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Predicting colour and flavour stabilities of meat from pre-slaughter assessments

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Abstract

To determine whether circulating levels of blood isoprostane can be utilised as an effective biomarker in prime lambs to predict changes in the colour and/or flavour stabilities of meat post-slaughter; the circulating level of blood isoprostane was determined in 84 lambs finished on four different finishing diets (lucerne, ryegrass, commercial feedlot ration and a combination of rye grass and commercial feedlot ration). The circulating levels of isoprostane for each animal was then correlated with traditional meat quality measurements for colour and/or flavour stabilities. This included lipid oxidation levels in meat (i.e. thiobarbituric acid reactive substances (TBARS) analysis), muscle vitamin E content and meat colour redness and brownness as determined by HunterLab.

We found that lambs finished on ryegrass had the highest level of muscle vitamin E content, 2.9 mg/kg; whereas lambs finished on the commercial feedlot ration had the lowest muscle vitamin E content, 0.73 mg/kg ($P < 0.001$). Lipid oxidation levels were lowest in lambs finished on the ryegrass diet, highest for lambs finished on the commercial feedlot ration and intermediate for lambs finished on lucerne and combination ration ($P < 0.001$). After 8 weeks finishing, blood isoprostane levels were positively corelated with the level of lipid oxidation of fresh (5 days) and aged (60 days) meat displayed for 96 hours under simulated retail conditions ($P < 0.01$). Also, there was negative linear relationship between isoprostane concentration and muscle vitamin E concentration ($P < 0.07$); and lipid oxidation and muscle vitamin E concentration ($P < 0.001$). Therefore, after 96 hours simulated retail display, lambs finished on a ryegrass contain muscle vitamin E concentrations that reduce lipid oxidation in both fresh and aged meat. Whilst, lambs finished on a commercial feedlot ration have a significantly lower muscle vitamin E concentration that promoted greater levels of lipid oxidation in fresh and aged meat under the same conditions. Increased levels of lipid oxidation post-farm gate influences the flavour/aroma of lamb and has a negative impact on the consumers eating experience. However, we did not observe a significant relationship between the overall redness of meat stored under simulated retail display conditions at 1 and 4 days with blood isoprostane concentrations at week 0, 4, 6 or 8 of finishing or the vitamin E concentration of muscle.

The overall results show great Industry promise and prove that blood isoprostane concentration can be used as a biomarker to predict oxidative stress in farm animals pre-farm gate. Increased oxidative stress is associated with reduced meat quality and flavour deterioration in lamb due to higher levels of lipid oxidation. This is the first known report to identify a link between a biomarker of oxidative stress (i.e. isoprostane concentration) and reduced meat quality in sheepmeat.

Executive Summary

Colour and its association with flavour is a key component of consumer meat purchase decision making and is extremely important in key export markets such as Japan and South Korea. Discolouration causes significant financial loss to the Australian Red Meat Industry so reducing meat deterioration caused by oxidative processes will improve consumer acceptance and purchasing choice resulting in increased profitability for producers and processors.

This study reviewed the biochemical systems responsible for oxidative stress to identify potential biomarkers that can be used as predictors of colour and flavour stabilities of meat pre-slaughter. The review identified one group of potential biomarkers, the isoprostanes, that are present in the complex biochemical reactions relating to the oxidative processes that take place in the biological systems of live animals (*in vivo*) that could contribute to post-mortem events in meat (*in vitro*). The review suggested that blood isoprostane concentration could be a valuable tool to predict the colour or flavour stabilities of meat by relating this to the redness of meat or lipid oxidation during retail display, respectively.

The objective of the project was to determine the relationship between isoprostane concentration in the blood of lambs on farm, vitamin E concentration in muscle at slaughter and colour stability (assessed by redness of meat) or flavour stability (assessed by lipid oxidation) of meat at retail display. Second cross lambs (Poll Dorset x Border Leicester x Merino) raised on ryegrass pasture with their mothers until weaning (10 weeks of age) were used in this study. The lambs were randomly allocated to four finishing diets; (1) lucerne pasture, (2) annual ryegrass with sub clover pasture); (3) standard commercial feedlot pellets ; and (4) a combination of annual ryegrass based pasture and feedlot pellets .

The study showed there were significant effects of finishing diet on muscle vitamin E concentrations and lipid oxidation of fresh meat and aged meat stored under simulated retail conditions. The blood isoprostane concentration was positively correlated to lipid oxidation of meat displayed under retail conditions. Lambs fed a feedlot diet had a significantly lower muscle vitamin E concentration, which lead to greater lipid oxidation; whilst, lambs fed a ryegrass diet had higher muscle vitamin E concentration that lead to significantly lower lipid oxidation in meat displayed for 96 h under simulated retail display.

These results show that blood isoprostane concentration is a useful biomarker for detecting oxidative stress in farm animals pre-farm gate. This is the first known report to identify a link between oxidative stress (i.e. blood isoprostane content) and reduced meat quality.

Although further validation is required, measuring blood isoprostane content pre-slaughter will provide producers and processors with valuable information on an animal's meat quality and likely deterioration under retail conditions. A further study to investigate the relationship of finishing diet, duration on finishing diet, blood isoprostane concentration, muscle vitamin E concentration and lipid oxidation of meat stored under retail conditions will enable producers to devise on-farm dietary interventions that would protect their animals from oxidative stress and reduce the production of meat with inferior flavour and aroma due to lipid oxidation under retail conditions. This will increase the shelf life of meat and the overall profitability of the Industry.

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1 Background

Meat discolouration causes significant financial losses to the meat industry (Bekhit et al., 2013) and the importance of meat colour and flavour is high in meat export markets such as Japan (Sanders et al., 1997). Increasing the shelf-life of meat by 1-2 days has been estimated to save the American meat industry between $175 million and $1 billion (US) annually (Williams et al., 1992). There is no published estimate of value losses due to meat discolouration and undesirable flavour for the Australian meat industry; although Coles Pty Ltd have estimated they lose $2 million per year due to discounting associated with discolouration. Meat colour and flavour in addition to tenderness have universally been associated with decision making and the purchase of meat. Furthermore, the ability to produce a unique meat product with consistently better colour and flavour (taste) attributes could be a key to several local and international markets. Recent research indicates that antioxidant status (vitamin E) and haem iron content in muscle are major determinants in retaining muscle redness at retail (Ponnampalam et al., 2012a). Loss of meat redness due to oxidation of haem pigments and lipids may substantially reduce the retail value of meat cuts or products. Due to colour deterioration at retail, the price of cuts may be reduced or they may be converted to mince, resulting in a loss in revenue to the retail sector of the meat industry. Maintenance of (lamb) flavour, freshness and integrity of meat at retail by reducing the quality deterioration (by oxidation) may also lift consumer acceptance and purchasing choice that in turn will improve the profitability of producers and retailers by increased sales.

Oxidative stress in humans or animals is characterised by an imbalance between increased exposure to free radicals, which can be generated endogenously (e.g., acute or chronic diseases) or from exogenous sources (e.g., environmental effect) (Halliwell & Gutteridge, 2007), and inadequate antioxidant defence systems. Producing lambs extensively year round is challenging due to many constraints, which affects the cost of production and quality of meat through supply chains. Farm animals can undergo several challenges, such as a poor immune status, imbalance in feed nutritive values, and heat/cold stress, and these can all influence the oxidative status of individual animals to various degrees. Any variation in the oxidative status of the animal can induce the formation of free radicals, which can lead to further damage to DNA, lipids, and protein (Bagchi & Puri, 1998). It is logical to think that the oxidative processes in meat across the supply chain can be related to the oxidative stress status of animals on-farm which is mostly reflected by changes in lipids or other chemical components in tissues leading to colour or flavour deterioration in meat.

Oxidation of meat occurs under post-mortem conditions and is inevitable. This oxidation includes the biochemical changes in meat leading to changes in colour pigments and lipids. As a consequence colour deteriorates and undesirable flavours and rancidity develop in meat thereby impacting on consumer appeal and satisfaction (Descalzo & Sancho, 2008). Consumer choice when purchasing lamb can be influenced directly by the appearance at point of retail sale or indirectly from the perception of the texture and taste/flavour of meat when consumed. There have been several methods used to identify the oxidative stress status of humans and laboratory animals, but to-date none are available to assess the oxidative stress of farm animals and thus relate this to oxidative processes of muscle and lipid oxidation in meat.

1.1 Meat Colour

A large proportion of lamb (43%) and mutton (75%) produced from Australia is exported to markets in North America, North Asia, the Middle East and Europe (MLA, 2007). Most exported lamb is vacuum packed, aged for 3 to 8 weeks at 4^{o}C and then processed for packing under retail conditions. In Australia, lamb is sold as retail fresh without packaging (within as little as 24 h post-mortem), vacuum packed or subjected to modified atmosphere packaging and then sold after a period of ageing. The colour of meat is one of the important quality attributes because meat colour strongly influences a consumer's decision to purchase meat. MacDougall (1982) stated that "attractiveness of the product, usually considered as redness, may have little relationship to the final assessment of the product as eaten, but it is usually the only criterion, other than cost, which influences the consumer in making a decision to purchase". As meat ages, it becomes less red and this has been indicated by decline in a*-values (Renerre, et al., 1996; Faustman & Phillips, 2001) and is due to the conversion of oxymyoglobin to metmyoglobin (Faustman & Cassens, 1990). Consumers will not purchase brown meat (Hood & Riordan, 1973). Thus, early identification of animals producing meat with degraded colour will help the meat industry (producers or processors) develop on-farm management strategies or on-line technologies that enhance or preserve meat colour throughout retail display.

1.2 Meat Flavour

The nutrient composition of the diet consumed by animals, including humans, directly influences the biochemical components and oxidative capacity of muscle tissue systems (Ames et al.,1993; Fang et al., 2002). When the oxidative potential of a muscle tissue system is suboptimal, this may cause an imbalance or instability in the functions of muscle biological systems (Bagchi & Puri, 1998; Frei, 2004). As such, this may affect the reactive capacity of chemical components within muscle systems leading to the formation of free radicals and secondary oxidative substrates in the muscle of living animals as well as in post-mortem muscle foods (Dabbagh et al.,1994; Brigelius-Flohe & Traber 1999; Bekhit et al., 2013). A number of studies have reported that hydrogen abstraction from PUFA by low molecular weight metal complexes such as iron chelated-adenosine diphosphate (Fe-ADP), haem proteins such as myoglobin and haemoglobin, and enzymes such as lipoxygenases "initiate" lipid oxidation. The resultant products of lipid oxidation can significantly affect the flavour or aroma of meat. Free radicals resulting from breakdown of preformed lipid hydroperoxides by metals and haem proteins can also initiate hydrogen abstraction, which can lead to quality deterioration in muscle foods during storage (Morrissey et al.,1998; Chaijan, 2008). The extent of lipid oxidation in muscle foods is dependent upon the balance of chemical components present; mainly unsaturated fatty acids, chelating trace elements (e.g. iron) and/or antioxidants. External factors such as light and oxygen can also affect oxidation in muscles (Jensen et al.,1998). Several reviews discuss the interrelationship between lipid- and pigment-oxidation in muscle quality deterioration (Chaijan, 2008; Faustman et al., 2010) and the importance of vitamin E (α-tocopherol) as an antioxidant in muscle systems (Ames et al.,1993; Brigelius-Flohe & Traber 1999); however, many of the results from these studies are inconclusive. Thus, identifying a biomarker in the blood that is able to predict lipid oxidation in muscle tissues pre-slaughter would be extremely beneficial to the meat industry and enable producers and processors to develop management

strategies or on-line processing techniques that reduce the impact of lipid oxidation over the supply chain.

1.3 Biomarkers to predict oxidative stress in animals

Oxidation of meat occurs under post-mortem conditions and is inevitable. This oxidation includes the biochemical changes in meat leading to changes in colour pigments and lipids. As a consequence, colour deteriorates, and undesirable flavours and rancidity develop in meat thereby impacting on consumer appeal and satisfaction. Across carcasses there is variation in the rate at which muscle undergoes chemical reactions under post-mortem conditions that reflects inherent variation at the biochemical level. It is expected that this underlying biochemical variation will be reflected in living muscle through oxidative processes. The oxidative process of muscle tissues will vary according to an animal's immunity status, temperament, and ability to cope with stress, with all these affected by nutrition, genetics, management practices, and environmental conditions (hot and cold seasons). Identification of biomarkers that indicate the oxidative status levels of animals or muscle tissues *in vivo* could provide insight as to how the muscle will respond to the anoxic conditions that produce undesirable results in meat.

Currently, there is no methodology available to measure the oxidative status of animals on-farm and whether the oxidative status of animals affects the quality of muscles or muscle foods. Prediction of oxidative status in live animals may provide an opportunity to improve animal productivity and profitability through on-farm nutritional management. Therefore, the development of non-destructive techniques is an important priority (Wold & Mielnik 2000). One of the most important markers used for oxidative stress in human and laboratory animals is isoprostanes. Many of the methods that are traditionally used for the evaluation of oxidative stress *in vivo* are deficient in several important aspects, such as specificity and/or sensitivity, which make them unreliable (Wood et al., 2006). Isoprostanes are regarded as the "gold standard" and "the most reliable" marker of oxidative stress in humans (Morrow 2005; Lykkesfeldt & Svendsen 2007).

The formation of isoprostane (specifically 8-iso-PGF2α, also known as 15-F2t- isoprostane) was suggested as an indirect marker for *in vivo* lipid oxidation (Morrow 2000; Roberts & Milne 2009; Halliwell & Lee 2010) due to its abundant production. These compounds have the merit of being specific products of lipid oxidation and are relatively stable compounds that provide information about the oxidative status of an individual. This compound is found in body fluids such as blood and urine at detectable levels, and therefore, invasive procedures are not required for measurement (Morrow 2000, 2005). F2-isops are widely distributed in the body and their measurements have been reported in several biological fluids (plasma, urine, exhaled breath condensate, saliva, bile, cerebrospinal, seminal, and pericardial fluids) and tissues (including skeletal muscle) (Nikolaidis et al., 2011). Urine is the most common human biological fluid used for the detection of isoprostanes (Roberts & Milne 2009). However, collection of urine on-farm or at slaughter from live animals is not feasible. In this manner, the collection of blood for the determination of F2-isoprostane, as a marker for oxidative stress, is a viable pathway.

A review was conducted to identify the potential blood biomarkers that can be used as a predictor of colour and flavour stabilities of meat pre-slaughter. The literature review formulated by Ponnampalam, Bekhit, Fahri & Hopkins has been published in the

Comprehensive Reviews in Food Science and Food Safety (Bekhit et al., 2013). This review identified the potential use of one group of biomarkers, the isoprostanes, in the context of complex biochemical reactions relating to oxidative processes that take place in the biological systems of live animals (*in vivo*) and subsequently in meat (*in vitro*). The blood isoprostane concentration can be a valuable tool to predict the colour or flavour stabilities of meat by relating the concentration to the redness of meat (a*-value) or lipid oxidation (TBARS) during retail display, respectively.

2 Project Objectives

2.1 To determine the relationship between isoprostane in blood at 2 days to 6 weeks pre-slaughter and vitamin E in muscle at slaughter for lambs.

2.2 To determine how this relationship differs between lambs with different finishing (diets).

2.3 To determine how these effects (in points 1 and 2) are reflected in colour stability and lipid oxidation (TBARS) during retail display.

3 Design, Measurements and Outcomes

3.1 Experimental design

All animal experimentation was conducted at the Victorian Department of Environment and Primary Industries Research facility at Rutherglen and approved by the Animal Ethics Committee (AEC: 2012-19). Eighty-four crossbred lambs of mixed gender (wethers and females) were allocated to four finishing diets based on live weight. Table 2 shows the mean live weights and standard deviations for replicates at the commencement of the study. The four finishing diets were: (1) Lucerne pasture (n = 24), (2) Annual ryegrass with sub clover pasture (n = 18), (3) Standard commercial feedlot pellets (n = 24) and (4) Annual ryegrass based pasture and feedlot pellets (500 g/day/head) (n = 18).

3.2 Pasture and live weight measurements

The experiment was conducted during the Spring, 2012 (i.e. September – November). Animals were maintained under extensive finishing systems for a period of 8 weeks which included a 2 week adaptation period. Paddock details, treatments, replicates and number of lambs allocated to each block are listed in Table 1. During week 1 to 4 of feeding the lambs in the lucerne and annual ryegrass treatments were allocated a 6 m x 12 m grazing plot. Lambs were shifted each week onto a new grazing plot to offer new pasture. Animals finished on diet 4 had access to half the grazing area when compared to other grazing treatments (3 m x 12 m). From week 5 to 6 the grazing plot area doubled to 12 m x 12 m for the annual ryegrass and lucerne treatments and half the grazing area for diet 4 (12 m x 6 m).

Feed samples and lamb live weights were collected weekly for the analysis of chemical composition and animal performance, respectively. Each week from the pasture paddocks shown in Table 2, six 0.25 m x 0.25 m quadrats were cut and homogeneous samples were

taken for the determination of dry matter (DM) content of the pasture, chemical composition of the pasture and vitamin E content of the pasture. This was undertaken before introduction of lambs to each pasture paddock (pre-grazing) and after removal of lambs from each pasture paddock (post-grazing) weekly. Feedlot samples were also collected weekly for the determination of above mentioned parameters. One set of samples were dried at 100^{o}C for 24 h for the determination of DM content. Another set were dried at 65^{o}C for 48 h for the determination of chemical composition. The third set of samples were freeze dried for the determination of vitamin E (α-tocopherol) content. Figure 1 shows the weekly live weights mean for each treatment group.

3.3 Blood sample collection and isoprostane determination in live lambs

At the commencement of the study (week 0), 4, 6 and 8 weeks (i.e. 2 days prior to slaughter), blood samples were collected from all lambs. For each blood collection, lambs were brought into a shed as replicates in random starting at 9:00 am and left for 30 minutes to settle. Blood samples [10 ml] were then collected in lithium heparin vacutainer tubes by jugular venepuncture and maintained on ice for 2 h until centrifuging. After centrifuging at 3000 x g for 10 minutes at 4^{o}C, the plasma was separated into 3 aliquots and stored at -80^{o}C for analysis of isoprostane content and other lipid components. To one aliquot, 10 µl of 0.22 µM BHT prepared in ethanol was added to each mL of plasma sample. Isoprostanes analysis will be performed on this aliquot.

Isoprostane plasma sample preparation:

The pH of plasma was adjusted to pH 3 with 1N HCl and 1 mL of the sample was diluted with 1 mL of PBS. A positive control was included by spiking a sample to a final concentration of 5 ng/mL and the positive control was analysis in parallel with the samples. The samples and the positive control were subjected to a clean-up step using a C18 Sep Pak plate on vacuum manifold without the attachment of the 96 well polypropylene collection plate. The clean-up routine was carried out according to the protocol instruction (successive washing steps with deionised water (pH 3) and heptane, followed by elution with ethyl acetate : heptane (1:1) mixture in a 96 well plate. The collected eluate was transferred to Eppendorf tubes and the elution process was repeated 2 more times (the total eluate volume will be 1 mL). A 96 well plate was removed and the Sep Pak plate on manifold was reaffixed. A pre-wash step was carried out using methanol and ethyl acetate. Then the samples were loaded, and eluted at a flow rate of about 1 mL/min. The Sep Pak plate was washed again using methanol and ethyl acetate. The 96 well polypropylene was inserted and the sample was eluted with ethyl acetate : methanol (1:1) mixture. The eluted sample was transferred to a Eppendorf tube and the elution was repeated twice. The total eluted sample was evaporated under a stream of Nitrogen. The oily residue obtained was stored at -80^{o}C till analysis. The residue was reconstituted in a dilution buffer supplied with the kit. Half the sample was used for the enzyme immunoassay and the other half was used for the LCMS analysis.

Method 1: Determining isoprostane concentration by enzyme immunoassay

The plasma isoprostane concentration was determined using the Isoprostane Immunoassay Kit (Catalogue number: A84) from Oxford Biomedical Research according the manufacturer's instructions. One hundred microlitres of sample or standard (included in the kit) was added to each well of the coated plate supplied with the kit. A 100 µL of diluted 15-IsoP F2t HRP conjugate (8-isoprostane) was added to each well and the plate was incubated at room temperature for 2 hours. The wells were washed and a 300 µL of a washing buffer was added and the plate was left to stand for 2 - 3 minutes. The contents of the wells were removed by inversion of plate into an appropriate disposal device. The

procedure was repeated and 200 µL of TMB Substrate was added to each well. The plate was incubated for 30 minutes at room temperature until an appreciable blue hue was observed. 50 µL of 3 N H_2SO_4 was added to each well to stop the reaction and the colour changed from blue to yellow. The plate was read at 450 nm.

Method 2: Determining isoprostane concentration by LCMS analysis

The equipment used for the LCMS analysis is the Surveyor HPLC System from Thermo Finnigan, interfaced with a Surveyor MSQ single quadrupole mass spectrometer. A C-18 column (Alltech Altima HP C18, 100 mm x 2.1 mm, 3 µm particle size) was used for the separation of the isoprostane. The mass spectrometer was equipped with a single quadrupole mass analyser, an ESI probe, a turbo molecular pump, and a cone-wash system. The sample will be chromatographed with a linear gradient of acetonitrile in water (20% to 45% in 25 min) at a flow rate of 200 µL/min. The mass spectrometer conditions were as follows: negative ESI mode, drying gas flow (N2) at 650 L/h, needle voltage at 2.3 KeV, probe temperature at 400°C, cone voltage at 60 eV, and detector voltage at 1953 V. The analysis was carried out in SIM mode for the molecular ions of iPF2 (m/z 353.2). A pure standard (Cayman Chemical, Item No. 316350) was used for the identification and quantification of the isoprostane.

3.4 Measurements and sample (urine and muscle) collection at slaughter

At completion of the finishing period, lambs were transported to a commercial abattoir in Kyneton, Victoria and slaughtered after fasting for 18 h. The project team believed collecting urine at slaughter could be utilise as a potential biological fluid for determining isoprostane content. Thus, urine samples were collected 5 minutes post-slaughter. However, only 24 animals out of a possible 84 animals contained urine in their bladder. Thus, we did not process the 24 urine samples collected for isoprostane content.

At 1 h post-slaughter carcass weight, GR fat depth and m. *longissimus* (LL) pH were recorded on carcasses maintained in the chiller. Table 3 shows the slaughter weight, carcass weight, carcass yield (dressing percentage) and GR for lambs fed lucerne, ryegrass, ryegrass plus feedlot and feedlot treatments.

At 24 h post-mortem, each lamb carcass was split down the middle and a large (0.5 – 0.8 kg) portion of muscle LL (loin) and the *semimembranosus* muscle (SM; topside) were dissected from the left side of the carcass. Surface colour of muscle LL was recorded at the abattoir (approximately at 24 h) using a HunterLab meter (HunterLab Miniscan, TM XE Plus 45/10, Reston, VA, USA), with light source set at D65/10. Muscle samples also were collected for the determination of vitamin E. The LL and SM muscles were also used for the assessment of fresh (5 days) and aged (60 days) meat colour. These muscles were then vacuum packed and transported to NSW DPI Meat Research Laboratory, Cowra under chilled conditions. These LL (loin) and SM (leg) muscles were used for the evaluation of retail colour (shelf life) and tested (only LL at 1 & 4 days of display) using the thiobarbituric acid reactive substances (TBARS) assay. Retail colour was assessed in fresh and aged meat stored at 3^{o}C for 96 h after storage for 5 and 60 days, respectively.

Table 1: Average initial live weight (kg) of lambs allocated to each dietary treatment by replicate.

Paddocks	Mean live weight (kg)	Standard Deviation (kg)	Number of lambs
Feedlot Rep 1	31.78	1.74	6
Feedlot Rep 2	31.60	2.44	6
Feedlot Rep 3	31.50	2.13	6
Feedlot Rep 4	31.83	1.89	6
Lucerne 1e	31.17	1.72	6
Lucerne D1	31.35	2.31	6
Lucerne E	31.15	1.96	6
Lucerne J	31.90	1.57	6
Annual ryegrass/feedlot H	31.53	1.73	6
Annual ryegrass/feedlot P1	31.75	1.47	6
Annual ryegrass/feedlot P4	31.60	2.41	6
Annual ryegrass F	32.35	2.34	6
Annual ryegrass P3	31.68	2.02	6
Annual ryegrass V1	31.73	1.70	6

Table 2: Details of paddocks, treatments, replicates and number of lambs used in the study.

Paddock	Treatment	Replicate	Number of lambs
1E	Lucerne	1	6
D1	Lucerne	2	6
E	Lucerne	3	6
J	Lucerne	4	6
V1	Annual ryegrass	3	6
F	Annual ryegrass	2	6
P3	Annual ryegrass	1	6
H	Annual ryegrass / feedlot	1	6
P1	Annual ryegrass / feedlot	3	6
P4	Annual ryegrass / feedlot	2	6
Feedlot Rep 1	Feedlot	1	6
Feedlot Rep 2	Feedlot	2	6
Feedlot Rep 3	Feedlot	3	6
Feedlot Rep 4	Feedlot	4	6

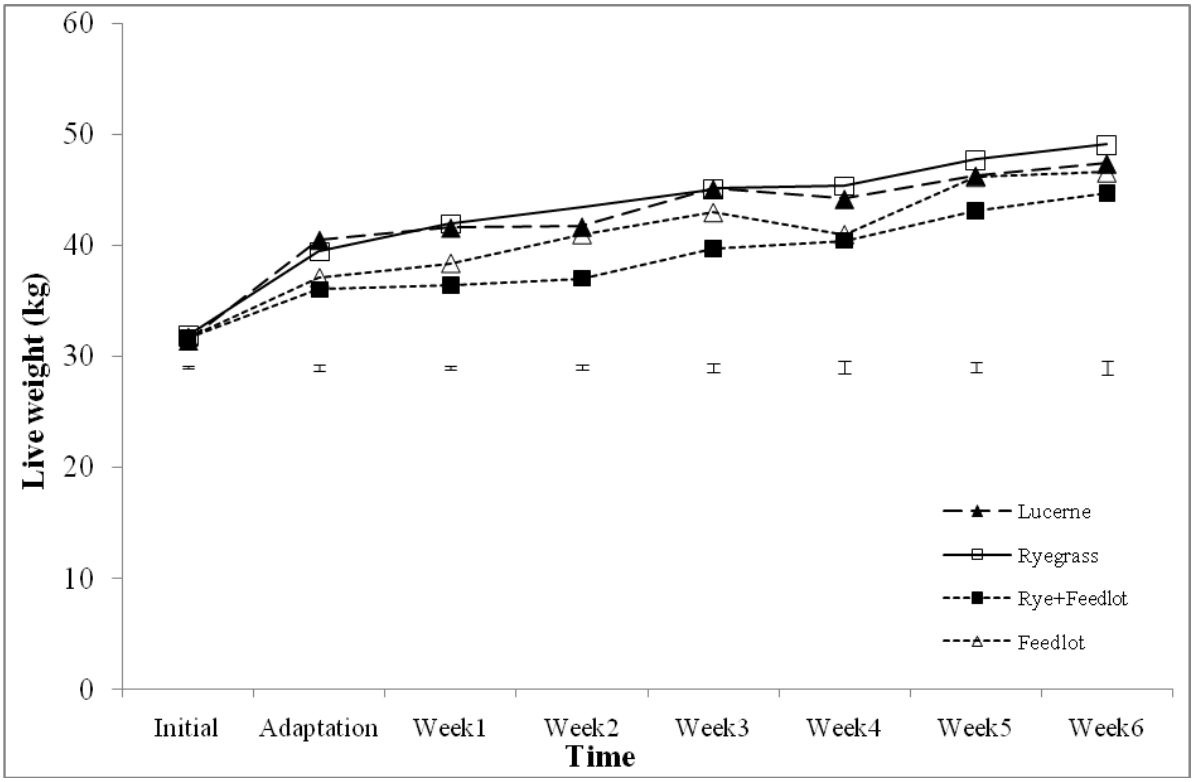

Figure 1: Treatment means for weekly live weights of lambs fed the lucerne, annual ryegrass, annual ryegrass plus feedlot and feedlot diets. Error bars indicate the standard errors of differences of means (SED) at each weighing point.

Table 3: Initial live weight, live weight prior to slaughter, carcass weight, dressing percentage and GR of lambs fed lucerne, ryegrass, ryegrass plus feedlot and feedlot diets.

	Lucerne	Ryegrass	RyeFeedlot	Feedlot	SED	P-value
Initial live weight (kg)	31.4	31.6	31.6	31.7	0.23	NS
Live weight prior to slaughter (kg)	47.4^{bc}	49.1^{c}	44.7^{a}	46.6^{ab}	1.22	0.04
Carcass weight (kg)	22.4^{b}	21.7^{b}	19.5^{a}	22.2^{b}	0.55	0.002
Dressing percentage (%)	47.2^{b}	44.2^{a}	43.6^{a}	47.8^{b}	0.72	0.001
GR (mm)	11.2^{bc}	10.6^{ab}	9.2^{a}	12.6^{c}	0.88	0.02

Mean followed by different superscripts are significantly different at $P < 0.05$.

3.5 Retail colour measurement (shelf life of meat)

Retail colour (shelf life) of fresh (5 day) and aged (60 days) meat was measured at day 1, 2, 3 and 4 of display (day 1 = day 0 of slicing) under simulated display as reported by Ponnampalam et al. (2010). The LL (loin) and SM (topside) muscles were cut into one slice of 3.0 cm thickness, placed on a plastic tray, over-wrapped with a 15-micron PVC film and displayed under refrigerated conditions (3-4^{o}C) with fluorescent lights set at 1100 lux. The meat colour was measured, using a HunterLab meter (HunterLab Miniscan, TM XEPlus 45/10, Reston, VA, USA), with light source set at D65/10. For each muscle, on the day of preparation, colour was recorded after a 30-min bloom at 3^{o}C.

Colour stability of fresh and aged meat was assessed by measuring the change in redness of the meat (a*-value) and the formation of brownness/redness (a proxy for the ratio of oxymyoglobin and metmyoglobin (oxy: met) in the meat surface, as determined by the reflectance ratio at 630: 580 nm wavelength). The reflectance ratio of 630 and 580 nm (R630/580) is an indirect measure of metmyoglobin formation (brownness) on the meat surface (MacDougall, 1995). Data were analysed using ANOVA with “display time” points as repeated measurements. Treatment and ageing period (5 days vs 60 days) were used as main effects and paddock was used as block structure in order to obtain the means of interaction between treatment by ageing by time and other main effects. At slicing (day1) and the end of retail colour measurement (day 4) for fresh and aged meat, samples were collected from all lambs for LL and stored at -20^{o}C for determination of lipid oxidation using TBARS assessment.

3.5.1 Retention of redness in meat as assessed by a*-value

The change in redness of meat as assessed by the a*-value for LL of fresh (5 days) and aged (60 days) muscles is shown in Figures 2A & 2B while for the SM it is shown in Figures 3A & 3B. With LL muscle, there was a treatment by ageing by display time interaction (P < 0.03) where the retention of redness in 60 day aged meat for feedlot ration fed lambs was not maintained as shown with fresh meat. A similar tendency (P = 0.06) was also shown with SM muscle in the retention of redness. It was clear that although the a*-values were higher on day 1 for 60 day aged meat in both LL and SM, there was a rapid decline over days 2 to 4 compared with fresh meat (5 days). For fresh meat, redness values of LL and SM at 4 days of simulated display were even higher than values found for aged meat (60 days) at 2 days of display. This dramatic decline was not observed in a previous study where meat was stored for 30 days at similar storage condition to this study, but where muscle vitamin E concentration was 3.2 mg/ kg meat or greater (Ponnampalam et al., 2013). In the current study, since the vitamin E concentration was less than 3.2 this could be a reason for the 60 day aged meat exhibiting a rapid decline in redness over the display time.

3.5.2 Brownness formation in meat as assessed by reflectance 630/580

Formation of brownness on the meat surface of the LL as assessed by the R630/580 ratio for fresh (5 days) and aged (60 days) muscles is shown in Figures 4A & 4B. The ratio for SM fresh (5 days) and aged (60 days) muscles are shown in Figures 5A & 5B. For both LL (P = 0.40) and SM (P = 0.21) muscles, there were no treatment by ageing time by display time interactions observed. For SM, there was a treatment by display time interaction (P < 0.02). With time, the formation of brownness in LL and SM muscles for aged meat rapidly

increased (i.e., lower reflectance of 630/580) and the values at day 2 for aged meat were lower than the values observed at day 4 for fresh meat. The rapid decline in retention of redness or marked increase in formation of brownness on the meat surface of meat aged for 60 days in all treatments for both LL and SM muscles could be associated with the muscle vitamin E concentration being less than 3.2 mg/kg meat. Other potential reasons for this rapid decline in the retention of redness or marked increase in the formation of brownness include reduced iron content in meat or fatty acid composition.

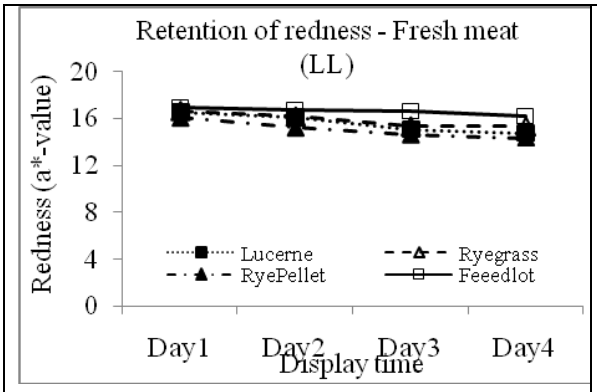

Figure 2A

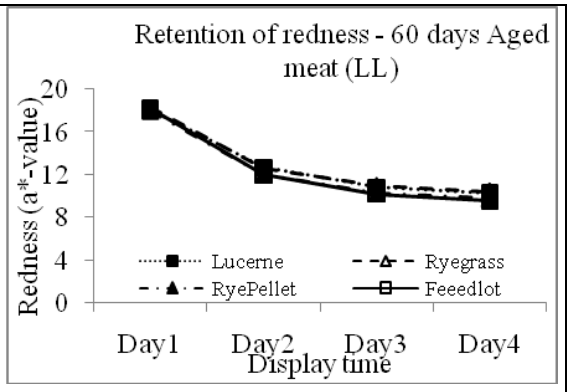

Figure 2B

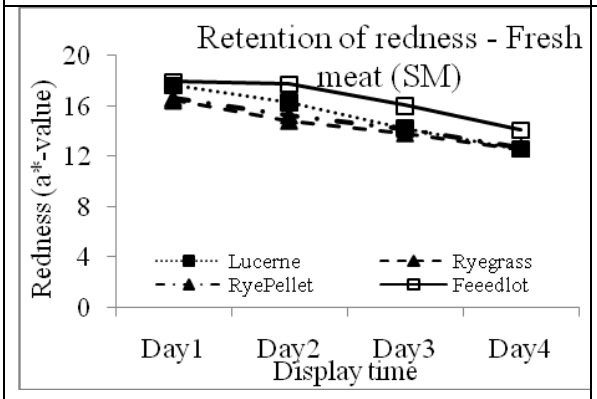

Figure 3A

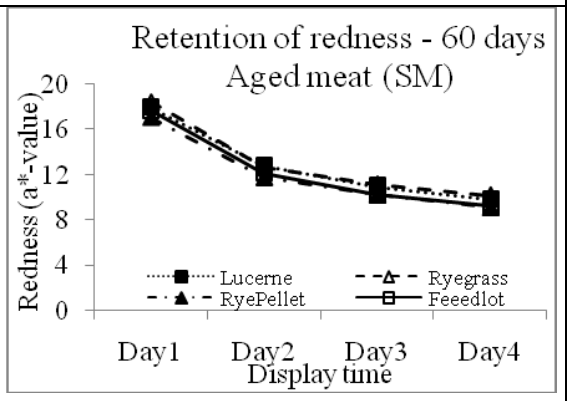

Figure 3B

Figures 2 and 3: The change in redness in LL (Fig. 2A & 2B) and SM (Fig. 3A & 3B) muscles as assessed by the a*-value for fresh (5 days) and aged (60 days) meat from lambs fed different diets.

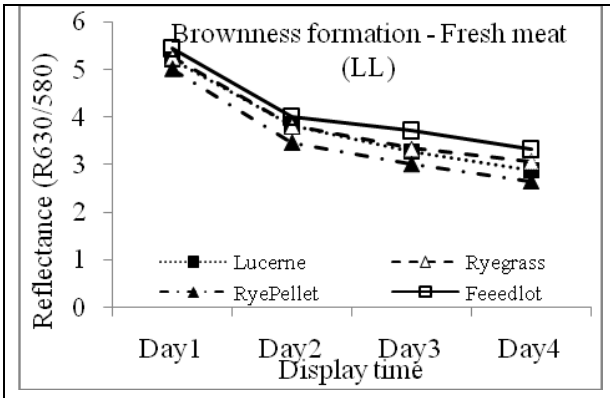

Figure 4A

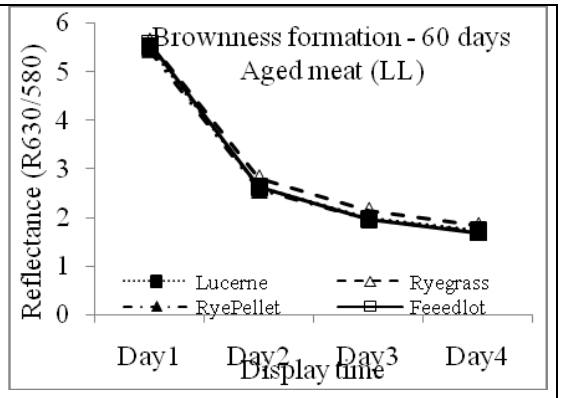

Figure 4B

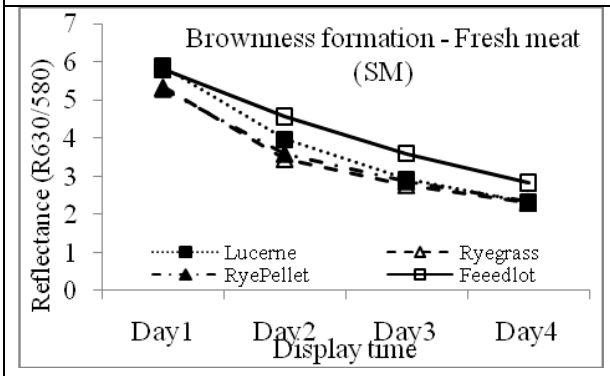

Figure 5A

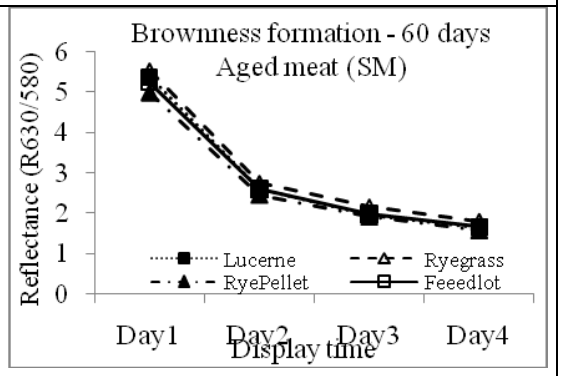

Figure 5B

Figures 4 and 5: The formation of brownness on the meat surface of the LL (Fig. 4A & 4B) and SM (Fig. 5A & 5B) muscles as assessed by the ratio R630/580 of fresh (5 days) and aged (60 days) meat from lambs fed different diets.

3.6 Lipid oxidation in meat

At the end of retail colour measurement (96 h) for fresh (5 days) and aged meat (60 days), samples of LL were collected from all lambs and stored at -20^{o}C for determination of lipid oxidation. The lipid oxidation in meat was assessed by the thiobarbituric acid reactive substances (TBARS) assay and the results are expressed in milligrams of malondialdehyde (MDA)/kg of muscle. There were significant treatment differences in lipid oxidation measured in 5 day (Figure 6A) and 60 day aged (Figure 6B) LL samples ($P < 0.001$; SED = 0.25). The results indicate that higher antioxidant potential (for example in the ryegrass treatment) in muscle provides resistance to oxidation as shown by lower levels of lipid oxidative substances. This was consistent for both 5 days and 60 days stored meat displayed for 96 hours under simulated retail display. When compared 5 day and 60 day stored samples over the 96 h (4 days) display time, the lipid oxidation levels showed a smaller increase in lucerne fed lambs (2.8 fold increase in lipid oxidation) compared with ryegrass, ryegrass/pellet and feedlot ration fed lambs (4.2, 3.3 & 3.1 fold increase, respectively).

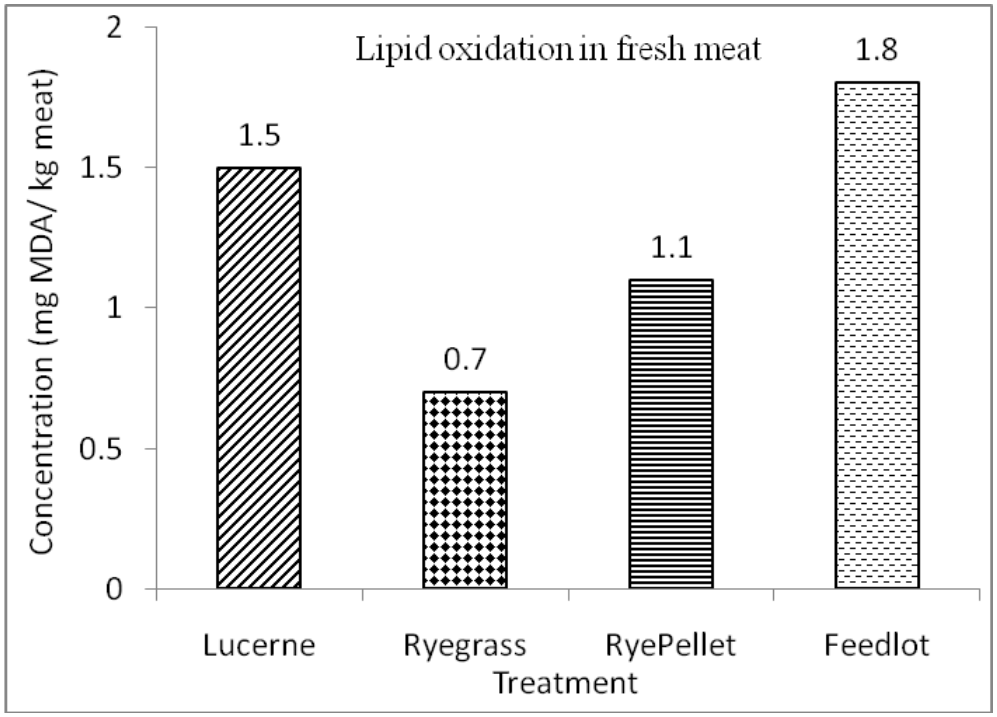

Figure 6A: Lipid oxidation in fresh meat (5 day aged LL) from lambs fed lucerne, ryegrass, ryegrass with pellet (RyePellet) and a feedlot ration. Muscle LL samples were collected after 96 h of simulated retail display at 3-4^{o}C.

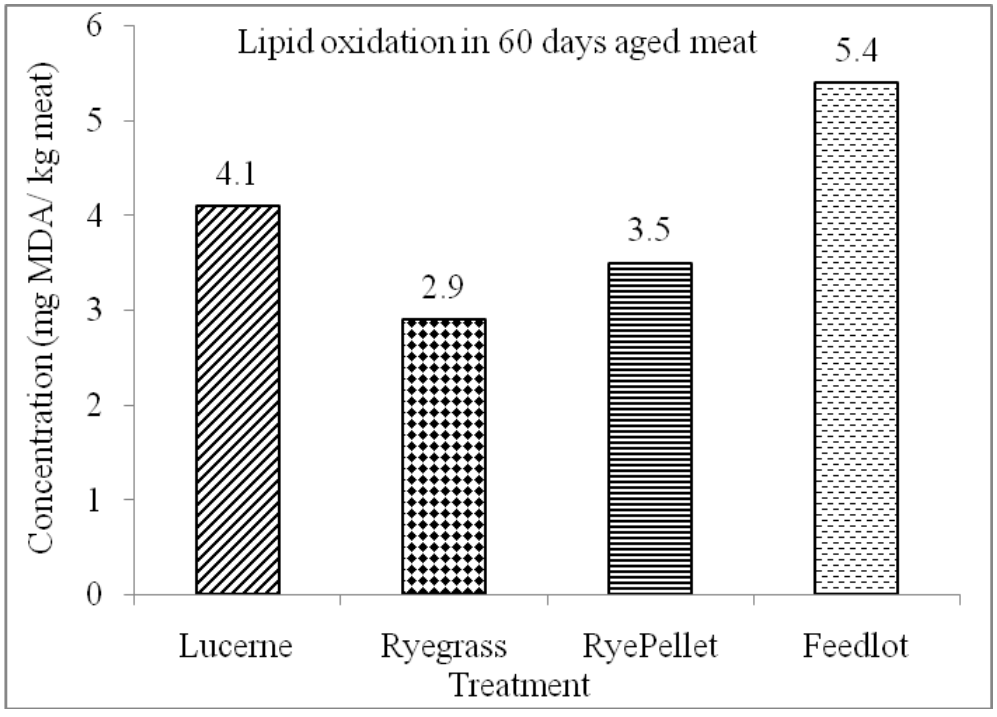

Figure 6B: Lipid oxidation in aged meat (60 day aged LL) from lambs fed lucerne, ryegrass, ryegrass with pellet (RyePellet) and a feedlot ration. Muscle LL samples were collected after 96 h of simulated retail display at 3-4^{o}C.

3.7 Muscle vitamin E concentration

Muscle longissimus lumborum (LL) samples collected at 24 h post mortem were used for the determination of vitamin E concentration. Figure 7 shows that the muscle vitamin E concentration of lambs fed ryegrass was the highest and lowest for lambs fed the commercial feedlot ration ($P < 0.001$; SED = 0.11). The vitamin E concentration of muscle for all treatments was below 3.2 mg/ kg meat. This is probably due to the fact that the animals utilised in this study were slaughtered at 4.5 months of age and the feeding period was only 6 weeks. Thus, the animals did not have sufficient time to accumulate reasonable stores of vitamin E in muscle.

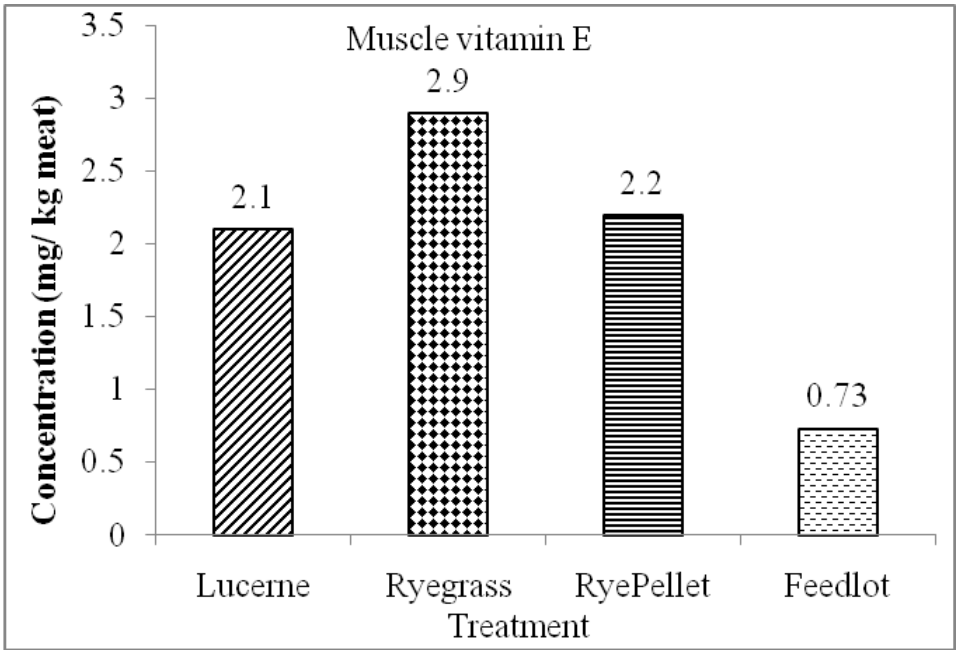

Figure 7: Muscle vitamin E concentration of lambs fed lucerne, ryegrass, ryegrass with pellet (RyePellet) and a feedlot ration.

3.8 Isoprostane formation

Isoprostane formation is initiated by the oxidation of arachidonic acid mediated by free radical attack followed by several steps of oxygen insertion and cyclization (Roberts & Milne 2009). This will generate a complex mixture of 64 enantiomers that are grouped into 4 regioisomeric families of prostanes (see Morrow 2000; 2005) that are commonly referred to as F2-isop 5-, 8-, 12-, or 15-series (see Morrow 2000; 2005; Roberts & Milne 2009). The series-naming refers to the carbon atom position where the hydroxyl side chain is attached with series F2-isop 5-, 8-, and 12-, or 15- derived from the attack at C7, C10, and C13, respectively. The isoprostane 8-epi-PGF2α (aka 8-iso- PGF2α or 15-F2t-isoprostane) is the most studied member of the isoprostanes. The degradation of components such as lipids, protein, or DNA *in vivo* can lead to the formation of several compounds that can be used as markers for oxidative stress. For example, the formation of isoprostanes and isofurans from arachidonic acid (F2-isoprostanes), and neuroprostanes (F4-neuroIsop) from docosahexaenoic acid (DHA) that can be present in different isoforms (Montine et al., 2004; Roberts & Milne 2009). Isoprostanes can be generated from peroxidation of linolenic acid

(F1-isop) and eicosapentaenoic acid (F3-isop) also (Roberts and Milne 2009); however, this is dependent on the antioxidant status of the animal. The majority of the isoprostanes detected in plasma and urine samples are the product of nonenzymatic oxidation of arachidonic acid via RS attack (Roberts & Milne 2009; Halliwell & Lee 2010). The oxidation products are designated as A, E, D, or J that will have a number reflecting the origin of the compounds. For example, those from arachidonic (F2-isop) will be A2-, E2-, D2, and J2-isop. Roberts and Milne (2009) suggested that derived oxidation products from different fatty acids can potentially have different functions. These authors cited a study where EPA derived isop (15-F3t-isop) demonstrated different activity from the equivalent AA-derived (15-F2t-isop) and the supplementation of EPA reduced F2-isop in mouse heart tissues by 60%. Similarly, the supplementation of EPA and DHA to subjects reduced levels of 8-iso-PGF2α to their baseline levels (Higdon et al., 2000; Barden et al., 2004; Mas et al., 2010). The latter suggests that the supplementation of EPA and/or DHA may reduce the oxidative stress in animals by lowering the F2-isoprostane levels in the circulatory systems or body. Research indicated that plasma isop concentrations were not affected by the consumption of high-fat meals (Richelle et al., 1999; Gopaul et al., 2000); however, the type of fatty acid may be more important in modulating isoprostanes in plasma (Higdon et al., 2000; Barden et al., 2004; Iannone et al., 2009; Mas et al., 2010).

3.8.1 Measurement of F_2-Isoprostane

F_2-Isop-8 series can be isolated and measured in several biological fluids and tissues (see Bekhit et al., 2013 for details). The most common methods for the quantification of isoprostanes are: GC-MS, LC-MS, enzyme immunoassay (EIA), and ELISA (Table 13; Bekhit et al., 2013). Commercial EIA and ELISA kits are simple, cheap, and could be used for the rapid screening of animals under realistic commercial conditions. On the other hand, GC-MS and LC-MS methods are extremely sensitive (picogram levels of detection) and can detect several isomeric forms of isoprostanes. However, GC-MS and LC-MS methods are laborious, very expensive, require high technical skills, and are time-consuming that will be a significant hurdle for application to the commercial screening of animals. There are several modified techniques that use various GC-MS methodologies. Modifications of the GC-MS measurement system by Morrow et al. (1992) (namely, separation of isops via solid-phase extraction or affinity chromatography with or without the aid of a thin-layer chromatography followed by final quantification of the compounds of interest with spectrometric techniques such as GC–MS, HPLC-MS, or tandem MS) have been reported to cause the co-migration of various types of isoprostanes and can potentially produce different results (Montine et al., 2007). Therefore, even with the same technique, there is a need for validation of methods. A clear advantage of LC-MS based methods is that they are regarded as a simpler analysis technique due to sample preparation not requiring derivatisation of the molecule before analysis unlike the GC-MS technique. There is a strong correlation between EIA and the GC-MS results (Wang et al., 1995; Devaraj et al., 2001; Wood et al., 2005, 2006). A comparison between EIA and gas chromatography (electron capture)/negative ion mass spectrometry (GC-ECNIMS) showed that both assays provide good relative values (r = 0.91) among samples, but the absolute values were different with higher values found using EIA (Yeoh-Ellerton & Stacey 2003). However, other studies found no or low correlation coefficients (Proudfoot et al., 1999; Bessard et al., 2001; Saenger et al., 2007; Soffler et al., 2010) suggesting a cross validation may be required for the same set of samples (Morrow, 2000). The measurement of F_2-Isop-8 series in several animals has been investigated with

the aim of verifying the accuracy of the compound as a biomarker (Table 13; Bekhit et al., 2013). Generally, the measurements of isoprostane were able to distinguish between animals that had been exposed to toxic prooxidants, underwent surgery, were obese, or were deficient in important nutrients (Table 13, Bekhit et al., 2013). Therefore, some authors advocate the use of F_2-Isop-8 series as a prognostic indicator (Noschka et al., 2011). The majority of these studies used commercial kits that are promising for the effective screening of large numbers of farm animals. However, caution should be exercised as a recent study (Soffler et al., 2010) compared the determination of F2-isop across a range of farm animals using GC-NICIMS and glucuronidase (GL)-ELISA and found various outcomes. These authors found, since there was acceptable agreement between the 2 methods, that the use of GC-MS for some species is recommended.

3.8.2 Assessment of enzyme immunoassay and LC-MS methodology for determination of isoprostane in plasma samples

Isoprostane quantification was determined using enzyme immunoassay and/or LC-MS methodology. Using Oxford Biomedical Research immunoassay kit to quantify the isoprostane concentration on the same blood samples generated a different profile of isoprostane over the 4 bleeding time and quantitative values compared to the EIA kit supplied by Cayman Chemicals (Figure 8). For EIA kit assay, 84 samples per bleed (in total 84 x 4 bleed time) were used. Only 12 blood samples per each bleed (in total 12 x 4 bleed time) were used to compare the ELISA and LC-MS assays. The EIA kit shows a gradual increase in the isoprostane concentration, in the order of pictogram per millilitre, over the 4 bleeding periods with very low differences among the means, which were significant only at weeks 4 and 8. The ELISA kit shows fluctuating trend of isoprostane concentrations, in the order of nanograms per millilitre, over the 4 sampling time points. When the overall outcome from both assays are compared, a general conclusion of lower isoprostane is found in ryegrass feed lambs whereas high isoprostane concentration is found in feedlot lambs could be reached. This provide a circumstantial evidence for the role of Vitamin E in reducing oxidative stress since an inverse relationship for Vitamin E was found in these treatment groups (Figure 7).

LC-MS measurements did not progress as planned because the system did not detect 8-isoprostane in the samples despite the ability to detect and quantify pure standards. We were able to measure 90% recovery of spiked samples. The team is now looking at the degradation products of the 8-isoprostane in limited number of samples as the data generated by the LC-MS system is quite large and the chemistry of the degradation products is not streamlined in literature.

3.9 Relationship between blood isoprostane concentration, muscle vitamin E concentration and lipid oxidation

To examine the relationship between lipid oxidation at day five and different measurements of isoprostanes at bleeding 0, 4, 6 and 8 weeks, for the different diets, a series of restricted maximum likelihood (REML) mixed model analyses were used that included a priori random effect for paddocks within the treatment. In these REML analyses, the experimental unit was measurements taken from individual lambs in each paddock. For each analysis, a residuals verses fitted values plot was examined to determine any extreme outliers. Terms were either included or excluded in the model based on Wald's F-tests. From our preliminary analyses it

was found that diets and vitamin E measurements are strongly correlated to each other, thus making it unfeasible to fit a saturated model first to develop a parsimonious model. Thus initially, the relationship of these measurements with lipid oxidation at 96 h display was examined by fitting a term in the REML model individually. Once the terms that had a statistically detectable effect on lipid oxidation were established, a correlation matrix was generated to study the correlation structure among these terms. Then a sensible saturated model was created by including all the terms that did not have a statistically detectable effect individually plus those terms which had a statistically detectable effect, but were not significantly correlated to each other. A parsimonious model was developed by sequentially dropping individual terms based on Wald's F-tests.

There were no treatment differences on isoprostane concentration for blood collected at the commencement of the study (week 0), and at 6 weeks of feeding the experimental diets. Finishing diets significantly affected ($P < 0.01$ and $P < 0.05$) isoprostane concentration in blood collected after 4 and 8 weeks of feeding the experimental diets (Figure 8). Lambs on the Ryefeedlot treatment had the highest concentration and lambs on the feedlot diet had the lowest concentration of isoprostane in their blood after 4 weeks of feeding, but after 8 weeks of feeding, lambs fed the feedlot diet had the highest concentration of isoprostane while lambs fed the lucerne pasture had the lowest concentration and ryegrass and Ryefeedlot treatments had intermediate values.

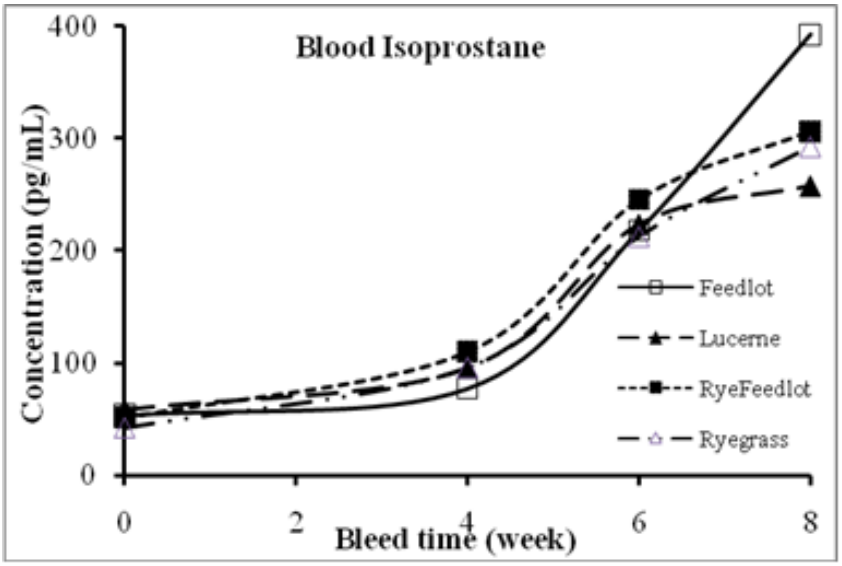

Figure 8. F_2-isoprostane (8-isoprostaglandin $F_{2\alpha}$) concentration in blood from lambs fed lucerne, ryegrass, ryegrass with pellets (Ryefeedlot) and a feedlot ration. Blood samples were collected at commencement (before adaptation to diets (Time = 0), after 4, 6 and 8 weeks of feeding the experimental diets. The assay was done using enzyme immunoassay (EIA) kit (Cayman Chemicals, USA).

There were no relationships observed between isoprostane and lipid oxidation for samples collected at commencement (Time = 0), 4 and 6 weeks of feeding. However, there was a linear relationship between lipid oxidation of meat in the simulated retail display was linearly related ($P < 0.008$) with isoprostane concentration in blood collected after 8 weeks of feeding (i.e., blood samples collected in the paddock 2 days prior to slaughter) (Figure 9). There was a negative linear relationship ($P < 0.001$) found between lipid oxidation at 96 h display and muscle vitamin E concentration (Figure 10).

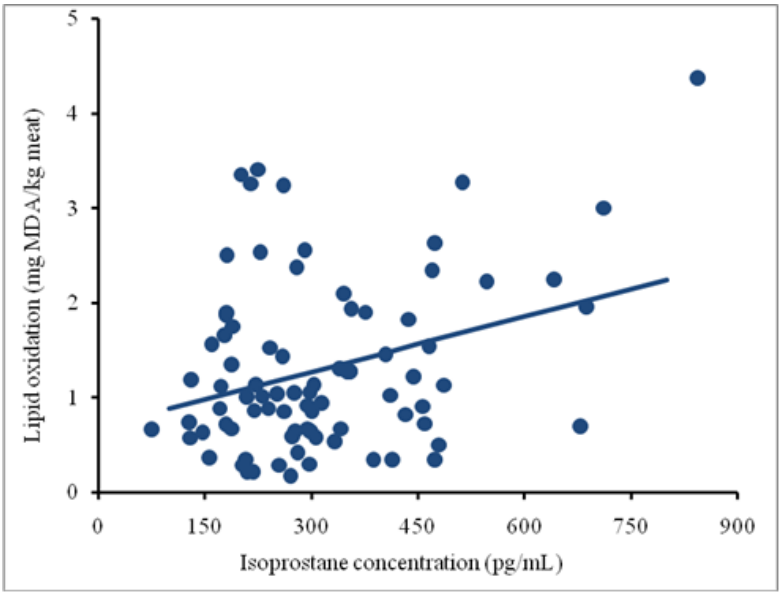

Figure 9. Relationship between F_2-isoprostane (8-isoprostaglandin $F_{2\alpha}$) concentration in blood from lambs fed lucerne, ryegrass, ryegrass with pellet (Ryefeedlot) and a feedlot ration. Data from blood samples (n = 84) collected after 8 weeks of feeding the experimental diets.

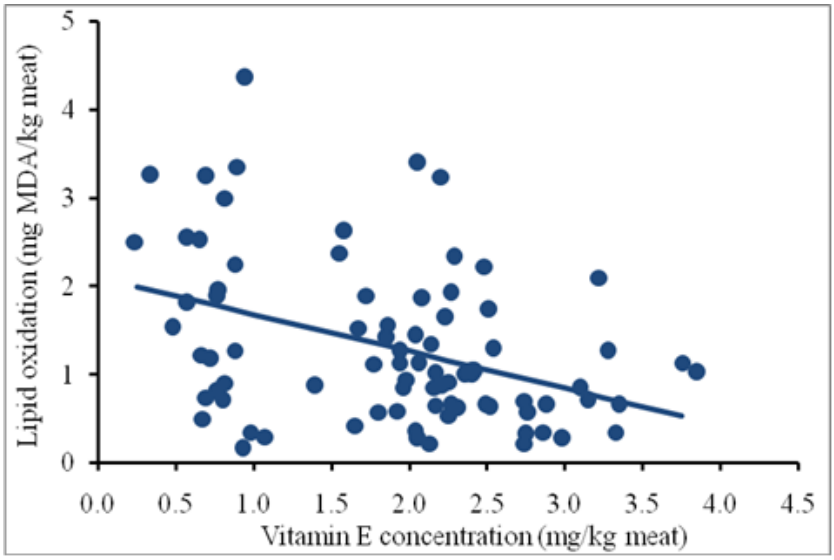

Figure 10. Relationship between F_2-isoprostane (8-isoprostaglandin $F_{2\alpha}$) at bleed 4 and muscle vitamin E concentration and lipid oxidation in meat from lambs fed lucerne, ryegrass, ryegrass with pellet (RyeFeedlot) and a feedlot ration. Data from muscle samples (n = 84) collected at 96 h retail display.

The strong influence of diet on Vitamin E was also confirmed by this study as diet explained 85% of variation in the vitamin E when fitted as a linear regression model. There was a linear negative relationship ($P < 0.07$) found between isoprostane concentration at bleed 4 (blood sample collected 2 days prior to slaughter) and muscle vitamin E concentration (Figure 11). However, there were no relationship between muscle vitamin E and isoprostane concentration at week 0, 4 or 6. In the parsimonious model for lipid oxidation, achieved by fitting a saturated REML model with multiple variables such as isoprostane concentration, vitamin E, lipid oxidation and diet first and sequentially dropping individual terms based on Wald's F-tests, the effect of vitamin E on lipid oxidation disappeared (Table 4).

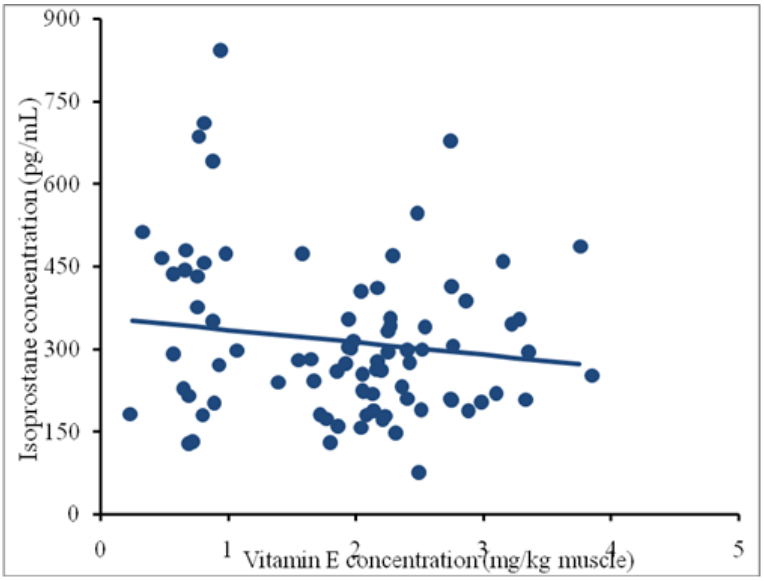

Figure 11. Relationship between muscle vitamin E concentration and F_2-isoprostanes (8-isoprostaglandin $F_{2\alpha}$) concentration in blood at bleed 4 from lambs fed lucerne, ryegrass, ryegrass with pellet (RyeFeedlot) and a feedlot ration. Data from blood samples (n = 84) collected after 8 weeks of feeding the experimental diets. There was a negative linear relationship between isoprostane concentration and muscle vitamin E concentration ($P < 0.07$).

Table 4: *P* - values for the terms that were included and excluded from the parsimonious model for lipid oxidation at retail display

Terms	F statistic	n.d.f	d.d.f	*P* - value
Terms included				
Treatment	10.41	3	10.7	**0.002**
Isoprostane 8 week (2 days before kill)	7.56	1	64.1	**0.008**
Terms excluded				
Isoprostane 0 week (day 0)	0.00	1	71.6	0.94
Isoprostane 4 week	1.37	1	71.0	0.25
Isoprostane 6 week	1.15	1	65.4	0.29
Vitamin E	0.96	1	76.6	0.33

3.10 Relationship between blood isoprostane concentration, muscle vitamin E concentration and retail colour

The meat colour assessed as redness of meat at days 1 and 4 was not significantly related to blood isoprostane concentration at weeks 0, 4, 6 and 8 (2 days prior to slaughter) or the vitamin E concentration of muscle (Table 5). Figures 12 and 13 show the relationship between blood isoprostane concentration (2 days prior to slaughter) and retail colour as assessed by redness of meat at day of display (day 1) or at 96 h retail display, respectively at simulated retail condition. Figures 14 and 15 show the relationship between muscle vitamin E concentration and retail colour as assessed by redness of meat at day of display (day 1) or at 96 h retail display, respectively at simulated retail condition.

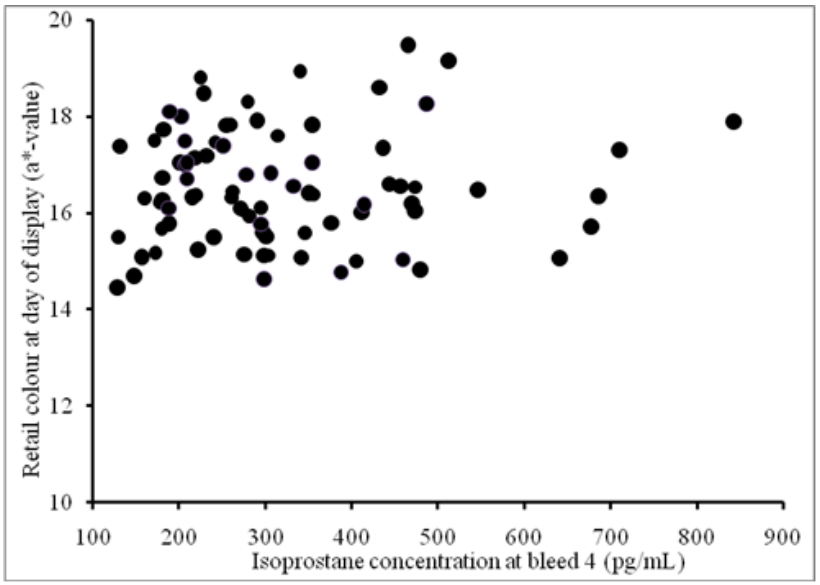

Figure 12: Relationship between blood isoprostane concentration (2 days prior to slaughter, n = 84) and retail colour as assessed by redness of meat at day of display (day 1) at simulated retail condition.

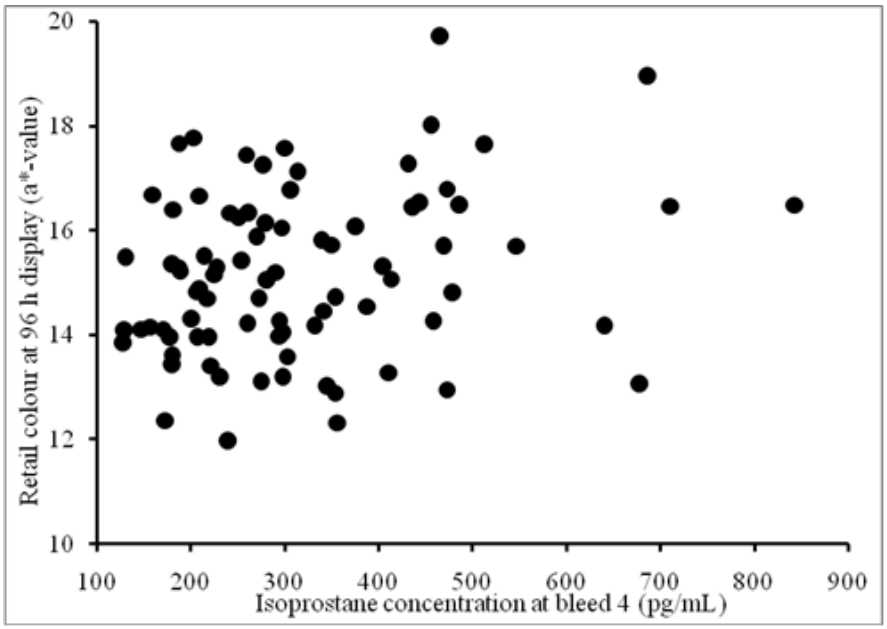

Figure 13: Relationship between blood isoprostane concentration (2 days prior to slaughter, n = 84) and retail colour as assessed by redness of meat at 96 h retail display at simulated retail condition.

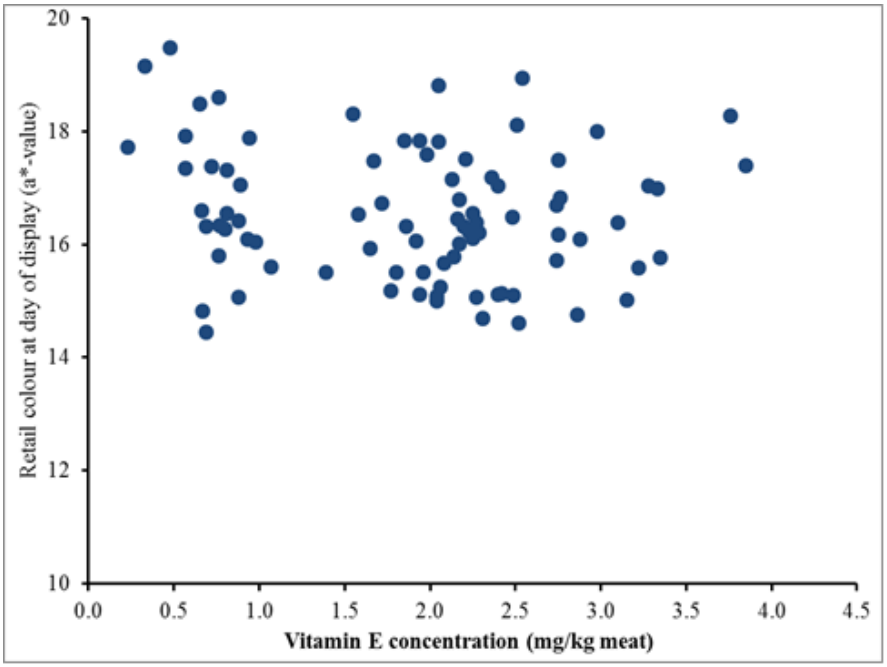

Figure 14: Relationship between muscle vitamin E concentration and retail colour as assessed by redness of meat at day of display (day 1) or at 96 h retail display at simulated retail condition.

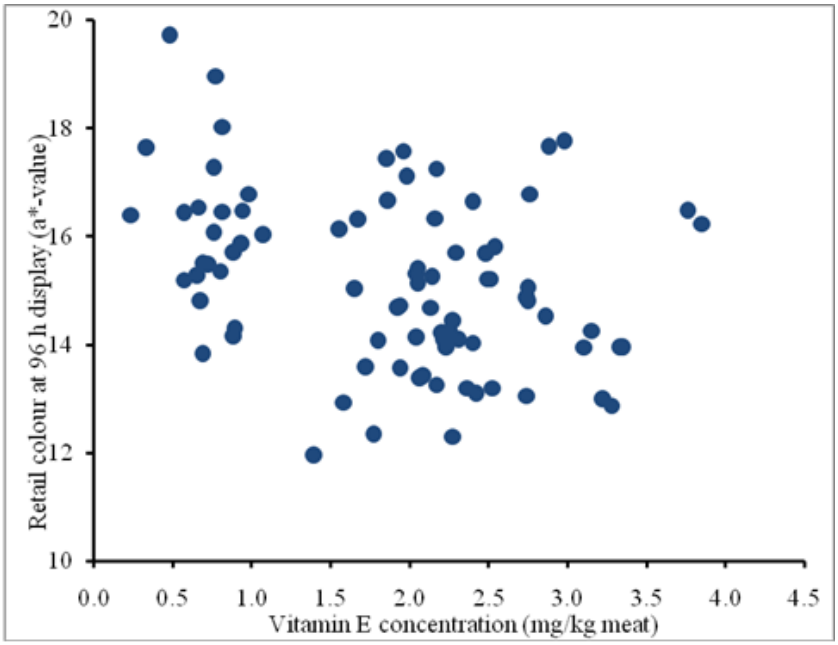

Figure 15: Relationship between muscle vitamin E concentration and retail colour as assessed by redness of meat at 96 h retail display at simulated retail condition.

Table 5: *P*-values for the terms that did not have statistically detectable effect when fitted individually (one at a time) for their relationship with redness of meat at day 1 (day of display)

Terms	F statistic	n.d.f	d.d.f	*P*-value
Isoprostane 0 week	0.34	1	79.6	0.56
Isoprostane 4 weeks	0.11	1	77.4	0.74
Isoprostane 6 weeks	0.01	1	79.7	0.93
Isoprostane 8 weeks	0.71	1	73.4	0.40
Treatment	2.22	3	10.0	0.15
Vitamin E	1.91	1	17.3	0.18

4 Discussion

4.1 Flavour and colour of meat

Lipid oxidation and retail colour deterioration of meat throughout the supply chain and retail display reduces the profitability of the red meat industry. Increased lipid oxidation in meat can affect flavour, taste and nutritional value of meat. Previous studies conducted by DEPI (Ponnampalam et al., 2014) showed that lipid oxidation was significantly impaired when vitamin E levels were maintained above 2.95 mg/kg muscle in lamb LL stored at 2^{o}C for 4 weeks before retail display. This current study determined muscle vitamin E content and lipid oxidation of lambs finished on 4 commonly used lamb finishing diets. We found that mean muscle vitamin E concentration for each finishing diet was less than 2.95 mg/kg resulting in higher levels of lipid oxidation.

Grass-feeding not only improves the level n-3 PUFAs, but also provides higher vitamin E and β-carotene levels that protect these PUFAs in the meat post-mortem (Descalzo and

Sancho 2008; Garcia et al.,2008; Ponnampalam et al., 2006; Ponnampalam 2012b; Warren et al., 2008a,b,). The influence of a pasture diet compared with a concentrate diet on the balance of oxidation and anti-oxidative capacities was evaluated in lamb LL muscle (Santé-Lhoutellier et al.,2008). In the latter study, samples from pasture-fed lambs had higher vitamin E content than their concentrate fed lamb counterparts with 6.42 vs 1.61 mg/kg meat in pasture and concentrate treatments, respectively. Meat from pasture-fed lambs has higher PUFA, n-3 PUFA, and CLA contents compared to concentrate-fed lambs. Despite the high level of PUFA, lipid oxidation during post-mortem storage was inhibited and was significantly lower in pasture-fed lambs compared with concentrate-fed group due to the protective effects of vitamin E (Santé-Lhoutellier et al., 2008). The current study also showed that lambs fed a feedlot diet had a significantly lower muscle vitamin E concentration, which lead to greater lipid oxidation while lambs fed a ryegrass diet had higher muscle vitamin E concentration that lead to significantly lower lipid oxidation for both fresh and aged meat displayed for 96 hours under simulated retail display.

At the same time Najafi et al. (2012) have reported that goats fed with a 3% fish oil supplement in the diet compared to palm oil or soybean oil had significantly higher levels of long chain omega-3 fatty acid in muscles. However, in this study, the meat colour, assessed by L*, a*, and b* values or sensory properties evaluated by flavour intensity, was not affected, although the vitamin E concentration in meat was not reported. Karami et al. (2013) have recently shown that the inclusion of canola oil into the diet of goats increased blood, muscle, and liver omega-3 fatty acid contents, but lipid oxidation assessed by the levels of TBARS was significantly lowered in blood and muscle. This suggests that lipid oxidation of meat is affected by muscle vitamin E concentration or long chain polyunsaturated fatty acid concentration such as omega-3 fatty acid or is it due to the omega-6 fatty acid concentration in muscle that influences muscle membrane integrity via inflammation in the muscles of living animals and therefore enhances lipid oxidation in meat.

The colour of meat at retail display can change the consumer purchasing decision. Coles Pty Ltd has previously estimated that they lose about $2 million per year due to discounting and removal of meat from the shelves due to discolouration. Vitamin E is considered as a safe supplement. The incorporation of vitamin E into animal diets is an effective method to increase the level in the muscles and subsequently in meat (Faustman et al., 1989). A proper duration of vitamin E supplementation via diets and the concentration used are very important for maximizing the colour stability of fresh meat (Mitsumoto et al.,1993). Previous studies conducted overseas and in Australia showed that deterioration of meat colour at retail display can be minimised by elevating the muscle vitamin E concentration above 3.2 mg/ kg meat. All lambs in the current study had a vitamin E concentration less than 2.91 mg/kg meat and there were minimal treatment differences observed in meat colour assessed as the redness of meat when compared between 5 days (fresh meat) and 60 days (aged meat) storage over 96 hours of simulated retail display.

Dietary supplementation with *α*-tocopheryl acetate is an effective method to improve the stability of the meat post-mortem without exogenous intervention (Faustman et al.,1998). Significant improvements in beef colour have also been achieved through dietary supplementation with vitamin E (Faustman et al.,1998; Lanari et al.,1993, O'Grady et al.,2001; Mitsumoto et al.,1993). For example, steaks from cattle supplemented with dietary vitamin E exhibited better appearances than controls and were preferred over controls during display by 91% of surveyed Japanese consumers (*n* = 10,941) (Sanders et al.,1997).

It was found that vitamin E influenced the length of the metmyoglobin induction period (Sanders et al.,1997) and diminished the adverse effect of temperature abuse on colour stability (Chan et al.,1995). This effect was dependent on dose (the higher the vitamin E supplement the longer the induction period) and muscle type (LL had a longer induction period than the colour-labile GM).

It should be noted that the current experiment was conducted in weaner lambs (~10 weeks old) fed for 6 weeks with two additional weeks of adaptation to the diets. The lambs were slaughtered at 4.5 months and reached a market live weight (45-50 kg) that provided carcass weights suitable for the local market (18-22 kg). However, muscle vitamin E concentration (average for treatments) was not achieved above 3.0 mg/kg meat and lipid oxidation in meat both stored for 5 days (fresh) and 60 days (aged) was affected under simulated retail conditions. Perhaps a longer duration of feeding i.e., 10 or 12 weeks might have lifted the muscle vitamin E concentration above 3 mg/kg meat for ryegrass and lucerne treatments. The results demonstrate that finishing diets can influence the muscle vitamin E concentration which in turn can affect lipid oxidation in meat during retail display. Further investigation is required to identify the effect of different finishing diets used to finish lambs over the summer - autumn period. These might be formulated rations or summer active pastures that can elevate muscle vitamin E and other nutritional characteristics in meat which then avoid lipid oxidation and colour deterioration at the retail level. Most importantly the levels of lipid oxidation achieved in this study provided the variation needed to test the ability of isoprostanes for use as biomarkers for oxidative stress. This enabled us to test the experimental hypothesis.

4.2 Blood isoprostane, muscle vitamin E concentration and lipid oxidation in meat

In Australia, economic losses due to meat discolouration and undesirable flavour is unknown. Nonetheless, these two factors in addition to tenderness have universally been associated with decision making and the purchase of meat. The stability of colour and the flavour of lamb meat are dictated by the rate of oxidative processes post-slaughter, which are regulated by the antioxidant capacity and the reducing systems of muscle tissues at slaughter. The latter can be influenced by nutritional background, genetics, chiller management and packaging of which diet has the major effect. Several studies have indicated lipid oxidation of meat at retail display or during storage was influenced by PUFA and this affected flavour due to the formation of rancidity or secondary compounds in meat (Descalzo & Sancho, 2008). Others have reported lipid soluble antioxidants incorporated (e.g., vitamin E) in muscle membranes are the major factor that slows down or avoids oxidation by quenching free radicals produced by post-mortem processing and storage of meat (Ponnampalam et al., 2014).

The results show that finishing diets affected the oxidative stress of lambs as assessed by isoprostane concentration in blood and lipid oxidation of meat during retail display. These latter effects could be due to levels of PUFA and/or vitamin E concentration in the muscles. The interesting and main outcome of the present study was the relationship between oxidative stress and lipid oxidation in meat after retail display. This implies that flavour/aroma deterioration due to the lipid oxidation process in meat post-farm gate can be detected by measuring the isoprostane concentration in live animals on-farm. This provides

valuable information for devising on-farm dietary interventions that would avoid the production of meat with inferior flavour and aroma due to lipid oxidation at the retail level.

There was a negative linear relationship observed between blood isoprostane concentration prior to slaughter and muscle vitamin E concentration and between lipid oxidation in meat and muscle vitamin E concentration. In the parsimonious model for lipid oxidation, achieved by fitting a saturated REML model with multiple variables such as isoprostane concentration, vitamin E, lipid oxidation and diet first and then sequentially dropping individual terms based on Wald's F-tests, the effect of vitamin E on lipid oxidation disappeared. This means blood isoprostane concentration prior to slaughter (bleed 4) is strongly related and directly influencing lipid oxidation. However, if blood isoprostane was not measured and not included in the model, vitamin E would show a significant linear effect on lipid oxidation. It indicates that isoprostane concentration explains more about lipid oxidation in meat than the effect of muscle vitamin E concentration on lipid oxidation in the current study. There was also some evidence in the data to suggest that the oxidative stress in live animals (predicted by blood isoprostane concentration) was low when vitamin E concentration in muscle is higher. This is the first investigation reported in farm animals that links oxidative stress (i.e. isoprostane concentration) pre-farm gate with reduced meat quality post-farm gate. However, further investigation is required to validate these results with larger cohorts of animals covering different species, genetics, production seasons and feeding duration.

4.3 Blood isoprostane, muscle vitamin E concentration and redness of meat

Faustman et al. (1989) and Arnold et al. 1993) reported that to gain the maximum protection against discolouration, 3 - 3.5 mg vitamin E/kg muscle was needed depending on the muscle type. In the current study, all dietary treatments delivered meat with muscle vitamin E concentration (treatment average) below 2.9 and therefore differences in redness or discolouration between treatments would not be expected. In supporting this, another study has shown (Ponnampalam et al.,2012a) a significant difference in retention of redness at 3 and 4 days of display for fresh meat between lambs that had grazed annual pasture having a vitamin E concentration of 3.42 mg/kg muscle compared with similar lambs that had been supplemented with a hay and grain diet at 1.69 mg/kg muscle.

The current study show that there was no relationship between meat colour and blood isoprostane concentration or meat colour and vitamin E concentration of muscle. This means that the redness of the meat was not influenced either by blood isoprostane concentration or vitamin E concentration of muscle. Further study is needed with large number of animals fed for longer durations to test the relationship between blood isoprostane concentration, muscle vitamin E concentration and redness of meat when muscle vitamin E concentration has a wider range i.e., below 2 to above 4 mg/kg muscle.

5 Conclusion

- Lambs fed a feedlot diet had significantly lower muscle vitamin E concentrations that promoted greater levels of lipid oxidation in fresh and aged meat displayed for 96 hours under simulated retail display conditions. Whilst lambs fed a ryegrass diet had a higher muscle vitamin E concentration that lead to significantly lower lipid oxidation when stored under the same conditions.

- Finishing diets affected the oxidative stress of lambs as assessed by isoprostane concentration in blood and lipid oxidation of meat under simulated retail display conditions.
- Lipid oxidation of fresh and aged meat stored under simulated retail display was linearly related to isoprostane concentration in blood collected 2 days prior to slaughter.
- Muscle vitamin E content showed a significant negative linear relationship with lipid oxidation. However, if blood isoprostane concentration was included in the model, we do not observe this negative relationship. These results indicate that blood isoprostane concentration explain more about lipid oxidation in meat than the effect of muscle vitamin E concentration on lipid oxidation.
- It is likely that oxidative stress in live animals, as predicted by blood isoprostane concentration is inversely related to muscle vitamin E content. For example, animals with low blood isoprostane concentration had a high muscle vitamin E concentration.
- The study suggests that flavour/aroma deterioration due to the lipid oxidation process in meat post-farm gate can be detected by measuring the isoprostane concentration in live animals on-farm.
- Blood isoprostane as a pre-slaughter biomarker can provide valuable information for devising on-farm dietary interventions that would avoid the production of meat with inferior flavour and aroma due to reduce lipid oxidation under retail storage conditions.
- There were no treatment differences in redness or discolouration of meat. The results suggest that the redness of the meat stored at retail conditions was not influenced either by blood isoprostane concentration or muscle vitamin E concentration.
- The Cayman EIA kit has better practical use compared with LC-MS or ELISA kit supplied by Oxford Biomedical Research. This kit can be a better option for commercial use.

6 Recommendations

This is the first known report to identify a link between a biomarker of oxidative stress (i.e. isoprostane concentration) and reduced meat quality in sheepmeat. However, these results require further validation as the current study was conducted on weaner lambs fed a variety of finishing diets for a period of 8 weeks. The lambs were slaughtered when they reached a target live weight (45-50 kg) which meet a target specification of 21 kg carcass weight. Also, the average muscle vitamin E concentration did not exceed 3.0 mg/kg meat for each of the diet groups. Thus, a further study is required to examine the influence of feeding duration (i.e. 10 or 12 weeks) and muscle vitamin E concentrations greater than 3 mg/kg. These findings also needs to be tested in other species that produce meat from various genetic backgrounds, produced over a variety of different seasons and of different ages. Future work may also investigate whether alternative methodology to measure isoprostane content in blood can be developed that is faster and more applicable to the sheepmeat industry.

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8 Appendices

Publications – Papers arising from this project

1. Bekhit, A.E.A., Hopkins, D.L., Fahri, F.T., & Ponnampalam, E.N. (2013). Oxidative processes in muscle systems and fresh meat: sources, markers, and remedies. Comprehensive Reviews in Food Science and Food Safety 12:565-597.

2. Ponnampalam, E.N., Bekhit, A.A., Fahri, T. F., Linden, N, and Hopkins, D.L. (2013). Muscle antioxidant potential and oxidative processes in lambs fed traditional diets. Proceedings of the Nutrition Society of Australia, 37; pp 25.

3. E. N. Ponnampalam, A. E. A. Bekhit, G. Khageswor, P. Lewandowski, F. T. Fahri, & D. L. Hopkins (2014). Isoprostanes: Potential biomarkers of oxidative stress in muscle. 60th International Congress of Meat Science and Technology, 17-22nd August 2014, Punta Del Este, Uruguay (will be submitted in May 2014).