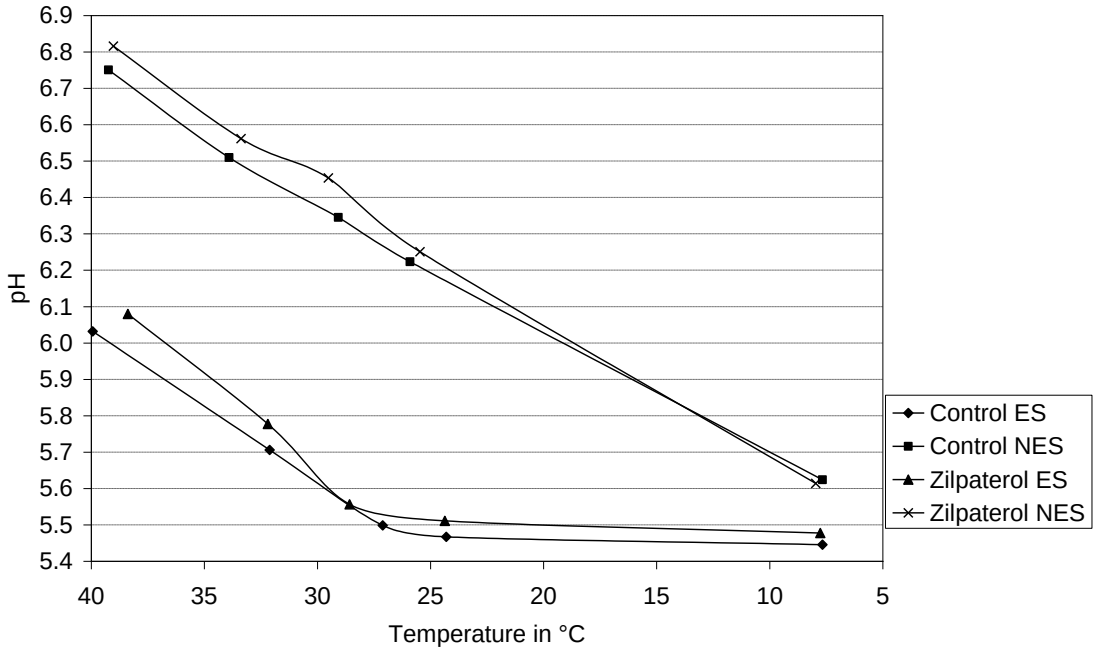

Fig. 9. pH/temperature dynamics of the *M. longissimus lumborum* for different combinations of treatment and stimulation (ES and NES = stimulated and non-stimulated; data points represent recordings at 1, 2, 3, 4 and 18 hours *post mortem*)

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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