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Expression of ovine ubiquitin C-terminal hydroxylase 1, pH and colour of variety meats from head-stunned Dohne Merino sheep

P. O. Fayemi[#] & V. Muchenje
Department of Livestock and Pasture Science, Faculty of Science and Agriculture, University of Fort Hare, Private Mail Bag X1314, Alice 5700, Eastern Cape Province, South Africa

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Abstract

Ubiquitin C-terminal hydroxylase (UCH-L1) has been identified in few transcriptome studies as a biomarker coding for trauma and perception of pain in non-meat species. For the first time, real-time polymerase chain reaction (qPCR) assay was used to quantitate the expression of ovine ubiquitin C-terminal hydroxylase 1 (ovUCH-L1 mRNA) from head-stunned Dohne Merino ewes and lambs presented for slaughter at a high-throughput abattoir. The correlation between ambient environment and quality of variety meats from those ovine species was also determined. The level of ovUCH-L1 mRNA expression found in lambs was high, based on the outcome of qPCR quantification. The effect of head stunning shows that ewes exhibited higher capacity to impede electric insults than lambs. Except for the trachea and heart, ambient conditions had a negative correlation with pH of most variety meats. Similarly, a negative correlation was observed between total colour difference (ΔE^*) for fillet and dew point. Saturation index of a few variety meats showed a moderate relationship with ambient temperature. Industrially, the results on ambient conditions are important for post-mortem control of pH, colour, and preservation of other physiochemical properties of variety meats. Findings from qPCR quantification indicated that ovUCH-L1 is a novel candidate marker for pain detection in head-stunned ovine species.

Keywords: Biomarker, edible offal, halal slaughter, ovine species, stunning
[#] Corresponding author: pfayemi@ufh.ac.za / topepeter@gmail.com

Introduction

Ubiquitin C-terminal hydroxylase 1 (UCH-L1) is a member of the deubiquitinating enzyme family known as deubiquitinases. UCH-L1 is a neuronal-specific protein gene product consisting of 223 amino acids encoded by nine exons (Setsuie & Wada, 2007). It contains 1–5% total neuronal protein and conserved peptidase C12 superfamily catalytic domian with short N- and C-terminal extensions (Bishop *et al*., 2016; Das *et al*., 2006). This cytosolic neuronal marker forms 5–10% of the cytoplasmic protein (Bishop *et al*., 2016). UCH-L1 acts as a hydrolytic enzyme in its monomeric form, and as ligase in its dimeric state (Day & Thompson, 2010). UCH-L1 is highly expressed throughout the central and peripheral nervous systems, but only in tracal quantities in the large intestine, kidney, ovary, and testis (Orr *et al*., 2011). This marker is involved in protein binding, proteasome-mediated ubiquitination, sensory pain perception, maintenance of the axonal integrity, axon target recognition, neuroendocrine cell protection, proteolysis, neuromuscular process, muscle fibre development, and traumatic brain injury (Papa *et al*., 2010; Blyth *et al*., 2011; Wilson *et al*., 2016). Therefore, the ubiquitin system plays significant roles in the regulation of many cellular and biological processes. Some ubiquitin- related genes also influence muscle and meat quality (Damon *et al*., 2012). Similarly, stunning and pre-slaughter conditions affect the physico-chemical properties of mutton and lamb meat (Chulayo & Muchenje, 2013).

However, there is no evidence of molecular quantification of ovine ubiquitin C-terminal hydroxylase1 (ovUCH-L1) as a candidate marker for detecting slaughter pain in electro-stunned Dohne Merino sheep. Dohne Merino is a product of the cross between pure Merino and German mutton Merino. This Merino strain is reportedly one of the leading registered composite breeds, which constitute nearly 55% of the total sheep

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population in South Africa. It is a dual-purpose Merino sheep that is raised extensively for wool and meat production (Cloete *et al*., 2012; Fayemi & Muchenje, 2013). Dohne Merino is acceptable in halal abattoir where the electrical stunning method is used to render the animal immobile or insensitive to pain before slaughter. Routinely, the animal to be slaughtered is restrained by placing the stunner on the 'whole body', 'head and chest', 'head and leg' or 'head only' to induce unconsciousness (Prinz *et al*., 2010; Weaver & Wotton, 2008). Agitation, injury, avoidable pain and stress are thereby prevented before the carotid artery, jugular vein, trachea, and oesophagus are severed with a sharp knife (Lambooij *et al*., 2012; Nowak *et al*., 2007).

Electrical stunning is permissible for halal meat production. If done in compliance with statutory regulations and religious tenet on humane slaughter, the process induces a temporary loss of consciousness without causing physical disability, or damage to the heart or brain of the animal (Farouk, 2013; Nakyinsige *et al*., 2013). An animal presented for slaughter can experience some avoidable stress and pain if the process of stunning is not managed efficiently. In comparison with other sentient creatures, sheep have sensory receptors to perceive unpleasant signals associated with tissue damage. The current passing through the brain during head stunning causes substantial depolarization of the neurons and production of brain status that is similar to grand mal epilepsy (Gregory, 2007). Electric insults being generated therefore trigger the release of neurotransmitters at nerve endings, which bind to receptors on neuronal surface for communication from one neuron to another. Consequently, the imbalance caused by electric insults from the stunner alters brain function and induces sensory or neuronal excitability across the brain cells, which is expressed as pain (George *et al*., 2011; Gregory, 2007). Nonetheless, there is no evidence about quantification of pain inflicted on sheep during head stunning and its correlative effects on quality of variety meats during halal slaughter at the abattoir.

The objective of the study was to use real-time polymerase chain reaction (qPCR) to quantitate the expressions of ovine ubiquitin C-terminal hydroxylase-1 as a biomarker coding for slaughter pain in electrically head-stunned Dohne Merino sheep and determine the relationship between ambient environment and quality of variety meats.

Materials and Methods

This study was reviewed and approved by the Research Ethics Committee of the University of Fort Hare (UFH/UREC, 7-REC-270710-028). Samples were sourced from a halal-compliant high-throughput abattoir in which the 'head-only' electrical stunning method is used for inducing unconsciousness in sheep before slaughter. As required by Sharia laws, all the sheep were alive prior to stunning and neck cutting was done with a sharp knife by a single swipe. A range of 5–10 ml of blood samples were collected from the jugular veins of Dohne Merino castrated lambs (n = 30) and ewes (n = 30) into heparinized vacutainers during exsanguination. Blood samples were stored in ultra-low freezer (-80 °C) conditions prior to ovUCH-L1 mRNA assay. The sheep were between 11 and 36 months old at slaughter. After evisceration and cleaning, 20–40 g of variety meats were excised from each animal with a sharp scalpel for meat quality determination. Variety meats sampled included the fillet, liver, lung, heart, kidney, oesophagus, tongue, and spleen.

Muscle pH and temperature of variety meats were measured with a digital pH metre (Crison pH25 instruments S.A., Alella, Spain) equipped with a penetrating electrode. The pH metre was first calibrated with standard solutions (Crison Instruments, S.A., Spain) before taking measurements at 1, 6, 24, and 30 hours. A data acquisition system (MT668 Major Tech Pvt Ltd, South Africa) connected to a computer was used for logging ambient temperature, relative humidity and DPs of the environment surrounding the meat samples. Readings were taken from the excised variety meats with a data logger every 60 minutes from 1 hour to 30 hours. Minolta colour-guide $45^0/0^0$ colorimeter with illuminant $D_{65,}10^o$ observation angle and 20 mm aperture size (BYK-Gardener GmbH, USA) was used for colour measurement. Colour (L*for lightness, a* for green/redness, b* and blue/yellowness) coordinates were determined on each variety meat as described by the Commission Internationale de l'Eclairage (1976). Readings were taken in triplicates on each variety meat at 1, 6, 24, and 30 hours post mortem. The procedure reported by Saricoban &Yilmaz (2010) was followed to calculate whiteness index (WI), saturation index (SI) and total colour difference (ΔE*) as shown below:

a) Whiteness index (WI) $=100-\sqrt{(100-L^*)^2 + a^{*2} + b^{*2}}$

b) Saturation index (SI) $=\sqrt{a^{*2} + b^{*2}}$

c)Total colour difference (ΔE^*) $= \sqrt{(L_o-L^*)^2 + (a_o-a^*)^2 + (b_o-b^*)^2}$

where subscript 'o' refers to the colour reading from the control sample used as the reference and a larger ∆E* indicates greater colour change from the reference sample.

Total mRNA was rapidly extracted from the collected blood samples using Zymo whole-blood RNA MiniPrep™ kit (Zymo Research Corporation, Irvine, CA 92614, U.S.A.). This kit was chosen for having the ability to extract high quality RNA (A260/A280 >1.8, A260/A230 >1.8), which is suitable for all downstream RNA-based manipulations. A total of 600-µl blood RNA Buffer™ was added to 200-µl whole-blood sample following red blood cell lysis, transferred into a Zymo-Spin IIIC™ column in a collection tube and centrifuged at ≥ 12000-x g for 2 minutes. Subsequently, 400 µl of RNA pre-wash buffer was added to the column and centrifuged at ≥ 12 000-x g for 30 seconds and 100 µl RNA recovery buffer was then added to the Zymo-Spin™ IIIC column and centrifuged at ≥ 12,000 x g for 30 seconds. Following this, 100-µl ethanol (95–100%) was added to the flow-through in the RNase-free tube and mixed well by pipetting. The mixture was then transferred to a Zymo-Spin™ IC column in a collection tube and centrifuged at ≥ 12000 x g for 30 seconds. Before discarding the flow-through, 400 µl RNA prep buffer was added to the column and centrifuged at ≥ 12,000 x g for 1 minute. For a second time, 800-µl RNA wash buffer was added to the column and centrifuged at ≥ 12,000 x g for 30 seconds and the flow-through was discarded. The wash step was then repeated with 400 µl RNA wash buffer. and the Zymo-Spin™ IC column was centrifuged in an emptied collection tube at ≥ 12,000 x g for 2 minutes. The Zymo-Spin™ IC column was carefully removed from the collection tube and transferred into an RNase-free tube. The DNase/RNase-free water (≥ 6 µl) was added directly to the column matrix and centrifuged at 10000x g for 30 seconds to finally elute RNA.

Reverse transcription was done by optimising the Maxima SYBR Green/ROX qPCR Master Mix for its ability to produce sensitive and specific quantification of genomic, plasmid and cDNA templates. Thermo-stable hot-start DNA polymerase, buffer, dNTPs, SYBR® Green dye, KCl and $(NH4)_2SO4$ in the Master Mixes were used to provide high specificity of primer annealing. ROX passive reference dye in the Master Mix serves as an internal reference for normalising SYBR Green 1 fluorescent signal to allow for correction of well-to-well variation that might occur due to pipetting inaccuracies and fluorescence fluctuations. Excitation and emission maxima of SYBR Green I were well suited to real-time cycler and were within 494 nm and 521 nm. Master Mix was used with the real-time thermal cyclers (LightCycler® 480 SYBR Green I Master LightCycler® 480 instrument). Two steps qPCR was used in thermal cycling for quantitative determination of ovUCH-L1 mRNA. Primer design for ovUCH-L1mRNA did not follow a conventional method since the sheep genome is not fully sequenced and no reference sequence data is available for the genes of interest (Figure 1). Partial mRNA sequences from GenBank accession (*Ovis aries* UCH L1-S27), a protein mRNA and partial cds (Accession AY566307, version: AY566307.1) were used to BLAST the long Expressed Sequence Tags (EST) to represent nearly the full length of mRNA sequences. This approach was chosen to cater for exon-exon boundaries within the EST sequence as the EST data were not fully curetted. This approach was necessary to prevent erroneous amplification that might result from contaminating genomic DNA in the cDNA samples. The primers were thus designed to generate PCR products of approximately 200–350 base pairs (bp) long to maximize primer potential to bind in different exons (Table 1). Primers for qPCR were used on the cDNA samples, but were first tested to determine their capacity to bind and amplify sheep genomic DNA. Necessary precautions were taken to get rid of residual genomic DNA and purify the DNA-free RNA before it was synthesized into cDNA. PROC CANCORR procedures of the Statistical Analysis System (SAS version 9.1.3 of 2007) were computed to determine the relationships among all the tested parametres. Significant difference level of P <0.05 was used.

Results

The result of pimer sequence for ovine Ubiquitin C-terminal hydroxylase-1 is presented in Figure 1. The forward primer of the sequence (5'-TCCGGGTCTCATCTGTCTCCTCCT-3') was within 9-42 and the reverse primer (3'-CGTCCATCTTCCAGTTGCTTGCCA-5') was in the range of 231-208. As shown in Table 1, the Guanine-Cytosine (GC) content of 58.33% for forward primer and 54.17% for reverse primer were within the normal range of 45–60%. This GC range clearly shows a high annealing strength with the primer length at 24 bp.

The melt curve analysis demonstrates a typical primer-dimer formation by using SYBR Green Master mix in the temperature-dependent dissociation between DNA strands (Figure 2A). The derivative reporter (-Rn) value for the standard records the highest melting point with the Tm value of 83.00 °C. This result implies that at a higher inflection point, 50% of the primer was annealed. Also, it was observed that a high degree of interactive stability between the primer-target gene and a rise in absorbance intensity (hyperchromicity) produced an ideal single stranded UCH-L1mRNA amplicon.This result invariably agrees with the fundamental principle that fluorescence intensity inceases proportionally with the concentration of the amplicon (dsDNA) under real-time ploymerase chain reaction. Further results showed that the biggest change in fluorescence produced visible peaks with corresponding denaturation temperatures of the double-

stranded DNA (dsDNA) fragments (Figures 2B & C). However, the fluorescence signal produced was characteristic of a curve consisting of baseline region, exponential growth and linear phases. Therefore, the dissociation curve in Figure 2B indicates that 50% of dsDNA fragment for sheep that exhibited slaughter pain was attained at 82.13 °C melting point (Tm). Figure 2B also indicates that positive amplification occurred at 11.25ΔR after successive thermal cycling. The presence of single curve at the peak of amplification showed the absence of contaminating products such as contaminating DNA or primer-dimer that could have appeared as additional peaks, different from the desired amplicon. In Figure 2C, the melting curve showing absence of ovUCH-L1 mRNA expression in slaughtered sheep has a Tm value of 76.1 °C, which is typical of dissociation reaction with a baseline fluorescence signal. Generally, these findings have established the assertion that Tm value depends on length, sequence order, and GC content of the dsDNA fragment in qPCR quantification.

1 TCTCGCCCTT CCTGTGTC**TCCGGGTCTCATCTGTCTCCTCCT**TTTCCTCA CCCTCAGGTG
61 GAACCGCCGC CAGCATGCAG ATTTTCGTGA AGACCCTGAC GGGGAAGACC ATCACTCTTG
121 AGGTCGAGCC CTCGGATACA ATAGAAAATG TGAAGGCCAA GATCCAGGAT AAGGAAGGAA
181 TTCCTCCTGA CCAGCAAAGA CTGATCTTTG C**TGGCAAGCAACTGGAAGATGGACG**TACTT
241 TGTCTGACTA CAACATTCAA AAGGAGTCCA CTCTTCATCT AGTGTTGAGA CTTCGTGGTG
301 GCGCTAAGAA AAGGAAGAAG AAGTCTTACA CCACTCCCAA GAAGAACAAG CATAAGAGAA
361 AGAAGGTTAA ATTGGCTGTT CTGAAATACT ATAAGGTGGA TGAGAATGGC AAAATCAGTC
421 GCCTTCGCCG GGAGTGTCCC TCAGATGAAT GTGGTGCTGG AGTTTTTATG GCCAGTCACT
481 TTGACAGACA TTATTGTGGC AAATGTTGTC TGACCTATTG TTTCAACAAA CCAGAAGACA
541 AGTAATTGTA CATTGGTTAA TAAACATATG AGCTAACATT TAAAAAAAAA AAAAAAAAAA
601 AAAAAAAAAA GCTCGCTCAG CCAGCTTGCC CTGCTTTCTG AGACATATGA CCTCTGGCCC
661 CAGCCGCTAG ACCTCTCCCG ACCTCACCTC TGACTTCAGC AGCCAAGTGT GAATGCAGAG
721 AGCAAAGCCC CAAGGAGGAA GCTCGGGCCT GAGCATAGCA GAGGGCTCCT TGCTGGGTTA
781 GGATGGAGCT CCCCAAGTTT TCCCAGCAGA AGGGATGACC TTTCATTCTG TTTTC

Note**: Red and bright green colours above denote forward and backward primers, respectively.**

Figure 1 Primer sequence (>*Ovis aries* UCH-L1-S27a EST: > TC65096 TC34746) for ovine ubiquitin C-terminal hydroxylase-1

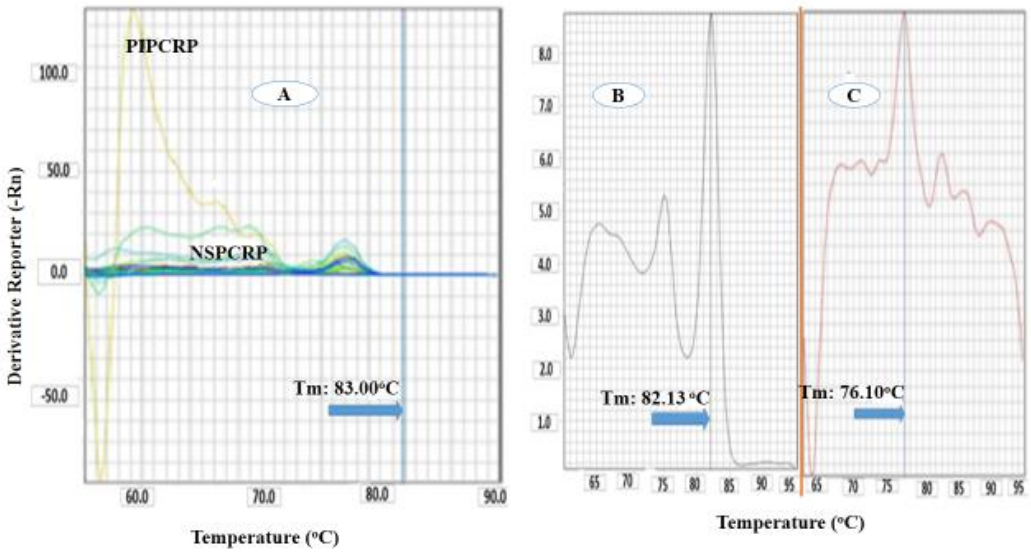
Table 1 Primer characteristics and sequence for ovine ubiquitin C-terminal hydroxylase-1

Primer Characteristics	Template strand	Length (bp)	Start	Stop	[a]Tm	[b]GC (%)
Forward primer	Plus	24	19	42	59.69	58.33
Reverse primer	Minus	24	231	208	59.71	54.17
Product length	217					
Two primers complementarily	Max complementarity in continuous: 3 bp, free energy= 1.50 Kcal/mol 5'-TCCGGGTCTCATCTGTCTCCTCCT-3' ||| 3'-ACCGTTCGTTGACCTTCTACCTGC-5'					
Two primers complementarily	Max complementarity in discontinuous: 8 bp 5'-TCCGGGTCTCATCTGTCTCCTCCT-3' || || ||| | 3'-ACCGTTCGTTGACCTTCTACCTGC-5'					

[a]Tm: Melting temperature; [b]GC: guanine-cytosine

The post-slaughter expression of the marker (ovine UCH-L1 mRNA) from the animals showed its implication for detecting slaughter pain from meat species such as Dhone Merino sheep. The interactions between animals' age and sex contributed to varied expressions significantly (P <0.001) of pain signals from the lambs and ewes (Figure 3). This suggests that a single factor was not responsible for the expression of specifc target (ovUCH-L1 mRNA) from the slaughtered animals. Observation prior to slaughter indicated that each animal was restrained in an upright position to prevent flight or fight tendencies and to minimize wrong

placement of electrodes during stunning. Regardless of the humaneness of the process, it is still practically difficult for animals to experience zero stress or pain-free slaughter procedures.



*NSPCRP: Non-Specific Polymerase Chain Reaction Products & PIPCRP: Peak of Interest for Polymerase Chain Reaction Products

Figure 2 Melt curves showing the standard (A), presence (B) and absence (C) of ovine ubiquitin C-terminal hydroxylase-1 expressions in head-stunned Dohne Merino sheep

Cases of extended vocalization, natural spontaneous blinking and rhythmic breathing do occur owing to ineffective stunning or post-stunning activity of the brain stem. Further observations showed that some sheep were stunned more than once due to mis-stunning, emotional excitability, poor head-stunner conductivity or improper contact between the animal and head-stunner. As shown in Figure 3, more painful effects were noticed from Dhone Merino lambs than ewes that are older. This result possibly suggests that sensory receptors in ewes have higher capacity to impede electric insults than lambs. The resultant neuronal excitability during slaughter might have elicited sensory transduction by the receptor through an 'action or graded potential' phenomenon. Reflecting on the embeded nature of pain and nociception, pain sensation can sometimes be partly or totally blocked by natural endogenous pain-relieving chemicals (opioids). It is therefore possible that ewes produced more opioids than lambs and this innate capacity might have triggered pain-suppressive pathways (or descending pathways) from their brain stem during slaughter.

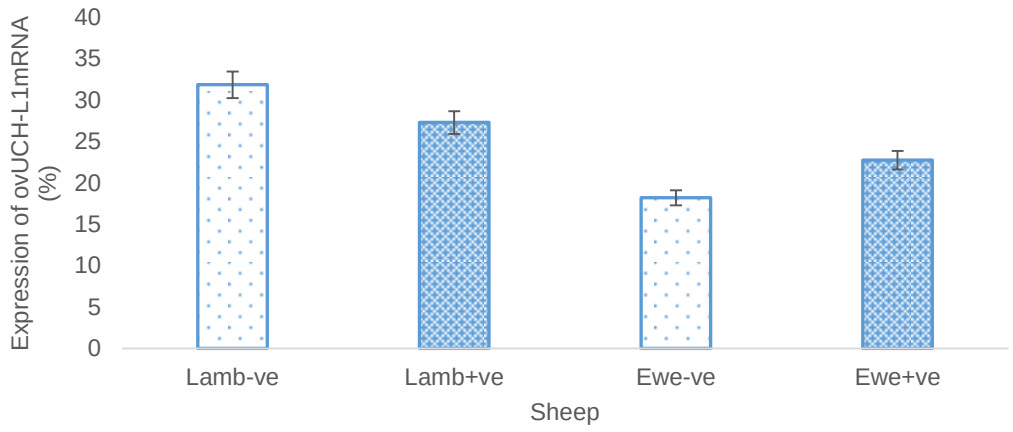


Figure 3 Post-stunning expressions of ovine ubiquitin C-terminal hydroxylase-1 from Dohne Merino castrates and ewes

According to Table 2, there was no significant effect (P >0.05) of age on post-mortem development of colour in the kidneys, lungs and oesophagus from lambs and ewes. Redness values for fillet from lambs were significantly higher (P <0.05) than those obtained from ewes. From 1 to 30 hours post-slaughter, no colour development (P >0.05) was noticed on fillet, heart and lung. Only the kidney, liver and spleen attained their ultimate blooming at 30 hours post mortem among all the non-intestinal variety meats.The results in Table 3 showed that ambient conditions had higher influence on the whiteness index of most variety meats. In particular, a positive correlation was observed between the pH and muscle temperature of trachea and heart. An inverse relationship was found between the relative humidity and the whiteness index of all the variety meats. Dew point indicated a negative correlation with the total colour difference (∆E*) for the fillet. Saturation index (SI) of few non-intestinal offal showed moderate relationship with the ambient temperature (AT). Ambient conditions showed negative correlation (P >0.05) with pH and muscle temperature (MT) of most variety meats except trachea (r = 0.5060*) and heart (r = 0.5272*). The extent of colour deviation from an ideal white suggested the existence of inverse relationship between relative humidity (RH) and whiteness index (WI) of all variety meats. As evident by the intensity of their colours, a moderate correlation (P <0.05) was found between ambient temperature (AT), saturation index (SI) of trachea (r = 0.5087*) and heart (r = 0.5315*). There was a consistent trend observed from ambient temperature (AT) at 1 hour and 24 hours post mortem. The effects of dew points on colour coordinates followed a similar pattern between 12 and 24 hours post mortem.

Table 2 Least square means (±SE) for post-slaughter timing and redness (CIEa*) of variety meats from head-stunned Dohne Merino lambs and ewes

Variety meat	Sheep class		Post-slaughter timing			
	Lamb	Ewe	1h	6h	24h	30h
Fillet	13.3 ^{a} ± 0.49	10.9 ^{b} ± 0.49	10.1 ± 0.68	11.9 ± 0.70	12.0 ± 0.70	12.0 ± 0.70
Heart	11.4 ^{b} ± 0.74	15.4 ^{a} ± 0.75	12.8 ± 1.04	14.1 ± 1.06	12.6 ± 1.06	14.1 ± 1.10
Kidney	14.0 ± 0.82	13.4 ± 0.83	13.8 ^{ba} ± 1.15	13.3 ^{ba} ± 1.17	11.8 ^{b} ± 1.70	15.9 ^{a} ± 1.2
Liver	12.6 ^{a} ± 0.61	10.5 ^{b} ± 0.61	12.7 ^{ba} ± 0.85	10.4 ^{bc} ± 0.87	2.4 ^{c} ± 0.87	13.7 ^{a} ± 0.9
Lung	18.7 ± 1.53	22.6 ± 1.55	19.5 ± 2.14	22.6 ± 2.19	21.4 ± 2.19	19.1 ± 2.19
Oesophagus	7.9 ± 1.34	11.2 ± 1.36	6.7 ^{b} ± 1.88	13.4 ^{a} ± 1.92	8.7 ^{ba} ± 1.92	9.5 ^{ba} ± 1.92
Spleen	10.4 ± 0.65	9.1 ± 0.66	8.9 ^{b} ± 0.91	7.6 ^{c} ± 0.93	8.2 ^{b} ± 0.93	14.2 ^{a} ± 0.93
Trachea	10.7 ^{b} ± 1.10	13.5 ^{a} ± 1.10	14.3 ^{a} ± 1.50	10.3 ^{b} ± 1.5	11.9 ^{b} ± 1.50	11.8 ^{b} ± 1.50
Tongue	4.9 ^{b} ± 1.60	9.7 ^{a} ± 1.60	12.0 ^{a} ± 2.30	6.9 ^{b} ± 2.31	4.9 ^{b} ± 2.31	5.3 ^{b} ± 2.30

^{a,b,c} Means in the same row with different superscripts are significantly different at P <0.05

Discussion

As indicated above, halal slaughter requirements were followed strictly at the abattoir where data were generated for this study. For instance, the abattoir has a proof of halal certification to attest to their mode of operation.The stunning device and actual slaughter was performed by a trained Muslim slaughterman. The lairage and slaughter premises for animals regarded as ‘haram’ or forbidden were widely seperated from those that are acceptable in a halal abattoir. There was no evidence that the stunning procedure caused permanent injury or death of the animals. All these conditions clearly agreed with fundamental prerequisites for stunning to be acceptable in halal meat production (Nakyinsige *et al.,* 2013). Additionally, quantitative analysis with qPCR provided evidence of presence or absence of pain expression in head-stunned sheep during halal slaughter. This partly suggests that congenital traits or mutant genes in some animal species can manifest in form of insensitivity to pain during stunning or slaughter. In a similar study by Wilhelm & Pingoud (2003), the melt peak-resolution and amount of ovUCH-L1 mRNA product detected from the animals followed a pattern that was expected to validate the expression of this marker from any biological sample. In agreement with Aniko & Delano (2006), the generation of a single product, especially the ovUCH-L1mRNA during a melt run, suggested an association between ‘time-temperature binding pattern’ of the SYBR Green 1 and growth of the peak. Similarly, the use of intercalating dyes such as SYBR Green 1 for melting curve analysis was in tandem with a report on amplicon detection and differentiation by Papa *et al.* (2010).

Table 3 Correlation between ambient conditions and colour parametres of variety meats from Dohne Merino sheep

Variety Meat	Ambient Conditions	L*	a*	b*	WI	SI	∆E*
	AT	0.254	0.1926	0.1169	-0.639*	0.1988	0.6067*
Liver	RH	0.0669	-0.2786	-0.037	-0.4346	0.0918	0.2394
	Dew point	0.1985	0.1951	0.0604	-0.48	0.1565	0.4806
	AT	0.2274	-0.0473	0.0074	0.6367*	0.4012	0.2185
Spleen	RH	-0.0673	-0.4033	-0.1692	-0.5286*	-0.0213	0.0588
	Dew point	0.2417	0.0093	0.0148	0.6990*	0.3530	0.2000
	AT	0.1528	-0.0672	-0.0739	0.8909*	0.4168	0.0474
Lung	RH	0.0352	0.1180	0.2954	-0.5840*	-0.0438	0.1442
	Dew point	0.1247	-0.0820	-0.1114	0.9366*	0.3669	0.0098
	AT	0.3657	0.1814	-0.0062	0.6610*	0.5087*	0.2398
Trachea	RH	-0.1015	-0.1178	-0.0495	-0.4691	-0.1489	-0.0037
	Dew point	0.3575	0.1970	-0.0027	0.7061*	0.4717	0.2254
	AT	0.1868	-0.1666	-0.0323	0.8785*	0.4139	-0.4269
Kidney	RH	0.1114	0.0770	-0.2644	-0.5645*	-0.1603	0.0297
	Dew point	0.1508	-0.1994	0.0096	0.9214*	0.3816	-0.3992
	AT	0.3234	-0.1927	0.2802	0.9508*	0.0879	0.1808
Oesophagus	RH	-0.0026	0.2297	0.0328	-0.5383*	-0.1232	0.0526
	Dew point	0.2966	-0.2301	0.2389	0.9745*	0.1037	0.1574
	AT	0.2180	-0.0713	-0.2168	0.9169*	0.5315	0.1919
Heart	RH	-0.2023	0.0562	0.0804	-0.5881*	-0.1306	-0.0676
	Dew point	0.2255	-0.0864	-0.2262	0.9627*	0.4753	0.1730
	AT	-0.3721	0.0599	0.0035	0.9359*	0.2717	-0.5955*
Fillet	RH	0.1102	-0.1066	-0.0997	-0.5578*	-0.0635	0.2758

AT: ambient temperature; RH: relative humidity; L^*: lightness; a^*: redness; WI: whiteness index; SI: saturation index; ∆E*: total colour difference; significantly correlated at *P <0.05

High annealing strength obtained from this study indicates an acceptable standard that the GC-content for forward primer and reverse primer were within the normal GC range of 45–60% expected in qPCR quantitation. Using SYBR Green Master mix in temperature-dependent dissociation between DNA strands gave a typical primer-dimer formation, which described the stability of interaction between the primer-target gene and hyperchromicity, which produced the desired single stranded UCH-L1 mRNA amplicon. It has been proven that investigating the effect of slaughter pain on head-stunned Dohne Merino did not yield a stunned state in all the animals. Vogel *et al.*(2011) also found that electrical stunning might not produce an instantaneous insensibility in stunned ovine species. As shown by ovUCH-L1 mRNA expression, almost 50% of the animals experienced pain during slaughter. This may be attributed to incidences of electroplectic shock, coupled with reflex reactions caused by severed nerves in the trachea, oesophagus, jugular veins and carotid arteries during throat cutting. The consequent manifestation of painful reactions in the form of prolonged vocalization, curling and moving of tongues with tendencies to return to normal rhythmic breathing may also be attributed to expression of pain marker or perhaps ineffective stunning. Lambs that exhibited such traits also recorded high levels of ovUCH-L1 mRNA expression based on the outcome of qPCR quantification.

Although few authors reported similar behavioural traits owing to ineffective stunning (Grandin, 2010; McKeegan *et al*., 2006; Velarde *et al*., 2002), a number of other factors could influence animal's sensitivity during stunning and bleeding. For example, slaughter weight, age of the animal, degree of fatness, degree of dehydration and amount of wool on the animal at slaughter could affect the impedance or amount of voltage-current required to cause grand mal seizure. Moreover, differences in heritability traits and adaptive response to traumatic brain injury or painful stimuli (hyperalgesia) could play some roles in these results

(Arnaoutakis *et al*., 2011; George *et al*., 2011). In contrast, Blyth *et al.* (2011) related the elevated level of UCH-L1 mRNA expression to incidence of traumatic brain injury due to abnormal blood-brain barrier function. According to Gottschalk & Smith (2001), the capacity of opioids to bind to signal receptors in the central nervous system could affect the descending pain pathway in the brain and spinal cord.This phenomenon might support the reason that ewes manisfested low pain expression and high bio-impedance than lambs.

Attainment of ultimate redness by variety meats showed some similarity with the skeletal muscles. The result is therefore consistent with that reported by Young *et al.* (1999), in which skeletal muscle from the beef reached its ultimate blooming at 30 hours post- laughter due to the levels of cytochrome and myoglobin pigments in the muscle. Since most of the organ meat attained ultimate redness beyond four-hour post mortem, it implies similarity in response between variety meats and *M*. *longisimuss* muscles from Merino crosses to ambient conditions (Farouk & Lovatt, 2000). Some slight differences between the results in the current study and those of skeletal muscles could be ascribed to the types of muscle, their location in the animal's body and the ante-mortem conditions of the animal. High concentration of pigment due to age, sex, gene-diet interactions and pre-slaughter stress may be attributed to the reason for variety meats from older animals recording a darker and redder colour (Kamenski, 2006). MacDougall (1982) used saturation index in discoloration studies to describe changes that are dependent on time, storage and display. As proposed by Abudullah & Matarneh (2010), the ability of each organ to reflect or absorb light could influence significantly the ultimate redness of organ meat. Furthermore, McNeil *et al.* (1987) postulated that at lower temperatures, oxygen penetrates deeper into meat and then thickens the oxymyoglobin layer. This postulation could be responsible for the accelerated change in colour of the fillet as ambient temperature rises to 18.65 °C in this study. The average relative humidity of 44.25% obtained in the present study is within the recommended range of 45–60% for meat packaging, boning and cutting rooms but lower than 80–95% range for meat chilling and ripening (FAO, 1991). With respect to reports by Arnau & Gou (2001); Arnau *et al*. (2012); Kenneth & Elovitz (1999), RH affects water activity on meat surface and thus the range of 50–55% RH obtained in this study has the potential to produce the desirable whiteness index on some meat parts. It may be inferred then that the relationship between RH of 44.25% (approved by the FAO) and moisture sorption isotherms for edible offal would give a better idea on stability of WI and shelf-life extension of variety meats.

Conclusion

Head stunning used in the present study confirms its acceptability in halal abattoir for the conversion of sheep into meat. Real-time quantification of ovUCH-L1 mRNA expression shows that the process did not produce zero pain sensation in most of the slaughtered Dohne Merino sheep. The gene region obtained from electro-stunned Dohne Merino sheep shows that ovUCH-L1 is a candidate marker for slaughter pain detection. Additionally, the impact of head stunning on the animal and its interraction with post-mortem ambient environment hold some significant relevance that can be adopted in slaughter houses, buctheries and meat outlets for optimal post mortem maintainance of muscle pH, colour, and other physiochemical properties of variety meats.

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Conflict of Interest Declaration

The authors declare that they have no conflict of interest.

Authors' Contributions

The work was designed by FPO and VM. FPO collected the data, did the analyses and wrote the manuscript. Both authors read and approved the final draft of the manuscript.

References

Abudullah, A.Y. & Matarneh, S.K., 2010. Broiler performance and the effects of carcass weight, broiler sex and post-chill carcass aging duration on breast fillet quality characteristics. J. Appl. Poultry Res. 19, 46-58.

Aniko, V. & Delano, J., 2006. Real-time RT-PCR and SYBR Green I melting curve analysis for the identification of *Plum pox virus* strains C, EA, and W: Effect of amplicon size, melt rate, and dye translocation. J. Virol. Method. 132, 146-153.

Arnaoutakis, G.J., George, T.J., Wang, K.K., Wilson, M.A., Allen, J.G., Robinson, C.W. Haggerty, K.A., Weiss, E.S., Blue, M. E., Talbot, C.C., Jr Troncoso, J.C., Johnston, M.V. & Baumgartner, W.A., 2011. Serum levels of neuron-specific ubiquitin carboxyl-terminal esterase-L1 predict brain injury in a canine model of hypothermic circulatory arrest. J. Thoracic Cardio. Surg.142 (4), 902-910.

Arnau, J. & Gou, P., 2001. Effect of air relative humidity on ham rind and subcutaneous salted fat during the resting period. Meat Sci, 58, 65-68.
Arnau, J., Muñoz, I. & Gou, P., 2012. The effect of air relative humidity on the appearance and structure of subcutaneous pork fat unsalted or treated with NaCl, KCl or K-lactate. LWT–Food Sci. Tech. 47,133-137.
Bishop, P. Rocca, D. & Henley, J.M., 2016. Ubiquitin C-terminal hydrolase L1 (UCH-L1): Structure, distribution and roles in brain function and dysfunction. Biochem. J. 473(16)2453-2462.
Blyth, B.J., Farahavar, A., He, H., Nayak, A., Yang, C., Shaw, G. & Bazarian, J.J., 2011. Elevated serum ubiquitin carboxy-terminal hydrolase L1 is associated with abnormal blood-brain barrier function after traumatic brain injury. J. Neurotrauma 28 (12), 2453-2462.
Chulayo, A.Y. & Muchenje,V., 2013. Effect of pre-salughter conditions on physico-chemical characteristics of mutton from three sheep breeds slaughtered at a smallholder rural abattoir. S. Afr. J. Anim. Sci. 43(5), 64-68.
Cloete, J. J. E., Hoffman, L. C., Cloete, S. W. P. & Fourie, J. E., 2004. Slaughter traits of Merino sheep divergently selected for multiple rearing ability. S. Afr. J. Anim. Sci. 34, 189-196.
Cloete, J.J.E., Hoffman, L.C. & Cloete, S.W.P., 2012. A comparison between slaughter traits and meat quality of various sheep breeds: wool, dual-purpose and mutton. Meat Sci. 91, 318-324.
Commission International De L' Eclairage. 1976. Colorimetry. 2nd edition. CIE, Vienna.
Damon, M., Wyszunska-Koko, J., Vincent, A., Herault, F. & Lebret, B., 2012. Comparison of muscle transcriptome between pigs with divergent meat quality phenotypes identifies genes related to muscle metabolism and structure. PLOS One 7(3), e33763.
Das, C., Hoang, Q.Q, Kreinbring, C.A. Luchansky, S.J.Meay, R.K., Ray S.S, Lansbury, P.T, Ringe, D. & Petsko, G.A., 2006. Structural basis for conformational plasticity of the Parkinson's disease-associated ubiquitin hydrolase UCH-L1. Proc. National Acad. Sci., USA 103, 4675-4680.
Day, I.N. & Thompson, R.J., 2010. UCH L1 (PGP 9.5): Neuronal biomarker and ubiquitin system protein Epub90(3), 327-362.
Farouk, M.M. & Lovatt, S.J., 2000. Initial chilling rate of pre-rigor beef muscles as an indicator of colour of thawed meat. Meat Sci. 56,139-144.
Farouk, M.M., 2013. Advances in the industrial production of halal and kosher red meat. 59th Int. Cong. Meat Sci. Tech. 95 (4), 805-820.
Fayemi, P.O. & Muchenje, V., 2013. Biomarkers coding for ovPAG-1 mRNA expression and pregnancy status in Dohne Merino ewes at an abattoir. Trop. Anim. Health & Prod. *4*5(7), 1597-1602.
Food and Agricultural Organisation (FAO). 1991. Manual on meat cold store operation and management. FAO Animal Production and Health Paper, p 92.
George, J.A., Timothy, J.G., Kevin, K.W., Mary, A.W., Jeremiah, G.A., Chase, W.R., Kara, A.H., Eric, S.W., Mary, E.B., Talbot, C.C., Juan, C.T., Michael, V.J. & William, A.B., 2011. Serum levels of neuron-specific UCHL1 predict brain injury in a canine model of hypothermic circulatory arrest. J. Thorac. Cardio. Surg. 142 (4), 902-910.
Gottschalk, A. & Smith, D.S., 2001. New concepts in acute pain therapy: pre-emptive analgesia Ame. Family Phy. 63(10).
Grandin, T., 2010. Improving animal welfare: A practical approach. 2010, CABI. Wallingford, Oxfordshire, United Kingdom.
Gregory, N.G., 2007. Animal welfare and meat production. CABI. Wallingford, Oxfordshire, United Kingdom.
Kamenski, C., 2006. Variety meats vital to boosting United States beef exports. Issues Update, March-April 2006. Available at: www.beefusa.org/uDocs/varietymeatsvitaltoexports.pdf.
Kenneth, M. & Elovitz, P.E., 1999. Understanding what humidity does and why. American Society of Heating, Refrigeration and Air-conditioning Engineers (ASHRAE),1-7. Accessed 26 July 2012.
Lambooij, E., Van der Werf, J.T.N., Reimert, H..G.M. & Hindle, V.A., 2012. Restraining and neck cutting or stunning and neck cutting of veal calves. Meat Sci. 87 (10), 2160-2165.
MacDougall, D.B., 1982. Changes in the color and opacity of meat. Food Chem. 9,75
McKeegan, D.E.F., McIntyre, J., Demmers, T.G.M., Wathes, C.M. & Bryan-Jones, R., 2006. Behavioural responses of broiler chickens during acute exposure to gaseous stimulation. Appl.Anim. Behav. Sci. 99, 271-286.
McNeil, I., McPhail, N.G. & Macfarlane, D., 1987. Carcass chilling. Available at: http://www.meatupdate.csiro.au. Accessed 22 July 2012.
Nakyinsige, K., Man, Y.B., Aghawan, Z.A., Zulkifli, I., Goh, Y.M., Abu-Bakar, F., Al-Kahtani, H.A. & Sazili, A. Q., 2013. Stunning and animal welfare from Islamic and scientific perspectives. Meat Sci. 95(2), 352-361.
Nowak, B., Mueffling, T.V,. & Hartung, J. 2007. Effect of different carbon dioxide concentrations and exposure times in stunning of slaughter pigs: Impact on animal welfare and meat quality. Meat Sci. 75, 290–298.
Orr, K.S., Shi, Z. Brown, W.M., O'Hagan, K.A., Lappin, T.R., Maxwell, P. & Percy, M.J. 2011. Potential prognostic marker ubiquitin carboxyl-terminal hyrolase-L1 does not predict patient survival in non-small cell lung carcinoma. J. Expt. Clinic. Cancer Res. 30 (79),1-12.
Papa, L., Akinyi, L., Liu, M.C., Pineda, J.A., Tepas, J.J., Oli, M.W., Zheng, W., Robinson, G., Robicsek, S.A., Gabrielli, A., Heaton, S.C., Hannay, H.J., Demery., J.A., Brophy, G.M., Layon, J., Robertson, C.S., Hayes, R.L. & Wang, K.K.W., 2010. Ubiquitin C-terminal hydrolase is a novel biomarker in humans for severe traumatic brain injury. Critic. Care Med. 38 (1), 138-144.
Prinz, S., Vanoijen, G., Bessei, W., Ehinger, F. & Cienen, A.M.L., 2010. The effects of amount and frequency of alternating current used in water bath stunning and of slaughter method on electroencephalograms in broilers. Anim. Welf. J. 15**,** 7-15.

Saricoban, C. & Yilmaz, M.T., 2010. Modelling the effects of processing factors on the changes in colour parameters of cooked meatballs using response surface methodology. World Appl. Sci. J. 9 (1), 14-22.
Setsuie, R. & Wada, K., 2007. The functions of UCH-L1 and its relation to neurodegenerative diseases. Neurochem. Inter. 51(2-4), 105-111.
Velarde, A., Ruiz-de-la-Torre, J.L., Roselló, C., Fàbrega, E., Diestre, A. & Manteca, X., 2002. Assessment of return to consciousness after electrical stunning in lambs. Anim. Welf, 11 (3), 333-341.
Vogel, K.D., Badtram, G., Claus, J.R., Grandin, T., Turpin, S., Weyker, R.E. & Voogd, E., 2011. Head-only followed by cardiac arrest electrical stunning is an effective alternative to head-only electrical stunning in pigs. J. Anim. Sci. 89 (5), 1412-1418.
Weaver, A.L. & Wotton, S.B., 2008. The Jarvis beef stunner: Effects of a prototype chest electrode. Meat Sci. 81, 51-56.
Wilhelm, J. & Pingoud, A., 2003. Real-time polymerase chain reaction. ChemBioChem. 4, 1120-1128.
Wilson, C.L., Murphy, L.B., Leslie,J., Kendrick, S., French, J., Fox, C.R., Sheerin,N.S., Fisher. A., Robinson, J.H., Tiniakos, D.G., Gray, D.A., Oakley, F. & Mann, D.A., 2016. Ubiquitin C-terminal hydrolase 1: A novel functional marker for liver myofibroblasts and a therapeutic target in chronic liver disease. J. Hepatol. 63(6), 1421-1428.
Young, O. A., Priolo, A., Simmons, N. J. & West, J., 1999. Effects of rigor attainment temperature on meat blooming and colour on display. Meat Sci. 52(1), 47-56.