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*)

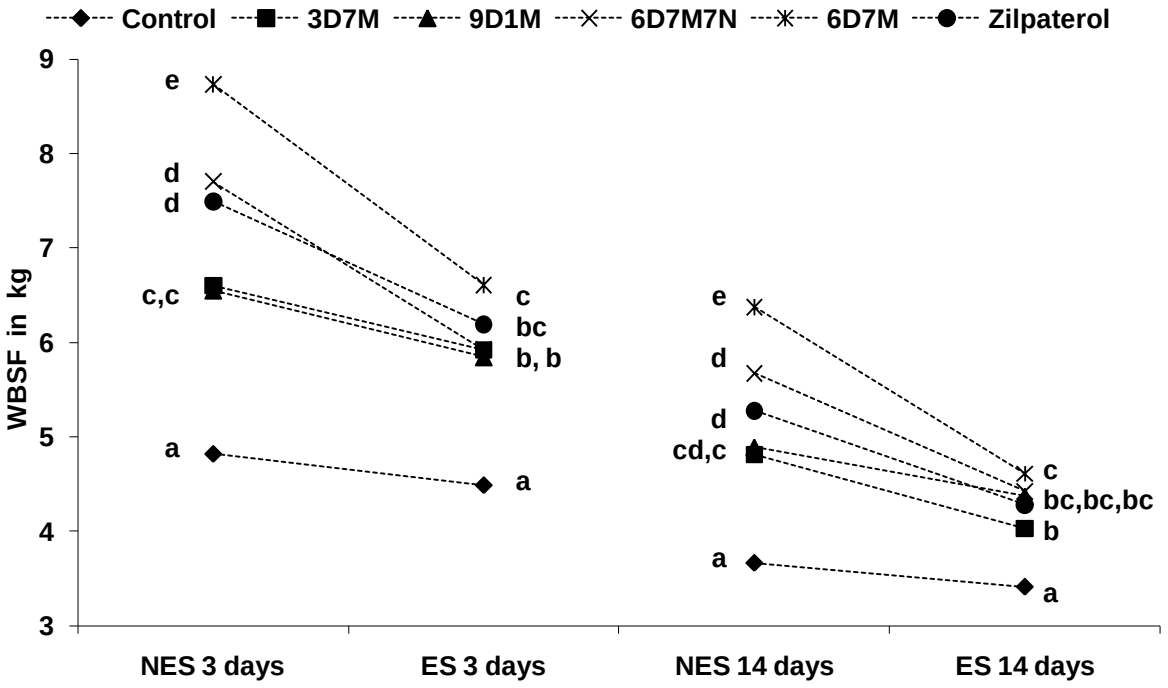

Fig. 1a. Interaction between treatment (Control, 3D7M, 9D1M, 6D7M7N, 6D7M, Zilpaterol), electrical stimulation (NES and ES) and *post mortem* aging (3 and 14 days) for Warner Bratzler shear force.

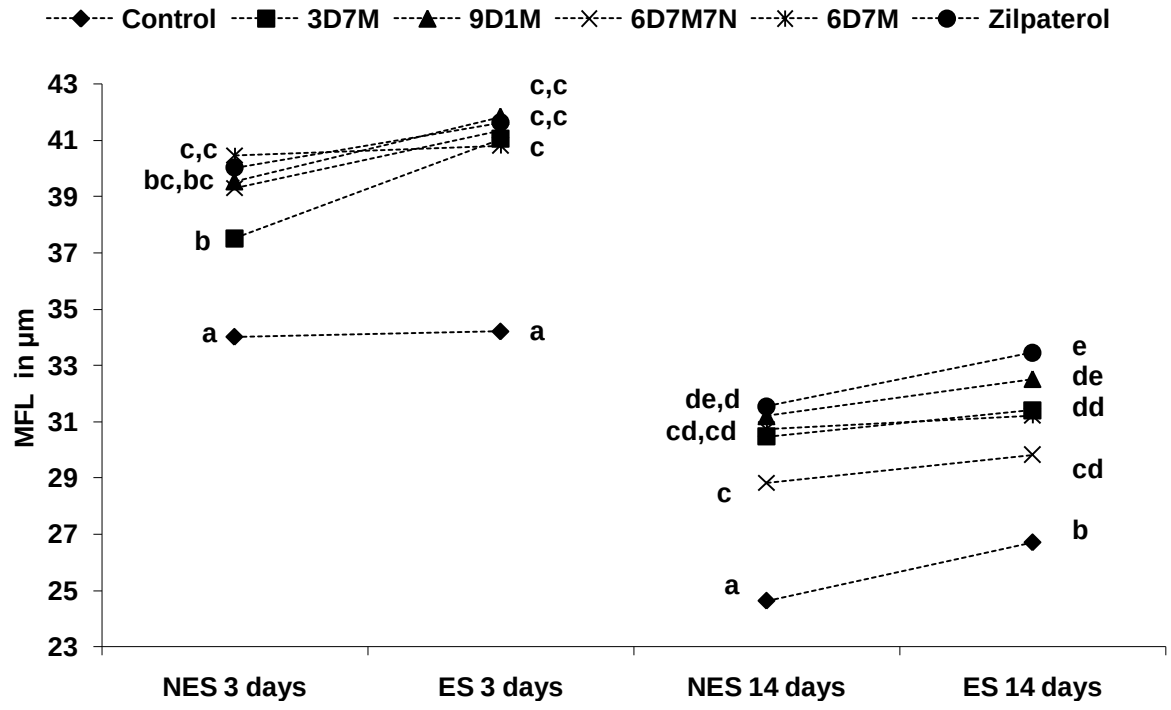

Fig. 1b. Interaction between treatment (Control, 3D7M, 9D1M, 6D7M7N, 6D7M, Zilpaterol), electrical stimulation (NES and ES) and *post mortem* aging (3 and 14 days) for Myofibril fragment length.

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*)

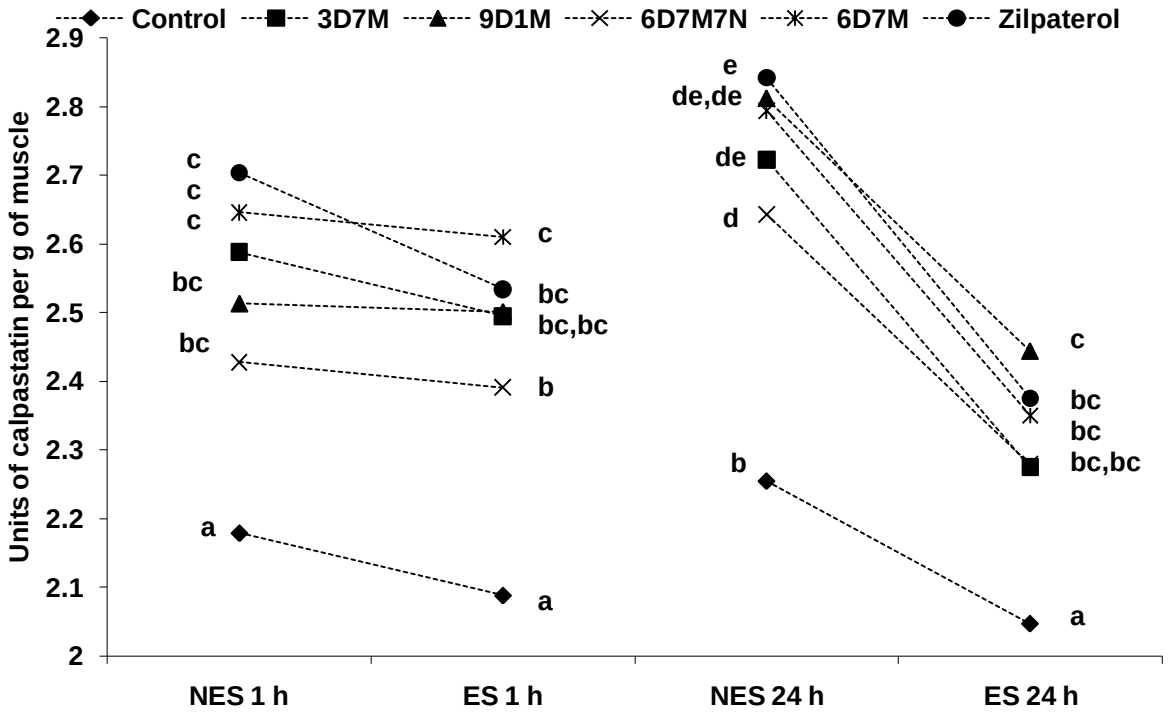

Fig. 1c. Interaction between treatment (Control, 3D7M, 9D1M, 6D7M7N, 6D7M, Zilpaterol), electrical stimulation (NES and ES) and time of measurement *post mortem* (1 h and 24 h) for Calpastatin activity.

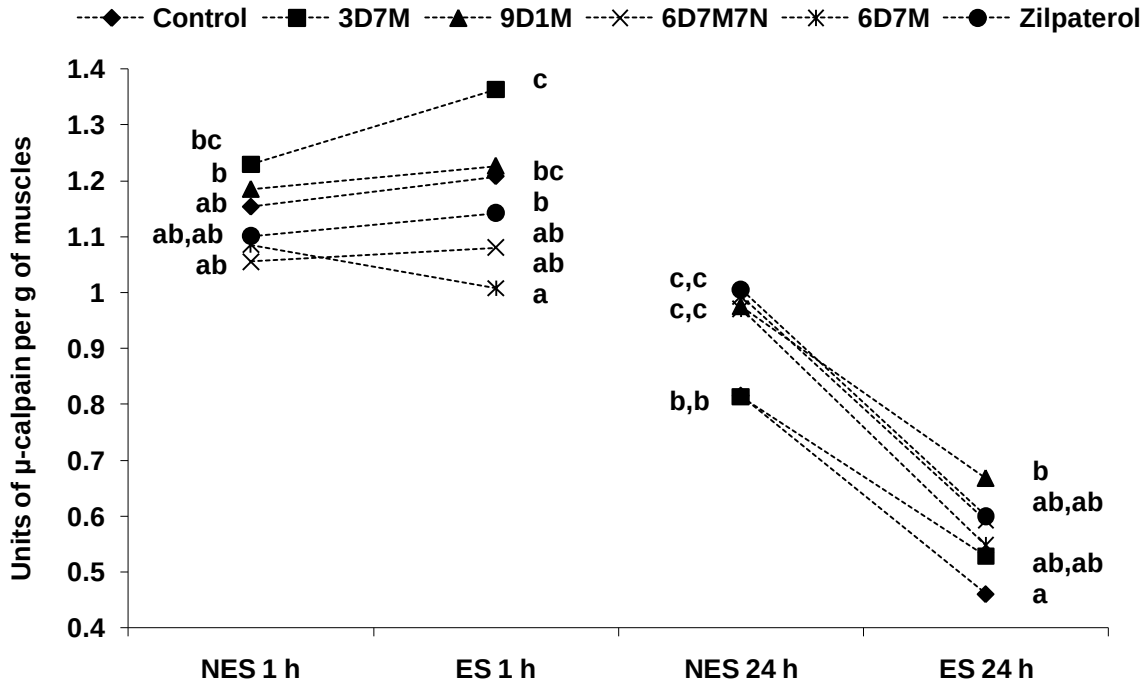

Fig. 1d. Interaction between treatment (Control, 3D7M, 9D1M, 6D7M7N, 6D7M, Zilpaterol), electrical stimulation (NES and ES) and time of measurement *post mortem* (1 h and 24 h) for µ-calpain activity.

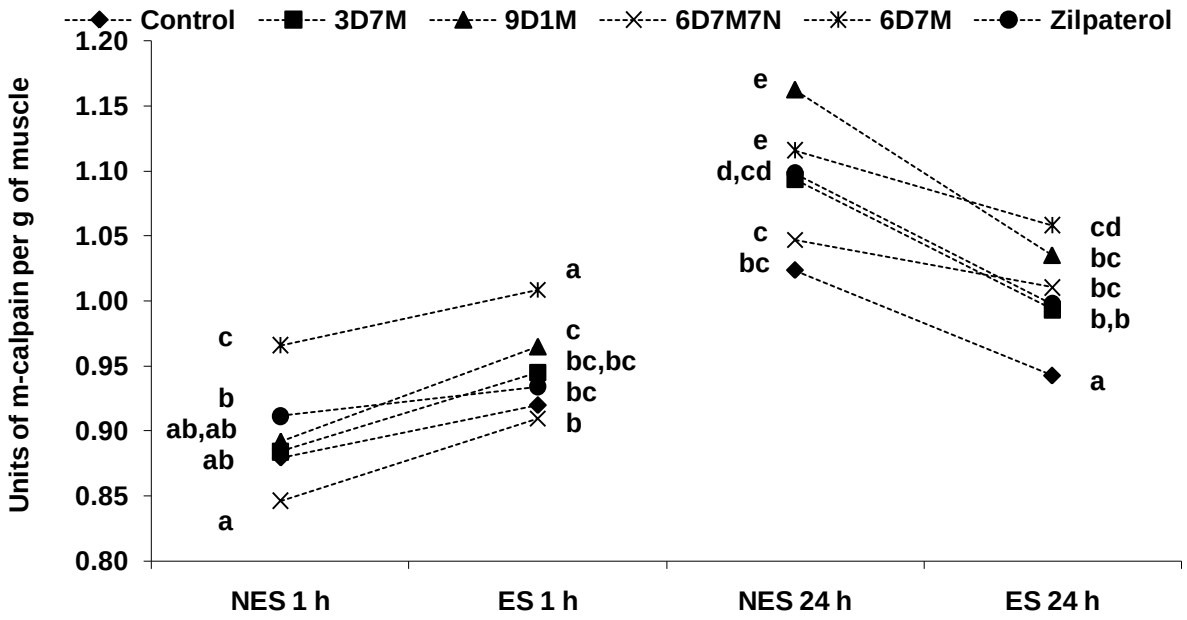

Fig. 1e. Interaction between treatment (Control, 3D7M, 9D1M, 6D7M7N, 6D7M, Zilpaterol), electrical stimulation (NES and ES) and time of measurement *post mortem* (1 h and 24 h) for m-calpain activity.

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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