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The influence of pre-slaughter stress on the meat quality and carcase yield of prime lambs

Abstract

Understanding the impact of acute and chronic stress incurred during the pre-slaughter period is important to maximise lamb meat quality and yield as well as maintaining high animal welfare. This study utilised 2877 lambs of the Meat and Livestock Australia Genetic resource flock. Plasma indicators of acute and chronic stress were quantified at slaughter and analysed for their association with meat quality and carcass yield traits. Production factors had the largest impact on indicators reflecting acute stress, feed deprivation, dehydration, muscle activity and damage, the majority of which were elevated above normal range at slaughter. Phenotypically leaner lambs also demonstrated greater fat metabolism and lower rates of glycogen turnover at slaughter. Loin ultimate pH demonstrated a positive association with slaughter levels of plasma glucose and lactate, indicating that acute pre-slaughter stress has an impact on glycogen turnover and ultimate pH in lamb. Kill order was associated with an increase in loin Warner Bratzler shear force, which indicates that a greater exposure to stress decreases tenderness. Feed deprivation up to 48 hours under resting and commercial conditions resulted in up to 35% higher adipose tissue response in Merino sired lambs compared to Terminal sired lambs, a response that was also influenced by selection for increased genetic growth and leanness. Feed deprivation beyond 36hrs had a negative impact on ultimate pH of the M. Semitendinosus in Merino lambs and caused a 3% reduction in carcass weight. Further work is required to understand methods of mitigating acute pre-slaughter stress in order to minimise high ultimate pH and reduced tenderness. In addition, it has been demonstrated that Merino genotypes have a significantly higher fat turnover in response to feed deprivation and stress, and indicates that shorter duration of curfew and lairage may be beneficial in minimise carcass losses and meat quality.

Executive Summary

Understanding the impact of acute and chronic stress incurred during the pre-slaughter period is important to maximise lamb meat quality and yield as well as maintaining high levels of animal welfare. This project utilised 2877 lambs of the Meat and Livestock Australia genetic resource flock from sites (Katanning, Western Australia and Armidale, New South Wales) over a two year period and quantified the level of acute and chronic stress at slaughter utilising plasma indicators reflecting acute stress, feed deprivation, dehydration, muscle activity and damage and then analysed their association with meat quality and carcass yield traits. Finally, the impact of genetic selection for increased carcass yield on the metabolic response to feed deprivation was examined under resting and commercial slaughter conditions.

Production factors including site, year and kill group had the largest impact on all stress indicators at slaughter, the majority of which were elevated above normal range at slaughter. There was also considerable variation in the concentration of many indicators (particularly those reflecting fat turnover) which are likely to reflect differences in pre-slaughter management, on-farm nutrition, feed and water deprivation and various acute stressors. In addition, levels of metabolic indicators at slaughter were found to relate to lamb carcass phenotype, with leaner lambs demonstrating greater fat mobilisation and lower rates of glycogen turnover at slaughter.

Further work is required to understand methods of mitigating acute pre-slaughter stress in order to minimise high ultimate pH and reduced tenderness. In addition, it was demonstrated that Merino genotypes have a significantly higher fat turnover in response to feed deprivation and stress. This indicates that a shorter duration of curfew and lairage may minimise carcass losses and improve meat quality.

Association between plasma metabolite levels and meat quality and carcase weight

An important finding of the project was the positive association between plasma glucose and lactate and ultimate pH in lamb. This represents the first large scale study in lamb which has shown that plasma indicators of acute stress at slaughter relate to ultimate pH. This result highlights that acute stress prior to slaughter is a significant driver of glycogen turnover which is having a negative influence on ultimate pH in lamb.

It is well established that to maximise the concentration of muscle glycogen at slaughter, thereby reducing the risk of dark cutting, pre-slaughter stress and associated adrenaline release must be minimised (Tarrant, 1981). A number of studies have shown that stress events such as handling (Sutherland et al., 2016), exercise (Gardner et al., 2001a; Warner et al., 2005), transport (Jacob et al., 2005a), and time in lairage (Toohey and Hopkins, 2006) may cause stress and increased glycogen turnover. However, previous work has shown that the immediate short term effects of stress before slaughter are unlikely to reduce muscle glycogen below the threshold for dark cutting if muscle glycogen levels are adequate (Immonen and Puolanne, 2000). Therefore, it is plausible in the current study that muscle glycogen levels were borderline, and combined with an acute stress response at slaughter, continuing to mobilise glycogen has resulted in an association being observed between ultimate pH and stress indicators. If glycogen concentrations had been adequate, it is possible that this experiment would not have exposed this association. A number of studies have shown that stress immediately before slaughter does not impact on ultimate pH (Warner et al., 2007).

An individual's stress susceptibility may also explain the association found between plasma lactate and glucose and ultimate pH. Lambs which are more susceptible to stress (e.g. poorer temperaments) may have greater rates of glycogenolysis in response to routine management practices such as mustering, handling and novel environments and therefore have lower resting muscle glycogen levels and be at risk of dark cutting (Coombes et al., 2014). The influence of temperament on the incidence of high ultimate pH has been well documented in beef (Tarrant, 1989; Coombes et al., 2014) but further work is required in lamb. A possibility in this study is that lambs with high glucose and lactate at slaughter due to stress, also had a greater adrenergic response throughout the pre-slaughter pathway due to handling, exposure to novel environments and loading/unloading. Thus, these peaks in adrenergic responses would have had the cumulative effect of gradually reducing muscle glycogen over the pre slaughter period, potentially leading to glycogen levels below the threshold required to reach a low ultimate pH. Future experiments may be required to determine whether the plasma lactate and glucose response throughout the pre-slaughter period is linked to the change in muscle glycogen from farm to slaughter and its impact on ultimate pH.

However, despite there being a significant association between plasma lactate and glucose at slaughter with ultimate pH, the effect of plasma indicators was very small in comparison to the production factors and only explained 10% of the variation in loin ultimate pH. Factors such as site, year and kill group had the largest impacts on ultimate pH, with these factors likely to reflect environmental and other circumstantial stressors. A key difference between sites, years and kill groups would have been nutrition, which has previously been shown to play a crucial role in determining muscle glycogen concentration (Pethick and Rowe, 1996). These differences could also have been explained by management practices within extensive grazing enterprises, with factors such as mustering, frequency of handling, location of watering points and environmental factors such as weather likely to have affected muscle glycogen levels (Gardner et al., 2014). Therefore, the ability to predict ultimate pH will be improved by better understanding the role of glycogen turnover throughout the pre-slaughter period.

The association between plasma indicators of acute and chronic stress and lamb loin tenderness was also investigated. This was an important study as tenderness is a key driver of consumer eating quality of lamb (Pannier et al., 2014), yet even the higher quality cuts such as the loin (M. longissimus lumborum) are known to vary markedly in tenderness. This was well demonstrated in a study by Pannier et al. (2014) who showed that approximately 40% of lamb loins rated as good every day (3 star) or lower by consumers under the Meat Standards Australia grading system with a significant proportion (7%) rated as unsatisfactory.

Contrary to the hypothesis, there was no association between WBSF and plasma indicators of stress, contradicting earlier studies in beef (Warner et al., 2007; Gruber et al., 2010; Pighin et al., 2015) which showed a link between acute stress and tenderness. This was unexpected, as a clear association was demonstrated between glucose and lactate and loin ultimate pH. In addition concentrations for plasma stress indicators were mostly above basal levels with a large range in concentration (Radostits et al., 2007) indicating that there was a degree of stress and variability in responsiveness in the lambs during the pre-slaughter period (Pighin et al., 2014).

Whilst this may indicate that there is no association between stress and tenderness, more likely it suggests that plasma indicators themselves are poor indicators of stress. The homeostatic regulation of glucose, fat and muscle metabolism, hydration status represents a normal physiological response to pre-slaughter stressors. Multiple factors such as physical activity, variable nutrition, and previous handling experiences would influence the slaughter responses (Ferguson and Warner, 2008), reducing the accuracy of plasma indicators as indicators of immediate pre-slaughter stress. Furthermore, the challenge with measuring indicators at slaughter and then relating them to meat quality traits is that the stress event, blood collection and physiological impact on shear force does not happen

simultaneously. This would have also uncoupled the link between acute stress, plasma indicators and shear force. Future work should focus on applying stress treatments under more controlled experimental conditions and examining whether acute stress affects eating quality as assessed by untrained consumers, rather than only objective measurements of tenderness.

One unexpected finding was the positive association between WBSF and kill-order, which may reflect the duration of exposure to immediate pre-slaughter processes during the lead up to slaughter. Previous behavioural studies have shown that cattle observed to be more nervous or more stressed tend to move through the race at the end of the group (Grandin, 1980; Orihuela and Solano, 1994; Stockman et al., 2012). This may apply in this experiment, where lambs that are killed later within a kill group were more stressed and/or were exposed to that stress for longer (due to the later kill-time), underpinning the link between kill-order, acute stress and the resulting impact on tenderness. However, a better understanding of the factors driving the kill-order effect are required. From a practical industry perspective animals must be killed one at a time. However, future work could be directed at understanding how different handling methodologies cause stress (Hargreaves and Hutson, 1997; Deiss et al., 2009; Sutherland et al., 2016) and influence both the kill-order effect on plasma metabolites and tenderness.

Production factors also had the greatest impact on loin WBSF in lamb. However there was still considerable variation not accounted for, with the base model only explaining 50% of the variation in WBSF. Considerable variation existed between years and kill groups, which varied by up to 20 N after correcting for other environmental factors such as site and genotype. This may be related to differences in growth rate or nutrition that have been shown to influence tenderness (Hopkins et al., 2005) as well as processing effects (Johnston et al., 2001). However, it may also reflect differences in stress experienced in these cohorts, which was not captured by plasma stress indicators. The results from this project clearly demonstrates that the use of plasma indicators of stress as a predictive tool to identify high pH carcasses or high WSBF carcases on a commercial basis is not practical or realistic and should be avoided in future research.

Importantly, the average WBSF values found in this study were higher than 27 N, which is associated with a 10% failure rate for lamb eating quality (Hopkins et al., 2006a). In addition, there was a clear positive association found between WBSF and ultimate pH. This highlights that significant improvement in WBSF can be achieved by implementing strategies to reduce the incidence of high ultimate pH in lamb.

The findings from this project demonstrate that further work is required on a large industry scale to identify factors from farm to slaughter that cause acute stress within the pre-slaughter period, and quantify their impact on muscle glycogen levels on farm and at slaughter and how this affects ultimate pH in Australian lamb. A limitation of the current study was that muscle glycogen levels were not collected pre- and post-slaughter, which would have provided a greater understanding of the impact of pre-slaughter management and stress on loin ultimate pH in this study. Future work investigating the role of nutrition, genotype and pre-slaughter management requires a more tightly controlled experimental design.

Relationship between dehydration and blood metabolites

Water restriction may cause hypertonic dehydration indicated by progressive hypernatremia and hyperproteinemia. Analysis of blood indicators shows that approximately 17% of lambs have plasma sodium concentrations indicative of dehydration. It is less clear in regards to total protein which appears to also be affected by animal factors such as breed, sex and birth type, reflecting its role in protein turnover. The results of the current study tend to indicate that the lambs in this study were

largely not dehydrated at slaughter, which probably reflects management under best practice pre-slaughter pathways in which water should be made available in lairage.

However, under commercial practice, work has indicated that large percentages (~50%) of lambs are sub-clinically dehydrated in the pre-slaughter period. The effectiveness of supplying water to rehydrate lambs prior to slaughter is not well known and not all lambs will drink water in unfamiliar environments. Therefore, future work should be directed at examining physiological and carcass yield benefits to providing water during on-farm curfew compared to in lairage.

Breed type and sire effects on meat quality and carcase yield

In addition to production factors, lamb genotype was also found to have an effect on loin ultimate pH. In line with previous studies, Merino genotypes had a higher ultimate pH in this experiment than Terminal and Maternal genotypes. Merino genotypes have been shown to have a higher stress susceptibility and greater risk for high ultimate pH (Gardner et al., 1999a). Genetic selection for muscling has been shown to reduce the propensity to mobilise muscle glycogen during acute stress and thus may have a protective effect against dark cutting. In this experiment it was shown that Terminal sired lambs, which are bred for increased lean meat yield (Anderson et al., 2015) had a lower ultimate pH than Merino sired lambs. Work by McGilchrist et al. (2011) and Martin et al. (2011) showed that high muscling genotypes have a reduced muscle response to adrenaline and therefore are less likely to mobilise muscle glycogen in response to acute stress. Further demonstrating this, McGilchrist et al. (2012) showed that cattle with larger eye-muscle areas had a reduced incidence of dark cutting.

The only impact of sire type on loin WBSF was observed in male lambs out of Merino dams, where Terminal sired lambs (34.6 ± 0.61N) had WBSF 2.5 N and 1.9 N higher than Maternal (32.1 ± 0.87N) and Merino (32.7 ± 0.64N) sired lambs respectively. However, when sire Australian Sheep breeding values (ASBVs) for Post-weaning weight (PWT), Post-weaning eye muscle depth (PEMD) and Post-weaning fat depth (PFAT) were included simultaneously as covariates in the base linear mixed effects models, the PEMD and PFAT ASBV demonstrated some association with WBSF (P<0.05). However, when ASBVs was included in core model separately, there was no effect (P>0.05) of PEMD on WBSF. There was no association (P>0.05) between PWT and WBSF. Decreasing PFAT was associated with increased loin WBSF values; however this effect was small in comparison to the production factors previously discussed. These results demonstrated a 5.6 N decrease in WBSF across the PFAT range; however this effect decreased when PFAT was included in the model alone, highlighting that PEMD and PFAT co-explain the negative impact on WBSF. Recent work by Pannier et al. (2014) showed that increased selection for leanness resulted in a reduction in lamb sensory tenderness, an effect which was largely independent of WBSF. These results highlight that genetic selection for increased leanness has a direct and negative impact on objective measures of tenderness in lamb.

The effect of curfew/lairage duration on meat quality and carcass yield

The metabolic response to feed deprivation in lambs genetically selected for high and low carcass yield characteristics under resting and pre-slaughter conditions were examined. Lambs from Merino dams with Merino or Terminal sires with a range in Australian Sheep Estimated Breeding Values (ASBVs) for breeding values for post-weaning weight (PWT), post-weaning fat depth (PFAT) and post-weaning eye-muscle area (PEMD) were used in this experiment.

Resting feed deprivation resulted in lower glucose concentrations and a greater NEFA and BHOB response in Merino sired lambs compared to Terminal sired lambs. In addition, selection for increased growth through the use of PWWT and leanness through reduced PFAT also altered the NEFA response

to feed deprivation and appeared to partly underpin the siretype difference. The results showed that significant differences in intermediary metabolism between Terminal and Merino sired lambs when undergoing metabolic adaptation to feed deprivation, warranting further investigation.

The aim of this study was to examine the metabolic response to feed deprivation up to 48 hours in low and high yielding lamb genotypes. It was hypothesised that Terminal sired lambs would have decreased plasma glucose and increased plasma non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHOB) concentrations in response to feed deprivation compared to Merino sired lambs. In addition, it was hypothesised that the metabolic changes due to feed deprivation would also be greater in progeny of sires with breeding values for greater growth, muscling, and leanness. Eighty nine lambs (45 ewes, 44 wethers) from Merino dams with Merino or Terminal sires with a range in Australian Sheep Breeding Values (ASBVs) for post-weaning weight (PWT), post-weaning eye muscle depth (PEMD) and post-weaning fat depth (PFAT) were used in this experiment. Blood samples were collected via jugular cannulas every 6 hours from time 0 to 48 hours of feed deprivation for the determination of plasma glucose, NEFA, BHOB and lactate concentration. From 12 hours to 48 hours of feed deprivation plasma glucose concentration decreased ($P<0.05$) by 25% from 4.04 ± 0.032 mmol/L to 3.04 ± 0.032 mmol/L. From 6 hours NEFA concentration increased ($P<0.05$) from 0.15 ± 0.021 mmol/L by almost 10-fold to 1.34 ± 0.021 mmol/L at 48 hours of feed deprivation. Feed deprivation also influenced BHOB concentrations and from 12 to 48 hours it increased ($P<0.05$) from 0.15 ± 0.010 mmol/L to 0.52 ± 0.010 mmol/L. Merino sired lambs had a 8% greater reduction in glucose and 29% and 10% higher NEFA and BHOB response respectively compared to Terminal sired lambs ($P<0.05$). In Merino sired lambs, increasing PWT was also associated with an increase in glucose and decline in NEFA and BHOB concentration ($P<0.05$). In Terminal sired lambs, increasing PFAT was associated with an increase in glucose and decline in NEFA concentration ($P<0.05$). Contrary to the hypothesis, Merino sired lambs showed the greatest metabolic response to fasting especially in regards to fat metabolism.

Under current Australian industry pre-slaughter guidelines, lambs may be off feed for up to 48 hours prior to slaughter. The purpose of this study was to examine what proportion of circulating metabolites at slaughter are due to feed deprivation compared to acute stress and if this response differs between Merino and Terminal genotypes. In addition the effect of feed deprivation on carcass weight and meat quality was examined. Jugular blood samples were collected from 88 Merino and Terminal sired lambs at rest and at slaughter following 24, 36 and 48 hours of feed deprivation and plasma analysed for glucose, lactate, non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHOB). From the same carcasses hot carcass weight (HCWT) were measured as well as a suite of meat quality traits measured such as M. longissimus lumborum (loin) and M. semitendinosus pH at 24 hours post mortem. Loin samples were also analysed for intramuscular fat content and Warner-Bratzler Shear Force. Merino sired lambs had a higher NEFA response compared to Terminal sired lambs at slaughter after 24, 36 and 48 hours of feed deprivation, with NEFA levels up to 35% higher than previously reported in the same animals at rest, whereas BHOB response to feed deprivation was not affected by sire type ($P>0.05$) and similar to previously reported at rest. In addition to the metabolic effects, increasing feed deprivation from 36 hours was associated with a 3% reduction in HCWT and dressing percentage as well as causing increased ultimate pH in the M. semitendinosus in Merino sired lambs. There was no effect of feed deprivation on intramuscular fat or Warner-Bratzler shear force. Findings from this study demonstrate that Merino and Terminal sired lambs differ in their metabolic response to feed deprivation under commercial slaughter conditions. In addition, commercial feed deprivation appears to have a negative effect on ultimate pH and carcass weight and warrants further investigation.

This project reveals some important impacts of acute stress and feed deprivation on plasma physiological responses at slaughter and the influence on lamb ultimate pH, tenderness and carcass weight. This was the one of the first comprehensive studies to quantify stress at slaughter using

plasma indicators of acute stress, feed and water deprivation and muscle damage. In addition, there are several key findings that are highly relevant and important to the Australian lamb industry. The association between plasma glucose and lactate with loin ultimate pH shows that acute stress is influencing glycogen concentrations in the immediate pre-slaughter period. The negative effects of kill order on tenderness further support this and warrant further investigation. Future work should be directed at developing new strategies to manage lambs during this period to reduce acute stress and to mitigate pH issues to maximise meat quality and food safety. Although the Merino breed continues to demonstrate greater responsiveness to feed deprivation and stress, the metabolic response to feed deprivation also appears to be driven by genetic potential for growth and leanness, indicating that recommendations for feed deprivation may need to be updated to accommodate more modern lamb genotypes. In addition, the negative impact of feed deprivation on M. semitendinosus ultimate pH and carcass weight is an important finding and indicates that best practice pre-slaughter management may need to be updated to include no more than 36 hours off feed before slaughter to maximise meat quality, carcass weights and animal wellbeing. However this would require further investigation at an industry level to validate the findings in the current study.

Background

Current best practice for the treatment of lambs pre-slaughter is that water available at all times (except during transport) and no more than 48 hours off feed from farm to stun (Jacob et al. 2005). Work in beef cattle has shown a relationship between elevated levels of acute stress close to stunning (as determined by blood lactate concentration) and tenderness (Gruber et al. 2010) building on previous results found by Warner et al. (2007). There is relatively little work in lamb to underpin the best practise recommendations to maximise meat quality and carcase yield together. A recent very small (48 lambs) European study using light weight indoor fed lambs (13.7kg HCW) found no difference between tail gate slaughter and 12 hours lairage with respect to a variety of meat quality parameters (shipped direct from farm and trucked for 1 hour (Liste et al. 2011)). However the same study did suggest lower levels of acute stress at stunning in the lambs in curfew for 12 hours. While of background interest, this study is not transferrable to Australian conditions due to different production systems, slaughter weights and transport/curfew times. In the context of this project carcase yield is affected largely by time off feed (0.1%/hr of carcase weight loss after 12 hours of fasting; Thompson et al. 1987) and dehydration (dehydrated lambs have a 2.5% reduction in carcase weight; Jacob et al 2006).

Studies to unravel cause and effect in this area are difficult and require large numbers of animals, of different genetic make-up and from different production systems. Utilisation of the Sheep CRC Information Nucleus lambs along with other commercial animals will underpin these requirements and represent the first large scale analysis in this area. Benefits will include a clearer understanding of the roles of acute and chronic stress on meat quality and carcase yield. It will also underpin best practise slaughter pathways from an animal welfare point of view.

Project Objectives

- Measure stress indicators in blood at exsanguination (e.g. lactate, non-esterified fatty acids)

- Relate the stress measures to objective measures of tenderness and meat colour and carcase yield including meat moisture and drip.

- Use MLA's resource flocks (previously known as the Sheep CRC Information Nucleus resource) so as to utilise a wide range of lamb genetics and production systems upon which to base conclusions.

- Make recommendations on best practise slaughter pathways to optimise animals welfare, carcase yield and meat quality.

- Undertake further targeted experimentation (e.g. consumer taste panels or alternative lairage scenarios) to consolidate the larger CRC data base conclusion

Abbreviations

ASBV	Australian Sheep Breeding Value
AST	Aspartate aminotransferase
AUC	Area under curve
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BHOB	B-hydroxybutyrate
BLM	Border-Leicester Merino
cAMP	3', 5'-cyclic adenosine monophosphate
CK	Creatine Kinase
CM	Commercial Maternal Dam
CRC	Cooperative Research Centre
CT	Computer tomography
DEXA	Dual x-ray absorptiometry
HCWT	Hot Carcass Weight
HSL	Hormone sensitive lipase
INF	Information Nucleus Flock
IMF	Intramuscular fat
LLWT	M. *longissimus lumborum* (loin) weight
LLFAT	M. *longissimus lumborum* (loin) fat weight
MSA	Meat Standards Australia
NAD/NADH	Nicotinamide adenine dinucleotide (oxidised and reduced forms)
NEFA	Non-esterified fatty acid
pH24LL	M. *longissimus lumborum* (loin) pH at 24 hours post-mortem
pH24ST	M. *semitendinosus* pH at 24 hours post-mortem
PFK	Phosphofructokinase
PWT	Post-weaning growth as assessed by live weight at approximately 240 days
PFAT	Post-weaning fat depth over the 12th rib, 45mm from the midline
PEMD	Post-weaning eye muscle depth measured at the 12th rib, 45mm from the midline
TCA	tricarboxylic acid cycle
WBSF	Warner-Bratzler Shear force

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Chapter 1 Introduction

The Australian Sheep meat industry for the 2015-16 year was worth approximately $4.83 billion dollars (source: Meat and Livestock Australia), producing 516,366 tonnes carcass weight of lamb and 196,040 tonnes carcass weight of mutton (source: Australian Bureau of Statistics). Australia is one of the largest and most efficient producers of lamb in the world, however in order to remain competitive both on domestic and international level it is critical to maintain a consistent, high yielding carcass while maintaining superior meat quality attributes.

In Australian lamb production systems, current best practice for the treatment of lambs pre-slaughter is that water is available at all times (except during transport) and there is no more than 48 hours off feed from farm to stun (Anonymous, 2015). In addition, handling stress should be minimised to prevent glycogen depletion and poor meat quality. However, there is relatively little evidence in lamb to underpin the best practice recommendations to maximise meat quality and carcass yield together.

Despite best practice recommendations, there is considerable variability in lamb tenderness, with consumers rating 40% of lamb loins as good every day (3 star) or lower under the Meat Standards Australia (MSA) grading system with a significant proportion (7%) rated as unsatisfactory (Pannier et al., 2014). Recent work suggests that immediate pre-slaughter acute stress may be a cause of variation in tenderness (Warner et al., 2007; Gruber et al., 2010; Pighin et al., 2015).

Chronic stress as a result of feed deprivation has been shown previously to affect carcass yields (Thompson et al., 1987; Warriss et al., 1987). However it is not well understood how modern genotypes deal with feed deprivation, with more recent studies indicating that feed deprivation has no impact on meat quality or yield in lambs (Daly et al., 2006). Work by Jacob et al. (2006c) showed that a large percentage of lambs are sub-clinically dehydrated at slaughter, which may have detrimental impacts on carcass yield.

Limited research has been conducted in prime lambs under Australian commercial conditions using physiological and metabolic plasma indicators to quantify acute and chronic pre-slaughter stress. Furthermore, studies assessing the influence of stress on meat quality and yield are difficult and require large numbers of animals, of different genetic make-up and from different production systems. This study will utilise lambs from the Meat and Livestock Australia genetic resource flock and will represent the first large scale analysis in this area of research.

The experiments in this thesis aim to firstly quantify the level of acute and chronic pre-slaughter stress using physiological and metabolic indicators in plasma at slaughter. Secondly, the association between lamb meat quality and pre-slaughter stress was examined by testing the association between plasma indicators and ultimate pH and shear force. The results from these experiments led to the development of hypotheses to test the metabolic response to feed deprivation in high yielding prime lambs under both resting and commercial conditions. These final two experiments facilitated a greater understanding of the relative impact of acute stress and feed deprivation on intermediary metabolism and effects on meat quality and yield.

Benefits afforded by this thesis include a clearer understanding of the level of acute and chronic stress at slaughter and its association with lamb meat quality and carcass yield. It also helps to underpin best practice slaughter pathways for the Australian lamb industry.

Chapter 2 Literature review

Whilst best practice pathways under Australian grading systems promote reduced stress during the pre-slaughter period, it is inevitable that stress may occur and have a negative impact on meat quality and carcass yield (Ferguson and Warner, 2008). Therefore, a greater understanding of the link between stress and meat quality and carcass yield is essential in order to inform and update best practice pre-slaughter pathways for Australian lamb.

2.1 Pre-slaughter acute stress

It is inevitable that lambs may experience periods of acute stress during the transfer from on farm to slaughter (Ferguson and Warner, 2008). Mustering, transport and handling during yarding can cause periods of acute stress as a result fear, physical exertion, fatigue and injury which several studies have implicated as a contributing factor in reduced meat quality and yield in lamb, as reviewed by Ferguson and Warner (2008). The immediate pre-slaughter period can also expose sheep to unfamiliar environments, increased handling and human contact and changes in social structure, all of which may cause acute stress.

Best practice guidelines in pathway grading systems such as Meat Standards Australia (MSA) assist in optimising the eating quality of lamb and sheep through reducing stress in the pre-slaughter period. These guidelines include; adopting quiet handling, especially of un-weaned "sucker" lambs, minimising the use of dogs during loading and mustering and avoiding transport during extreme weather conditions. Lambs should also be on a rising plane of nutrition in the two weeks leading up to slaughter. It is also important to ensure that lambs have access to water during curfew, transport and lairage (Anonymous, 2015).

The two main processes involved in the acute stress response include the autonomic nervous system and hypothalamic–pituitary–adrenal (HPA) axis. Adrenaline is the most abundant catecholamine hormone of the sympatho-adrenal medullary axes that is secreted by the adrenal medulla in response to acute stressors and is colloquially known as the "flight or fight" response that can be stimulated at various times during the pre-slaughter period (Ferguson and Warner, 2008). Physiological changes include tachycardia, increased respiration rate, elevated body temperature and redistribution of visceral blood volume towards skeletal muscle and the brain. Behavioural changes may also be evident including heightened alertness, immobilisation, and aggression and escape/avoidance behaviours.

Cortisol is a glucocorticoid that is secreted from the adrenal cortex via activation of the hypothalamic-pituitary-adrenal axis in response to physiological or psychological stress. A large number of non-specific stimuli can initiate cortisol secretion including trauma, temperature fluctuations, stimulation of the sympatho-adrenal system and restraint. Lambs within the pre-slaughter period may experience physical or psychological stress and as such, cortisol has been used extensively in livestock production research as a marker for stress and reduced animal welfare (Parrott et al., 1996).

Activation of the autonomic nervous system and HPA and the secretion of both adrenaline and cortisol results in significant changes in energy metabolism including lipolysis, glycogenolysis in muscle and gluconeogenesis (Kuchel, 1991). These changes in metabolism provide an immediate supply of glucose and lipid fuels for immediate and increased energy requirements during stress.

2.1.1　Metabolic changes during acute stress

The metabolic effects of adrenaline in muscle, hepatic and adipose tissue are primarily mediated via binding to β2-adrenergic receptors. Adrenaline binding activates adenylate cyclase, which is located on the inner surface of the cell membrane. Activation of adenylate cyclase results in formation of the second messenger cyclic AMP (cAMP) from adenosine triphosphate (ATP), which functions to amplify the signal from adrenaline. The messenger protein cAMP stimulates the activity of cAMP dependent protein kinase which has multiple enzymatic functions within glycogenolysis, glycolysis and lipolysis (Kaneko et al., 2008).

Secretion of cortisol is controlled almost entirely by adrenocorticotropic hormone (ACTH), which is secreted by the anterior pituitary gland. ACTH secretion is controlled by corticotrophin releasing hormone (CRF) released by the hypothalamus into the hypophysial portal system and carried to the anterior pituitary gland in response to stress (Guyton and Hall, 1992). Neural messages are transmitted towards the brainstem to the perifornical area of the hypothalamus, then into the paraventricular nucleus of the hypothalamus and to the median eminence, where CRF is secreted (Guyton and Hall, 1992). The role of cortisol is to then increase transcription of key enzymes such as phosphorylase, glucose-6-phosphatase, fructose 1,6-bisphosphatase and PEP-carboxylase. Cortisol has been found to have a stimulation-secretion delay of approximately three minutes (Pearson et al, 1977). Importantly, the HPA axis also operates independently of stressful situations in a circadian manner (Chrousos, 1998) and its baseline is essential for life. An important regulatory role of cortisol is to moderate the demand for glucose by some tissues whilst concomitantly stimulating hepatic gluconeogenesis to increase glucose delivery to skeletal muscle and the brain (Brockman and Laarveld, 1986).

2.1.1.1　Glycogenolysis

Adrenaline is a potent hyperglycaemic compound and increases hepatic and muscle glycogenolysis as well as enhancing gluconeogenesis due to increased substrate availability. It increases the hepatic extraction of lactate and allows the redistribution of glycogen during exercise (Brockman and Laarveld, 1986). Adrenaline can increase the rate of glycogenolysis in muscle and hepatic tissue via its action on β-receptors. Binding to β-receptors increases the rate of intracellular cAMP dependent protein kinase activity within hepatic and muscle cells which initiates a cascade of phosphorylating reactions within the cell. Phosphorlyase kinase is phosphorylated by cAMP dependent protein kinase, which then phosphorylates glycogen phosphorlyase. This leads to the activation of glycogen phosphorlyase which is the rate limiting step in glycogen degradation (Franch et al., 1999). Glycogen synthase is also phosphorylated by cAMP dependent protein kinase, which inhibits its action and thus reduces the rate of glycogenesis (Roach, 1990).

2.1.1.2　Hepatic glycolysis

Adrenergic stimulation and increased rate of glycogenolysis causes an increase in concentration of glucose-6-phosphate. This increases the rate of glycolysis, due to increased substrate available for conversion into fructose-6-phosphate and phosphofructokinase (PFK) activity (Kaneko et al., 2008).

The second mechanism by which adrenaline increases glycolysis is via increased activity of pyruvate kinase. Adrenaline activates cAMP-dependent protein kinase, which directly phosphorylates the activity of pyruvate kinase within muscle cells. This is opposite to the effect within the liver, whereby phosphorylation of pyruvate kinase inhibits its activity, shifting from a glycolytic to gluconeogenic pathway. Thus, an increase in pyruvate kinase activity, coupled with an increase in glucose from increased rates of hepatic glycogenolysis, will increase metabolism of glucose into large concentrations of pyruvate which can be channelled into the tricarboxylic acid (TCA) cycle via pyruvate

dehydrogenase. However, if energy demands are not high (e.g. at rest), then the ATP/ADP and oxidised and reduced forms of Nicotinamide adenine dinucleotide NADH/NAD ratios will increase and inhibit pyruvate dehydrogenase. Increasing concentrations of pyruvate are then converted into lactate, which is shuttled back to the liver for gluconeogenesis via the Cori cycle. Therefore, in animals at rest, adrenaline will increase plasma lactate concentrations (Kaneko et al., 2008).

2.1.1.3 **Lipolysis**

Likewise, adrenaline elicits its effects via binding to β-adrenergic receptors within adipose tissue. This increases cAMP-dependent protein kinase A, which phosphorylates hormone sensitive lipase (HSL). This allows HSL to catalyse the conversion of triacylglycerol into NEFAs and glycerol. Cortisol also promotes mobilisation of adipose tissue, which increases the plasma concentrations of NEFAs, increasing rates of β-oxidation and use of fatty acids in aerobic metabolism.

During psychological and exercise stress animals have the ability to mobilise adipose tissue to form non-esterified fatty acids which are then oxidised and used as an energy source for tissues (Chilliard et al., 2000). Adrenaline is also the most potent lipolytic agent in ruminants (Bassett, 1970) causing increased NEFA concentrations in blood (Shaw and Tume, 1992; Martin et al., 2011).

Adrenaline binds to β-adrenergic receptors within adipose tissue. This increases cAMP-dependent protein kinase A, which phosphorylates HSL. This allows HSL to catalyse the conversion of triacylglycerol into NEFAs and glycerol. Progressive removal of acetyl groups via β-oxidation then converts NEFAs into acetylCoA, which enters the Krebs cycle for aerobic metabolism. Via increased lipolysis, adrenaline assists to spare glucose for brain and skeletal muscle use (Hocquette et al., 1998).

However, the effects on glucose and fat metabolism persist only as long as elevated levels of adrenaline remain in plasma (Brockman and Laarveld, 1986). Interpreting levels of NEFA at slaughter is complicated further as increased lipolysis could be due to either increased mobilisation due to longer feed withdrawal and/or higher levels of stress experienced by those animals (Jarvis et al., 1996b)

Stress in the form of exercise in sheep causes a dramatic shift in fatty acid metabolism. Exercise will cause a shift in intermediary metabolism towards lipolysis (Pethick et al., 1987) as NEFAs have an important role in maintaining fuel homeostasis during exercise. Energy is initially derived from anaerobic glycolysis and glycogenolysis; however, as the duration of exercise increases, blood supply to muscles increases with a shift towards aerobic metabolism utilising glucose, free fatty acids and triglycerides. At low to moderate exercise intensities, fatty acid oxidation provides the major source of ATP. A single fatty acid molecule can provide up to 146 molecules of ATP, making it a very efficient metabolite for energy production (Kaneko et al., 2008). At lower work rates, the concentration of NEFA's gradually increases over time, however at higher levels of exercise peak NEFA concentration is seen within 30 minutes. Contributions of plasma NEFA's to skeletal muscle metabolism range from 40-70%, increasing as the intensity of work increases (Pethick, 1993).

Cortisol also promotes mobilisation of adipose tissue, which increases the plasma concentrations of NEFAs, increasing rates of β-oxidation and use of fatty acids in aerobic metabolism. Furthermore, cortisol increases the rates of oxidation within cells. This is particularly important during times of starvation or other long term stressors. The pathway of cortisol stimulated lipolysis is a much slower mechanism and requires several hours to be fully functional, but is important for long term conservation of glucose and glycogen (Guyton and Hall, 1992). Glucocorticoids (cortisol) can also potentiate the effect that adrenaline has on lipolysis (Brockman and Laarveld, 1986).

Interestingly, glucocorticoids such as cortisol must also be present for adrenaline to exert maximal effect on lipolysis of adipose tissue (Brockman and Laarveld, 1986). Studies in adrenalectomised animals found that the lipolytic response to adrenaline is reduced, indicating that glucocorticoids facilitate adrenaline stimulated lipolysis.

2.1.2 Factors causing and influencing the of acute stress response

2.1.2.1 Handling and Temperament

Pre-slaughter handling problems can affect both the welfare and efficiency of farm and abattoir operations. Pre-slaughter handling should allow animals to move forward freely but unhurried, minimising the risk of injury and keeping stress to a minimum (Grandin, 1980).

Delays in processing on slaughter lines can result in increasing length of time that animals may be exposed to humans which may cause acute stress. Cockram and Corley (1991) found that increases in plasma cortisol and creatine kinase were associated with delays in the slaughter line, the use of electric goads and restraint. Increases in plasma indicators of acute stress and muscle damage were also positively correlated with time spent within a race or trotting and struggling. Similarly, blood cortisol concentration were found to be significantly higher in sheep and cattle undergoing restraint and isolation compared to control group (Apple et al., 1995; Apple et al., 2005).

Plasma lactate also increases as a result of acute stress. Warner et al. (2007) found that stimulating cattle immediately pre-slaughter with electric prodders resulted in significant increases in plasma lactate concentrations, presumably through increased catecholamine stimulated muscle glycogenolysis. High plasma lactate levels are also observed in cattle with poor temperaments. Gruber et al. (2010) found that steers with adverse behavioural reactions to restraint and high flight scores had higher pre-slaughter levels of plasma adrenaline, heart rate and rectal temperatures. In addition, animals had high plasma lactate and creatine kinase levels, consistent with intense exercise and adrenergic stimulation. Similarly, Coombes et al. (2014) demonstrated that cattle exhibiting higher flight scores also had higher plasma lactate concentrations. This effect is also seen in pigs, where it was shown that as the level of stress experienced by the pigs increased, so did the average blood levels of lactate and creatine kinase at exsanguination (Warriss et al., 1994).

During transport, livestock experience periods of social change, environmental factors, restraint, loading and unloading all while exposed to a novel environment (Tarrant, 1990). Studies assessing the stress response to transport in livestock have found that loading and the initial few hours of transport is typically the most stressful (Knowles, 1998) which is shown by increased heart rates, muscle activity and plasma stress indicator concentrations. Eldridge et al. (1988) monitored heart rates of cattle during transport and found they were only 15% higher compared to those at grazing, highlighting that cattle become habituated to transport. Indicators of acute stress such as glucose, lactate and cortisol have been shown to increase during transport; many authors report that indicators return to basal levels within a short period, further supporting the general view that increased handling associated with loading, unloading and the beginning of a journey are the most stressful points for cattle and sheep (Knowles et al., 1995; Broom et al., 1996; Pettiford et al., 2008; De la Fuente et al., 2010; Fisher et al., 2010). Warriss et al. (1990) showed that the glucose concentrations increased within the first three hours of transport but had returned to basal levels by six hours. A similar result was found by Knowles et al. (1995) who demonstrated in sheep transported up to 24 hours, that plasma glucose also was increased after three hours to 6.5mmol/L but had returned to base levels after nine hours to 4.5mmol/L. Pettiford et al. (2008) also demonstrated that loading elevated cortisol levels in cattle, but

levels had returned to base line within six hours of transport. Similarly, (Pascual-Alonso et al., 2017) found that cortisol, glucose, non-esterified fatty acid (NEFA) concentrations were higher immediately after unloading in transported ewes but mostly returned to normal after four hours, with complete recovery after 24 hours.

This is similar to findings by De la Fuente et al. (2010) who showed that cortisol concentrations were significantly higher at slaughter after 30 minutes (7.29 ± 4.45 ng/mL) of transport compared to five hours (4.73 ± 4.45 ng/mL), although considerable variation existed between animals. The authors hypothesised that the lambs transported over the shorter duration had not had sufficient time to habituate to transport conditions.

Environmental conditions may also impact on plasma levels of stress at slaughter. Miranda-de la Lama et al. (2012) assessed the impact of transport direct to slaughter or via stop over type systems and found that plasma glucose, lactate and cortisol concentrations were significantly higher in lambs transported in winter compared to summer. This is likely due to increased adrenergic stimulation and muscle shivering increasing turnover of both liver and muscle glycogen.

2.1.2.2 Breed, genotype and sex

The Merino breed has been previously identified as more sensitive to stress (Gardner et al., 1999b) compared to other breeds and has a propensity to produce meat with a higher ultimate pH as a result of elevated rates of glycogen loss in response to exercise stress (Gardner et al., 2006). This was supported by Jacob et al. (2005a) who showed over a number of consignments that Merinos had greater glycogen loss from farm to slaughter as opposed to cross-bred sheep.

Genotype has also been shown to influence stress and may have a role in and lead to a risk of dark cutting. Several genetic factors also have an impact on muscle glycogen metabolism in ruminants, with one key effect being the impact of selection for muscling. For example, the muscle tissue of the greater muscular genotypes is less responsive to adrenaline. This was shown across several different studies; one in sheep using lambs sired by high or low muscling potential rams (Martin et al., 2011), and two in cattle with the first comparing the progeny of highly muscled Piedmontese with the progeny of Angus and Wagyu sires (Gardner et al., 2009), and the second comparing the extremes in an Angus selection line diverged for muscling (McGilchrist et al., 2011). This was further supported by McGilchrist et al. (2012) which showed that beef carcasses that had higher muscling, demonstrated by larger eye muscle areas, had a lower incidence of dark cutting, supporting the assertion that greater muscling reduces glycogen turnover as a result of stress (adrenaline).

Higher pHu has been reported in ewes compared to rams (Craigie et al., 2012) which may be attributed to higher stress susceptibility in females (Hernandez et al., 2010). Generally however, the literature tends to show that unlike in cattle, sex has a minimal impact on ultimate pH in lamb (Teixeira et al., 2005; Okeudo and Moss, 2008).

2.1.2.3 Magnesium

Magnesium is an essential co-factor for almost all metabolic and enzymatic pathways in the body (Fontenot et al., 1989). Plasma magnesium concentration is not regulated by a hormonal feedback system, instead being reflective of inflow via absorption from the gastrointestinal tract and outflow by endogenous secretion and tissue uptake (Martens and Schweigel, 2000).

Normal plasma magnesium levels in sheep range between 0.9mmol/L to 1.15mmol/L. Low levels of magnesium, known as hypomagnesaemia (0.6 to 0.8mmol/L) can affect cell membrane ion

sensitivity, accelerating nerve impulse transmission and increased nervousness (Sutherland et al., 1986; Goff, 1999). This may be more common in young and rapidly growing ruminants, as magnesium moves to the intracellular pool during bone formation and cellular growth and can have large effects on extracellular magnesium concentrations (Fontenot et al., 1989). Therefore, rapidly growing prime lambs destined for slaughter may be at greater risk of hypomagnesaemia and may also demonstrate greater adrenergic sensitivity.

Magnesium has been shown to depress neuromuscular stimulation (Hubbard, 1973) and when fed in the diet it resulted in an attenuation of glucocorticoids and catecholamine secretion (Kietzmann and Jablonski, 1985; Classen et al., 1986). This has been shown to reduce the stress induced glycogenolysis and promotes glycogen repletion after stress, improving post-mortem glycogen levels and meat ultimate pH (D'Souza et al., 1998; Gardner et al., 2001a). Previous work has shown that magnesium supplementation may be effective in reducing the effects of pre-slaughter stress and improving meat quality (Dunshea et al., 2005).

2.1.3   Impact of acute stress on ultimate pH

2.1.3.1   Muscle glycogen

The major determinant of ultimate pH is the quantity of muscle glycogen at slaughter, which in the post slaughter period, continues to be metabolised via anaerobic glycolysis into lactic acid. Glycogen is the major carbohydrate store in animals and is widely distributed throughout the body. In sheep, basal concentrations of glycogen range between 1.7-2.3g/100g of wet tissue in different skeletal muscle; 2.85g/100g in liver; and 0.06-0.14g/100g in skin, rumen, colon, duodenum and the kidney (Jacob et al., 2009). The muscle glycogen depot is of particular importance to the production animal industries due to its role as an anaerobic fuel driving the post-mortem acidification of meat (Gardner et al., 2014). The formation of lactic acid lowers the muscle pH from 7.0–7.2 in the living animal to approximately 5.5 in 24 to 48 hours post mortem (Tarrant, 1989).

However, if muscle glycogen levels at slaughter are below 0.40 – 0.57g/100g, insufficient lactic acid will be produced post mortem which leads to an ultimate pH greater than 5.7 (Howard, 1964; Tarrant, 1989). Meat with a high ultimate pH is generally darker in colour and is heavily discriminated against by consumers (Grunert et al., 2004).

Ultimate pH is also an important determinant of tenderness in lamb. High ultimate pH muscle results lead to variable tenderness in lamb as ultimate pH increases between 5.8 and 6.0 (Watanabe et al., 1996). Meat with an ultimate pH above 5.7 also leads to faster spoilage and reduced shelf life, as the meat surface is more conducive to bacterial growth. The breakdown of protein into ammonia by bacteria also results in the production of "off" flavours and odours, reducing consumer appeal (Newton and Gill, 1981).

The residual glycogen in normal meat contributes to its water content due to an increase in the water holding capacity as glycogen is a hydrophilic molecule (Olsson and Saltin, 1970). This assists in reducing drip loss when compared to dark cutting meat (Immonen et al., 2000) and may also contribute to the browning reactions (Pethick et al., 1995) and flavour enhancement in cooked meat. High ultimate pH meat is also considered dry in texture due to the reduced water content (Pethick et al., 1995).

For these reasons and other negative impacts on eating quality described below, beef carcasses with a ultimate pH greater than 5.7 are not eligible for grading under the MSA grading system (Ferguson et al., 1999). Currently the lamb industry does not have a ultimate pH cut-off, but rather a

pathways grading scheme which is designed to manage pre-slaughter stress and nutrition to prevent glycogen losses and high ultimate pH meat (Anonymous, 2015).

2.1.3.2   **Pre-slaughter nutrition**

Nutrition is an essential component of glycogen regulation and a clear relationship exists between muscle glycogen concentrations and the intake of metabolisable energy in the pre-slaughter period (Knee et al., 2004)(Knee, Cummins, Walker, & Warner, 2004). As well as ensuring meat quality is maintained, high muscle glycogen concentrations are essential for animals to buffer against environmental stressors including hypothermia, mixing with unfamiliar animals, exercise and psychological stress during the pre-slaughter period (Pethick et al., 1995).

Resting muscle glycogen storage levels of the animal on-farm is dependent on the quality and quantity of the nutrition that the animal has received before slaughter (McIntyre, 2006) and provision of a high energy diet prior to slaughter will afford animals an excess of energy which are then stored as muscle glycogen (McIntyre, 2006). One of the earlier studies that demonstrated this principle Pethick and Rowe (1996) compared muscle glycogen concentrations in sheep fed at 1, 1.3, 1.5, or 2.2 times maintenance, and found a clear positive relationship between metabolisable energy intake and muscle glycogen concentration. .This relationship is well established in beef and lamb where supplementary feeding in the finishing phase to slaughter increased muscle glycogen and reduced incidence of carcasses with high pHu and dark cutting (Immonen et al., 2000; Gardner et al., 2001b; Hopkins et al., 2005; McGilchrist et al., 2012).

Production system also has a major role in determining muscle glycogen at slaughter and animals fed in grass-fed systems have been shown to be particularly susceptible to seasonal fluctuations and the quality and quantity of feed availability. Variable levels of pasture metabolisable energy intake explains lower muscle glycogen concentration and higher incidence of dark cutting in cattle slaughtered in winter and late summer when feed quality or quantity are reduced (Pethick et al., 2000; Knee et al., 2004; McGilchrist et al., 2014). Likewise it also explains the contrast between pasture-fed cattle compared with feedlot cattle, with the latter having comparatively higher muscle glycogen concentrations and a reduced incidence of dark cutting at slaughter. This contrast between grass and grain fed is also present in the sheep industry (Jacob et al., 2005a).

In addition to high energy diets, other strategies to prevent stress induced glycogen losses have been evaluated, namely magnesium supplementation. Normal plasma magnesium levels in sheep range between 0.9 to 1.15mmol/L (Goff, 1999). Low to marginal levels of magnesium (0.6 to 0.8mmol/L) (Sutherland et al., 1986) can affect cell membrane ion function, accelerating nerve impulse transmission and increased nervousness (Goff, 1999). When fed in the diet, magnesium supplementation also resulted in an attenuation of glucocorticoids and catecholamine secretion (Kietzmann and Jablonski, 1985; Classen et al., 1986). Previous work has shown that magnesium supplementation reduced glycogenolysis and promotes glycogen repletion after stress, improving post-mortem glycogen levels and pHu (D'Souza et al., 1998; Gardner et al., 2001a). Alternatively, Lowe et al. (2002a) found no effect on muscle glycogen, ultimate pH or shear force values when lambs were fed magnesium as a bolus for 28 days prior to slaughter; however, it has been shown that supplementation should be short-term (D'Souza et al., 2002) to have an effect on meat quality as excess magnesium is excreted.

2.1.3.3   **Transport**

Many authors have concluded that transport per se has minimal impact on pHu (Knowles et al., 1999; Ferguson et al., 2001). However, it is likely that other environmental stressors in combination

with transport contribute to the incidence of dark cutting. Rough handling practices at loading and unloading may cause glycogen losses in sheep and cattle (Cockram and Corley, 1991; Warner et al., 2005; Warner et al., 2007).

Likewise, animal mixing during transport has been shown to increase the incidence of dark cutting in cattle (Mach et al., 2008; Warren et al., 2010). Environmental conditions can also have an effect. Road surface has been shown to have an impact on ultimate pH, with smoother road surfaces reducing the risk of high pHu meat in sheep (Ruiz-De-La-Torre et al., 2001; Miranda-de la Lama et al., 2012). Transport during extreme weather conditions can also increase the incidence of dark cutting. Kadim et al. (2007) reported higher ultimate pH in goats transported during hot conditions. Alternatively, Miranda-de la Lama et al. (2012) showed that lambs transported in winter had significantly higher ultimate pH values (5.83 ± 0.01) than lambs transported in summer months (5.51 ± 0.01). This effect coincided with greater plasma lactate and glucose concentrations in these lambs, likely due to greater glycogen turnover. Shorter transport times followed by immediate slaughter may also be detrimental to ultimate pH. De la Fuente et al. (2010) showed that immediate slaughter after 30 minutes of transport caused significant increases in ultimate pH compared to lambs that had been transported for five hours.

2.1.3.4 Lairage and pre-slaughter handling

There are contrasting reports in relation to the association of lairage, stress and ultimate pH. Several studies show that lairage time has no impact on the incidence of high ultimate pH meat and that shorter lairage times or even no lairage may even be detrimental to meat quality (Liste et al., 2011) as animals have not had time to recover from stress associated with transport and the novel environment of the abattoir. By contrast, some authors have reported an association between longer lairage times of up to two days and a greater incidence of high ultimate pH (Jacob et al., 2005a; Toohey and Hopkins, 2006). The negative effect of longer lairage times is most likely due to greater exposure to stress rather than feed and water deprivation, which have minimal effect on muscle glycogen and pHu levels (Daly et al., 2006).

Excessive physical activity has been shown to rapidly deplete glycogen stores in the muscle and liver (Harman and Pethick, 1994; Jacob et al., 2009). Previous work by Warner et al. (2005) also found that exercise immediately pre-slaughter resulted in higher ultimate pH levels in lamb. Likewise mixing animals and high stocking densities can also increase glycogen turnover and is a well-established risk factor for dark cutting in cattle (McVeigh et al., 1982; Mach et al., 2008). As well as increased movement, mixing also causes psychological stress due to fighting and changes in hierarchical structure (Tarrant, 1989).

Restraint and isolation stress in cattle and sheep has been found to elicit dramatic changes in plasma indicators of stress as well as influencing meat quality attributes, including causing increases in ultimate pH (Apple et al., 1995; Apple et al., 2005). It is likely that multiple stress events or prolonged stress are required to deplete enough muscle glycogen to have an impact on ultimate pH. In an early study by Bray et al. (1989), the cumulative effect of nutritional, shearing and pre-slaughter washing stressors on lamb meat quality were assessed. The most interesting aspect of this study was the impact that stress had on the ultimate pH of the M. *longissimus lumborum* muscle. Individually, these factors had no significant impact on ultimate pH; however, when combined the negative effects on ultimate pH were enhanced. Under commercial conditions, lambs will often be exposed to more than one stressor and because of the cumulative effects of these stressors on meat pH, it highlights that care needs to be taken in minimising stress at all levels of the consignment.

2.1.4   Impact of acute stress on tenderness

Tenderness is an important meat quality trait. It is determined by several factors including the amount and solubility of connective tissue, sarcomere shortening during rigor development, and post-mortem proteolysis of myofibrillar proteins (Koohmaraie and Geesink, 2006). It is indirectly influenced by the level of intramuscular fat (IMF) (Hocquette et al., 2010) with a minimum of 5% IMF required in order to achieve a failure rate of less than 10% for tenderness rated by consumers (Hopkins et al., 2006a). Another important factor in determining meat tenderness is the rate and extent of post-mortem energy metabolism, including ultimate pH (Thompson et al., 2006).

Despite significant improvements in lamb meat quality following implementation of the MSA pathways grading system, variation in tenderness in lamb meat quality still exists. Recent work from the Sheep Co-operative Research Centre (Sheep CRC) has shown that approximately 40% of lamb loins are rated as 3 star or less (Pannier et al., 2014) with significant variation in Warner Bratzler shear force (WBSF).

Environmental factors such as acute stress may play a role in affecting the tenderness of lamb meat. Previous work in beef (Warner et al., 2007; Gruber et al., 2010) and in pigs (D'Souza et al., 1998) has shown that increased stress immediately pre-slaughter results in a decrease in tenderness and increases in WBSF, however, the underlying mechanisms are not understood.

Pannier et al. (2014) found that date of consignment (kill group) was a significant cause of variation in loin tenderness. Factors that may contribute to variation in tenderness include; animal age, pre-slaughter nutrition, individual animal stress susceptibility and temperament, handling and handlers within the abattoir environment, transport distance or stress and weather conditions. These factors are likely to vary considerably between kill groups. These may also then interact with processing factors, further affecting tenderness (Warner et al., 2010a).

There is evidence that acute stress pre-slaughter negatively impacts on tenderness. The use of electric prodders immediately pre-slaughter has been found to impact on consumer sensory scores in beef. Warner et al. (2007) found the use of electric prodders in cattle immediately pre-slaughter caused a reduction in beef eating quality. Although WBSF was not affected by acute stress treatment, consumer scores for tenderness were significantly lower in the stress treatment group, as well as being rated as less juicy with a less acceptable flavour and overall liking compared to meat from the control group. Importantly, this effect was independent of pHu and pH decline, indicating that there are other mechanisms involved in stress induced changes to meat tenderness.

Similarly, Pighin et al. (2015) found that stress parameters including lactate and glucose were higher in cattle under conventional immediate pre-slaughter handling compared to reduced stress handling, which was associated with increased loin hardness, independent of ultimate pH. Gruber et al. (2010) also found that cattle with more excitable temperaments and higher flight scores had higher plasma lactate concentrations, which correlated to lower tenderness as shown by higher WBSF values.

2.2   Injury and bruising

Bruising is defined as tissue injury with the damage and localised rupture of blood vessels (Hoffman et al., 1998) and is the most common result of physical injury during the pre-slaughter period. Bruising can lead to losses in carcass weight and yields due to trimming.

A number of factors can influence the incidence of bruising and can provide an indication of the level of pre-slaughter stress. Inappropriate handling and high stocking densities throughout the pre-

slaughter period can cause muscle injury particularly during yarding, transport or immediately pre-slaughter (Jarvis et al., 1996a). In cattle, agonistic behaviour and mixing can lead to increased fighting and mounting behaviours (Tarrant, 1989)., which has been shown to be higher in heifers vs steers (Jarvis et al., 1996a).

The primary cause of bruising in livestock is considered to be rough pre-slaughter handling and in a study assessing 49 groups of commercially slaughtered sheep, 88% of the bruises were estimated to have occurred within the 24 hours prior to slaughter, indicating that bruising was mostly due to handling on farm, during transit or at markets (Cockram and Lee, 1991). Behavioural observations within the abattoir found significant correlations between carcass bruising and wool pulling, riding by other sheep when pushed and hits and squashes against yards when moved, with the highest incidence of bruising found in lambs compared to ewes (Cockram and Lee, 1991).

2.2.1   Plasma indicators of muscle stress and damage

With increased physical activity or trauma, muscle cell mitochondrial and cellular membrane permeability is increased (Highman and Altland, 1963; McNeil and Khakee, 1992) and muscle enzymes such as creatine kinase (CK) and aspartate aminotransferase (AST) are released into the vascular system.

Creatine kinase is an enzyme that is active in a variety of organs, including skeletal, cardiac and smooth muscle and brain (Kaneko et al., 2008) and is the most widely used serum enzyme determination for skeletal muscle damage (Kaneko et al., 2008). Creatine kinase is a cytoplasmic enzyme and catalyses a reversible reaction that involves the transfer of phosphate from creatine phosphate to adenosine diphosphate (ADP) to form adenosine triphosphate (ATP) and creatine, making ATP available for muscle contraction (Latimer, 2012; Stockham and Scott, 2013).

Aspartate aminotransferase (AST) is a cytoplasmic and mitochondrial enzyme that catalyses a reversible reaction which involves the deamination of L-aspartate to 2-oxoglutarate to form oxaloacetate and glutamate. Oxaloacetate can then enter the Krebs cycle (Latimer, 2012; Stockham and Scott, 2013). Although AST is not tissue specific, it is present in highest concentrations within skeletal muscle, followed by cardiac muscle and liver (Latimer, 2012; Thrall et al., 2012).

Elevations in CK are widely considered specific and sensitive indicators of acute muscle damage in ruminants (Russell and Roussel, 2007) due to their high concentration and activity in skeletal muscle. Subtle increases in CK can be seen with unaccustomed exercise and low-level trauma such as bruising (Tarrant, 1990) but can increase to very high levels as a result of excessive exercise, recumbancy, myopathies or severe trauma (Lefebvre et al., 1994).

There is evidence that transport causes a level of muscular fatigue and damage. Increasing plasma concentrations of CK are seen with increasing duration of transport. In a study by Fisher et al. (2010), increasing duration of transport of sheep for 12, 30 and 48 hours significantly increased CK concentrations and although the effect was small and concentrations were within published reference ranges, it does indicate that muscle damage occurs during transport. However, another study assessing the impacts of transport on sheep for up to 24 hours with treatment groups being transported for 3, 9, 15, 18 and 24 hours, no difference was detected in CK concentrations between these groups, possibly due to animals being able to lie down and rest (Knowles et al., 1995).

In a study assessing the impact of loading practices and six hour road transport on physiological responses in cattle, blood CK concentrations increased by 30% to levels above published normal reference ranges for cattle (Radostits et al., 2007) but had fallen to within normal limits within 17 hours following transport, indicating that the muscular damage was transient (Pettiford et al., 2008).

The change in CK concentration in this study were significantly lower than that reported in an earlier study by (Warriss et al., 1995) where cattle were transported for 5 - 15 hours had a 270% increase in CK concentrations. This may be due to stocking density; cattle in the study by Pettiford et al. (2008) had their own crate during transport, therefore minimising the amount of bruising and physical exertion required due to competition for space. Elevations in CK may also be due to increased muscular exertion as cattle attempt to maintain their balance (Warriss et al., 1995). In slaughter lambs transported from farm to market and then to abattoir, correlations exist between the mean number of carcass bruises per group and mean plasma creatine kinase (CK) concentration at exsanguination (Jarvis et al., 1996a). Alternatively, if transport times are short (De la Fuente et al., 2010) or carried out in good conditions (Cockram et al., 1999), the number of traumatic events may be too low to cause elevations in CK levels.

Published ranges for CK (U/L) are 102-358 (Dubreuil et al., 2005), 20-226 (Roubies et al., 2006), 180-454 (Lepherd et al., 2009). Variations in plasma CK may be due to stress associated with handling and restraint for blood sampling (Lepherd et al., 2009) and it has been suggested CK concentration is higher in younger animals due to greater metabolic activity (Roubies et al., 2006). CK concentrations may also be higher in serum tubes than plasma tubes due to the release of CK from platelets during clot formation (Latimer, 2012).

As opposed to CK, concentrations of AST in plasma increase more slowly and persist longer in plasma than CK, their half-lives approximately 20 hours and one hour respectively (Kaneko et al., 2008; Thrall et al., 2012; Stockham and Scott, 2013). Elevations in plasma AST above normal range may be present for weeks, unlike CK which only remains elevated up to three days post insult (Kaneko et al., 2008). Published reference ranges for AST (U/L) are varied at 72-101 (Dubreuil et al., 2005), 60-280 (Radostits et al., 2007) and 83-140 (Lepherd et al., 2009).

Limited research exists assessing the effect that pre-slaughter conditions have on AST in sheep. However Tollersrud et al. (1971) found that in housed and outdoor lambs which were transported and then herded, AST concentrations were significantly increased due to herding. In pig studies, AST concentrations significantly increased during a 60 minute journey during summer and although within reference ranges published, it indicates that a degree of muscular exertion and damage occurred (Sutherland et al., 2009).

2.3   Pre-slaughter feed deprivation

Feed deprivation represents a chronic stressor within the pre-slaughter period. The key points that this occurs along the pre-slaughter pathway are mustering and yarding prior to transport (curfew), transport, saleyards (if applicable) and lairage in the processing facility.

The main purpose of on-farm feed curfews is to reduce soiling during transport and optimise fleece cleanliness at slaughter, which assists in efficient dressing and lower carcass microbial counts. Under best-practice recommendation, feed deprivation should be kept below 48 hours to maximise carcass yields (Jacob et al., 2005a); however, the feed deprivation period may extend beyond this due to transport distances or if lambs are sold through saleyards. Feed deprivation results in significant shifts in energy metabolism, namely increased rates of lipolysis and hepatic glycogen turnover. Previous work has shown that feed deprivation causes reductions in live weight, carcass weight and liver weights in sheep (Thompson et al., 1987; Warriss et al., 1987; Jacob et al., 2009), However, most research appears to indicate that acute feed deprivation does not impose large disruptions to the physiology of the animal but rather the changes in metabolites reflect normal homeostatic responses to maintain euglycaemia and energy tissue balance.

2.3.1　Physiological impact of feed deprivation

2.3.1.1　Glucose and glycogen metabolism

During feed deprivation the major precursor for gluconeogenesis, propionate, declines. As a result, hypoglycaemia stimulates glucagon secretion (Brockman and Laarveld, 1986) from the α-cells of the pancreas with its site of action principally within the liver, where it simultaneously initiates lipolysis and importantly, glycogenolysis and gluconeogenesis, resulting in release of glucose into the vascular system (Clarenburg, 1992). The mechanism of action of glucagon culminates in the cleavage of α-1,4 glycosidic bonds of glycogen by glycogen phosphorylase, releasing sequential units of glucose-1-phosphate (Kaneko et al., 2008).

Hepatic glycogenolysis plays a key role in regulating glucose homeostasis by storing and mobilising glycogen in the fasted state. The mechanism of action of glucagon culminates in the cleavage of α-1,4 glycosidic bonds of glycogen by glycogen phosphorylase, releasing sequential units of glucose-1-phosphate (Kaneko et al., 2008). Glucose-1-phosphate is converted to glucose-6-phosphate by the reversible reaction catalysed by phosphoglucomutase. The glucose-6-phosphate is then irreversibly cleaved to free glucose and phosphate by the enzyme glucose-6-phosphotase which is found within the liver and kidney. Free glucose, unlike its phosphorylated intermediates, is transported out of the hepatic cell and enters the circulation and increases blood glucose concentrations maintaining glucose homeostasis (Kaneko et al., 2008).

Depletion of liver glycogen over a 12-24 hour period of feed deprivation in ruminants is also less than in simple stomached animals due to the continual supply of gluconeogenic carbon from the rumen in this period due to associated gut fill (Stangassinger and Giesecke, 1986). Work in sheep has shown a curvilinear reduction in liver weight with a total loss of 28% after 72 hours of fasting (Warriss et al., 1987). In conjunction with a decrease in hepatic glycogen content, plasma glucose concentrations also decline. Liste et al. (2011) showed that 12 hours of lairage with feed deprivation caused a significant decrease in plasma glucose concentration from 2.73 ± 0.22 mmol/L to 1.79 ± 0.22 mmol/L.

As feed deprivation continues and hepatic glycogen is depleted, glucose production by the liver, gut and kidney must source other substrates for gluconeogenesis; namely glycerol from lipolysis which contributes up to 40% of gluconeogenesis precursors during fasting (Bergman et al., 1968). This may be observed during periods of extended feed deprivation and was demonstrated by Warriss et al. (1989) where plasma glucose remained relatively stable in lambs undergoing feed deprivation from 0 up to 72 hours.

2.3.1.2　Lipolysis and ketogenesis

Feed deprivation is a potent stimulator of adipose tissue turnover and β-oxidation. Broadly, metabolic energy from the utilisation of glucose or oxidation of volatile fatty acids (VFAs) produced in the rumen ceases due to lack of exogenous substrate and is replaced by increasing levels of adipose tissue mobilisation (Heitmann et al., 1987). This results in an increase in circulating non-esterified fatty acids (NEFA) and a shift towards hepatic ketogenesis through β-oxidation of long chain fatty acids, producing β-hydroxybutyrate (BHOB) (Bergman, 1971; Chilliard et al., 2000; Pethick et al., 2005).

During feed deprivation, plasma insulin concentrations and pancreatic production of insulin decline, while plasma glucagon levels remain stable for up to two days of feed withdrawal (Rule et al., 1985). The changes in relative insulin and glucagon levels act to spare glucose by stimulating the

mobilisation of triacylglycerol stores via hormones sensitive lipase (HSL) stimulated lipolysis (Heitmann et al., 1986). This process yields NEFAs, glycerol and hydrogen ions (Engelking, 2015).

Glycerol is generally channelled towards hepatic production of glucose via gluconeogenesis (Brockman and Bergman, 1975). NEFA levels are normally very low at less than 0.l mmol/L in the resting, fed sheep (Pethick et al., 2005); however, fasting for 3-4 days may result in a ten-fold increase in blood NEFA concentrations (Pethick et al., 1983). In lambs fasted up to 72 hours, Warriss et al. (1989) showed that free fatty acid (FFA) concentration increased from 0.32mmol/L up to 2.25mmol/L at 36 hours after which FFA levels stabilised, while glucose remained relatively stable. Elevations in plasma NEFA may also be observed up to 24 hours. In lambs fasted for 12 hours, plasma NEFA concentrations increased from 0.33 ± 0.05mmol/L up to 1.09 ± 0.05mmol/L (Liste et al., 2011). Knowles et al. (1996) showed that NEFA increased from 0.4mmol/L up to 0.8mmol/L in lambs transported for 24 hours. This was lower than in an earlier study (Knowles et al., 1995) where FFA concentrations increased dramatically from approximately 600mmol/L to 1282mmol/L.

Physiologically, there are limitations to NEFA production and oxidation by tissues. Whether fed or fasted, NEFA oxidation is relatively low and generally only half that of other fatty acids such as acetone and ketones, with a substantial amount of NEFA entering non-oxidative pathways such as ketogenesis (Pethick et al., 2005). This process increases NEFA concentrations available for entry into the Krebs cycle for aerobic metabolism. NEFAs are degraded within body tissues by a series of four recurring reactions known as β-oxidation, which occurs within the mitochondria. β-oxidation also promotes gluconeogenesis by providing reducing agent NADH as well as stimulating pyruvate carboxylase, which converts acetylCoA to oxaloacetate. Furthermore, pyruvate dehydrogenase is inhibited, which prevents pyruvate entering the TCA cycle (Clarenburg, 1992).

The mitochondrial β-oxidation of NEFAs is a cyclic process, which involves four enzymes that sequentially remove acetylCoA from the carboxyl end of the fatty acid molecule. Each cycle of β-oxidation produces one molecule of acetylCoA, the reduced form of flavin adenine dinucleotide (FADH2) and NADH (Engelking, 2015).

Entry of FADH2 and NADH into the oxidative phosphorylation pathway results in the synthesis of five ATP molecules for each of the first seven acetylCoA molecules formed via complete β-oxidation of palmitate. Eight acetylCoA units are produced via this process each capable of producing 12 ATP molecules via the Krebs cycle, yielding a total net 129 molecules of ATP (Engelking, 2015).

The glycerol base formed from lipolysis of triglyceride is phosphorylated and then oxidised to form dihydroxyacetone phosphate, which is isomerised to form glyceraldehyde-3-phosphate. Glyceraldehyde-3-phosphate can be converted to either pyruvate or glucose is an intermediate of both the glycolytic and gluconeogenic pathway. During fasting and starvation this glycerol is generally channelled towards hepatic production of glucose via gluconeogenesis (Brockman and Bergman, 1975).

Ketogenesis is stimulated by a combination of several events including increased entry rates of NEFA into the liver, a declining insulin glucagon ratio and reduced feed intake (Heitmann et al., 1987). It is an essential pathway which allows increased fat mobilisation and utilisation to occur during times of excessive glucose demand or starvation, preventing accumulation of toxic levels of NEFA. Physiologically, there are limitations to NEFA production. At concentrations above 2mmol/L, NEFA become toxic to cell membranes and limits the amount of fat that can be mobilised from adipose tissue for utilisation by tissues (Pethick et al., 2005).

During high rates of fat mobilisation, high activities of Carnitine palmitoyltransferase I (CPT1) enables excess fat to enter mitochondria for β-oxidation to produce acetylCoA (Equation 1). The β-

oxidation pathway also produces large concentrations of NADH and FADH as well ATP, which inhibits the Krebs cycle. This subsequently pushes more acetylCoA into the ketogenesis pathway, thus sparing glucose for the brain and erythrocytes (Herdt, 1988).

Equation 1 The β-oxidation pathway

$$BHOB\ +\ NAD\ \rightarrow\ acetoacetate\ +\ NADH$$

$$SuccinylCoA\ +\ acetoacetate\ \rightarrow\ acetoacetylCoA\ +\ succinate\ (SuccinylCoA\ transferase)$$

$$AcetoacectylCoA\ +\ CoA\ \rightarrow\ 2\ acetylCoA$$
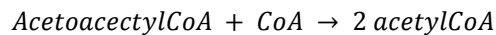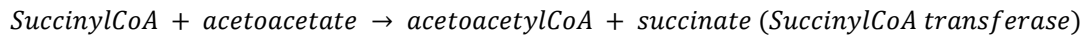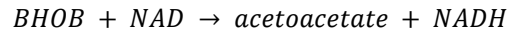
Ketone bodies are a group of compounds which are formed via the partial oxidation of fatty acids. Ketone bodies can be readily utilised by a number of tissues including muscle and kidney and essentially represent a less toxic form of fat. They include acetoacetate, β-hydroxybutyrate (BHOB) and acetate (Bergman, 1971).

Ketogenesis mainly occurs within the mitochondrial matrix within liver cells, however ruminants are unique in that they can synthesise β-hydroxybutyrate (BHOB) within the rumen wall from the volatile fatty acid butyrate. Thus the two pathways of BHOB production can make it difficult to utilise as a marker of nutritional stress because the ruminant receives a constant supply of ketone bodies whether extensive fatty acid oxidation is occurring or not (Herdt, 1988). However, during fasting, circulating concentration of BHOB has been shown to increase due to hepatic ketogenesis from NEFA with alimentary ketogenesis ceasing (Heitmann et al., 1986).

Normal physiological ranges for BHOB vary slightly but are generally within the range of 0.4-0.5mmol/L (Heitmann et al., 1987), 0.47-0.63mmol/L, (Radostits et al., 2007) and 0.2-0.7mmol/L (Lepherd et al., 2009). Increased circulating ketone bodies and BHOB may reduce the NEFA concentration; this is most likely achieved by increasing the rate of insulin secretion (Heitmann et al., 1987). Therefore, ketone body accumulation in plasma is a normal physiological adaption (Pethick et al., 2005). Previous work by Warriss et al. (1989) showed in lambs that plasma BHOB progressively increased over a 72 hour fasting period from 0.311mmol/L up to 0.717mmol. In a study assessing the effect of transport and feed deprivation up to 24 hours in slaughter sheep, FFA concentrations increased dramatically from approximately 600mmol/L to 1282mmol/L. This coincided with an increased in BHOB concentration from 0.28mmol/L up to 0.47 ± 0.027mmol//L (Knowles et al., 1995).

However, some studies have found no difference in BHOB levels between fed and fasted lambs. In lambs fasted and transported for 12 hours it was found that β-hydroxybutyrate concentrations were no different between transported lambs and control lambs given access to feed and water (Cockram et al., 1996). Similarly, Fisher et al. (2010) found that concurrent transport and feed deprivation had no effect on plasma BHOB concentrations in lambs, which indicates that in at least short to medium term transport and feed deprivation causes minimal fat mobilisation and hepatic ketogenesis.

Several authors have also suggested that feed deprivation may increase plasma cortisol levels. In Merino lambs undergoing fasting for 24 hours, plasma cortisol levels were significantly increased from 187nmol/L to 288nmol/L (Zimerman et al., 2013). Similarly, in dairy cattle feed deprivation up to 30 hours was accompanied with increased plasma cortisol concentrations. In addition, the emotional reactivity to a variety of stressors in this group was also higher, suggesting that feed deprivation caused increased psychological stress (Bourguet et al., 2011). More recently, cortisol concentrations were found to be significantly higher in lambs undergoing 24 hours (27.45nmol/L) and 48 hours (27.67nmol/L) of feed deprivation when compared to those not experiencing feed deprivation (11.04nmol/L) (Karaca et al., 2016).

2.3.2   Impact of feed deprivation on meat quality and yield

Although pre-slaughter feed deprivation results in significant shifts in energy metabolism, unlike adipose tissue, the role of glycogen in skeletal muscle is thought to be less influenced by nutrition and more by the effects of stress or by energy demands of muscle (Harris, 2006). In lambs fasted for up to 48 hours, it was found that feed deprivation had no significant impact on muscle glycogen levels and minimal effect on pHu or rate of pH decline (Daly et al., 2006). Furthermore, glycogen levels remained consistent over the duration of feed deprivation. Jacob et al. (2005a) showed that muscle glycogen loss occurred mainly during curfew on farm and transport and that the total time off feed was not correlated to pre-slaughter muscle glycogen loss. This indicates that the prior nutritional status on farm is likely to be the strongest driver of muscle glycogen levels at slaughter (Pethick and Rowe, 1996; Jacob et al., 2005a). However, stress caused by feed deprivation may impact muscle glycogen concentrations and ultimate pH. In a small study by Zimerman et al. (2013), Merino lambs fasted for 24 hours had significantly elevated cortisol levels as well as a trend for increased loin ultimate pH.

Unlike the minimal impact on meat quality, pre-slaughter feed deprivation has been shown in a number of studies to cause reductions in live weight, carcass weight and liver weights (Kirton et al., 1967; Carr et al., 1971; Thompson et al., 1987; Warriss et al., 1987). Knowles et al. (1993) suggested that recovery time of 96 hours or longer may be required to recover carcass weight losses following extended curfew times. The curvilinear association between carcass weight and time off feed indicates that duration of feed deprivation should be as short as possible to minimise the impact on carcass weight.

Previous work indicates that the rate of carcass loss is curvilinear with the greatest rate of hot carcass weight loss occurring during the initial part of the fast, with a 35kg lamb with a fat score of 2.4 losing 3.5, 5.9, 7.3 and 7.7% of initial hot carcass weight at 24, 48, 72 and 96 hours respectively (Thompson et al., 1987). Carcass losses have been shown to be correlated with reductions in fat, protein and water content of carcass joint sections including the leg, loin and shoulder (Kirton et al., 1967).

Daly et al. (2006) reported opposing results. In merino wethers deprived of feed for 0, 2 and 4 days under low stress pre-slaughter conditions, it was found that time off feed had no significant effect on carcass weights or GR tissue depth. Similarly, Jacob (2003) found inconsistent effects of increasing lairage times on carcass weight, with approximately half of consignments showing a significant effect from 4 to 48 hours time off feed.

Intrinsic factors including live weight, condition score, weaning status and pre-slaughter nutrition have been found to affect the rate of live animal and carcass weight loss (Thompson et al., 1987; Greenwood et al., 2008). Lighter and leaner animals have been shown to be most affected by time off feed. George et al. (1966) found that fasting lambs for 72 hours resulted in significantly higher pre-slaughter shrinkage, reduced carcass weight and dressing percentage compared to lambs fasted for 24 hours across different live weight groups ranging between from 33kg and 60kg, with the effect being greatest in the lighter animals. Likewise, Thompson et al. (1987) found that over a 48 hour fast, lambs that were leaner by one fat score lost approximately 0.5kg carcass weight more than fatter lambs of a similar live weight.

2.4   Pre-slaughter water deprivation

Under best practice industry recommendations, water should be made available during curfew and lairage. For sheep that are directly consigned from farm to abattoir the period of water deprivation is likely to be less than 24 hours as water should be made available at all times (except

during transport) (Anonymous, 2015). However, for stock that are sold through saleyards, there may be there may be situations where this period extends to 36–48 hours (Ferguson and Warner, 2008).

2.4.1 Water distribution in the body

Water represents 60-70% of body mass and lean muscle contains approximately 75% water (Offer and Knight, 1988). Total body water is distributed within two major body compartments; the extracellular compartment and intracellular fluid. Water deprivation can lead to hypertonic dehydration which occurs as a result of total water loss and an increase in osmolality and tonicity of both the intracellular and extracellular spaces (McKinley et al., 1983).

The extracellular compartment can be divided into two further sections, the intravascular fluid (plasma volume) which is approximately 5% of body weight, and the interstitial fluid which makes up 15% of body weight and consists of fluids surrounding cells, connective tissue and cerebrospinal fluid (Walz and Taylor, 2012). Unlike monogastric animals where the transcellular fluids may only contribute 5% of total body volume, the transcellular fluid compartment in ruminants may play an important role in water balance as rumen fluid volume can be the same volume as the vascular system (Hecker et al., 1964; Li et al., 2000).

Water movement across cell membranes is determined by osmolarity differences in the intracellular and extracellular space (DiBartola, 2006). Sodium and associated anions are responsible for more than 90% of the osmolarity of the extracellular fluid (ECF). The extracellular fluid volume must be regulated carefully to maintain blood volume and extracellular fluid osmolarity must be regulated to prevent cells from shrinking or swelling.

There are several mechanisms in place to regulate osmolarity and blood volume. These processes work in conjunction with one another and are activated during water deprivation. Plasma osmolarity is regulated by the anti-diuretic system and the thirst mechanism. Mechanisms that regulate blood volume include the renin-angiotensin2-aldosterone system (RAAS), peritubular capillary system and atrial naturetic peptide (ANP). There is also a reduction in faecal and urinary free water losses and a decrease in salivary flow rate and feed intake to help maintain electrolyte balance (McKinley et al., 1983; Silanikove, 1994). These processes act to causes withdrawal of fluid from the tissues into the vascular space attempt to maintain blood volume and osmolarity. During dehydration, intravascular fluid is lost resulting in haemoconcentration. If not corrected, loss of body fluid reaches a critical point and interferes with thermoregulation and cardiovascular function (DiBartola, 2006).

2.4.2 Regulating extracellular fluid osmolarity

2.4.2.1 Anti-diuretic hormone

Within the hypothalamus are osmoreceptors that are sensitive to changes in ECF osmolarity. Increased osmolarity within the ECF, due to water deprivation and hypertonic dehydration, leads to increased release of ADH from the posterior pituitary gland. The role of ADH is to reduce the water permeability of the distal tubules and collecting ducts of nephrons within the kidneys, reducing the water excreted in urine and reduction in urine outflow. Increased water retention combined with unchanged excretion of sodium and other solutes restores ECF osmolarity homeostasis (DiBartola, 2006).

Stress during the pre-slaughter period may also exacerbate these effects beyond limiting voluntary water intake. High cortisol concentrations associated with stress have been found to reduce

and in some cases result in complete abstinence from drinking (Parrott et al., 1987). Cortisol has been implicated in inhibiting the effect of ADH in dogs (Baas et al., 1984) and it may also inhibit the RAAS system (Coghlan et al., 1979), limiting the ability to concentrate urine and also increasing water loss through urination.

2.4.2.2 **Thirst**

There are several intrinsic and environmental factors which may impact on the overall hydration status of lambs.

Stress during the pre-slaughter period and within lairage may be a contributor to voluntary water intake. In an abattoir environment, lambs may not drink in lairage due to the unfamiliarity of the environment or there may be limited access to watering facilities due to high pen densities (Knowles et al., 1993; Ferguson and Warner, 2008). This effect may be greater in young lambs, particularly if they have been weaned just prior to consignment (sucker lambs) as they may have little experience using water troughs. Previous work by Jacob et al. (2006c) has shown that sucker lambs are more dehydrated than older lambs at slaughter. Alternatively, Jongman et al. (2008) found that while reduced space allowance did not negatively affect drinking behaviour during a 24 hour period in lairage, it was observed that approximately 20% of lambs did not drink.

Feed type is also likely to have an impact on thirst and water intake. Wilson (1974) showed that water consumption was significantly increased for sheep grazing saltbush compared to pasture, an effect which was higher in summer months. Alternatively, limiting access to feed can reduce water intake. Studies have shown that feed deprivation can have a negative impact on thirst, shown by Li et al. (2000) and Horton et al. (1996) where fasted lambs with access to water had reduced voluntary water intake. This may be a homeostatic mechanism to regulate electrolyte balance during reduced feed intake (DiBartola, 2006).

2.4.3 Regulating blood volume

Blood volume and pressure is tightly regulated by several physiological mechanisms. Decreases in plasma volume, which is essential for tissue perfusion, is closely related to sodium concentration within the plasma as this determines the ECF volume (Rose et al., 2001). As there is free exchange of water and ions across the capillary walls, increases or decreases in plasma volume will lead to changes in the rest of the ECF. Therefore if fluid is lost from the circulation, interstitial fluid will move into the blood, maintaining blood volume. Thus, the interstitial fluid acts as a buffer that allows for large changes in blood volume and pressure (DiBartola, 2006).

When blood volume decreases, stretch sensitive receptors in blood vessel walls and the atria of the heart detect changes in blood pressure. The frequency of action potentials generated in these receptors declines, which leads to increased activity in the sympathetic nervous system, resulting in vasoconstriction of the afferent and efferent arterioles of the kidney. When blood pressure declines, urinary excretion of salt and water also declines, with increased sodium reabsorption which is controlled by a number of homeostatic mechanism (DiBartola, 2006).

Ruminants are believed to be more resilient than monogastric species during periods of water restriction due to the amount of water contained within the rumen, which may act as a buffer to dehydration (Hecker et al., 1964; Fisher et al., 2009). However, work has shown that blood volume loss can be extreme during periods of water deprivation in ruminants. Weeth et al. (1967) showed cattle deprived of water for four days had decreased their blood volume by 28%.

In early studies assessing water and feed deprivation in sheep for up to eight days, it was found that the greatest decrease/shift in rumen fluid volume occurred during the first 2-3 days, with the rate of rumen fluid loss slowing as volume depleted (Hecker et al., 1964). This correlated with a decrease in urine volume but increase in urine osmolarity, which was also seen in other studies by (Macfarlane et al., 1961b). The absorption of rumen fluid during the first 2-3 days of feed and water deprivation may account for the increase in plasma volume recorded on the third day. This highlights that the rumen may act as a water "store" in sheep (Hecker et al., 1964) and may act to buffer against the effects of water restriction and dehydration (Silanikove, 1994). In support of this, sheep undergoing 72 hours of water deprivation in controlled, mild environmental conditions, showed total water body reduced by 17% but water content of their plasma only declined by 3% (Cole, 1995). Contrary to this, Jacob et al. (2006b) found that gastrointestinal weight did not differ between lambs with access to water and those deprived of water for 48 indicating that water absorption from the gastrointestinal tract was limited.

2.4.3.1 The renin-angiotensin2 aldosterone system and sodium reabsorption

A reduction in blood volume and pressure causes increased release of renin and increased production of angiotensin 2 and aldosterone (renin-angiotensin2-aldosterone system – RAAS) resulting in sodium reabsorption.

In addition, angiotensin 2 acts directly in the kidneys where it causes constriction of the afferent and efferent arterioles, which reduces renal blood flow. The increased vascular resistance reduces the pressure in peritubular capillaries, which results in increased tubular reabsorption of salts and water. Concurrently, angiotensin 2 also stimulates the thirst centre and anti-diuretic hormone production in the hypothalamus, therefore contributing to restore blood volume (DiBartola, 2006). Furthermore, angiotensin 2 acts on all arterioles within the body, causing vasoconstriction, thereby increasing total peripheral resistance, counteracting any decrease in arterial blood pressure (DiBartola, 2006).

2.4.3.2 Peritubular capillaries

Within the renal peritubular capillaries, hydrostatic pressure is lower and protein osmotic pressure is higher than elsewhere within the body. The pressures in these capillaries are important to the proximal tubule reabsorption because the tight junctions between nephron epithelial cells exert only moderate amounts of resistance on the transport of water and ions. Thus, if hydrostatic pressure is elevated and protein osmotic pressure is reduced in the peritubular capillaries surrounding the proximal tubules, water and ions will move into the proximal tubules, increasing urinary excretion, as may occur during large amounts of water consumption. During water deprivation, the opposite effect ensues; arterial blood pressure is lower, extracellular fluid volume is reduced and hydrostatic pressure is lower, resulting in reduced renal excretion (DiBartola, 2006).

2.4.3.3 Atrial naturetic peptide

When blood volume is increased and the atria are distended, atrial naturetic peptide (ANP) is released into the blood. The effect of ANP is to inhibit the reabsorption of sodium and increase its excretion in urine (DiBartola, 2006). This homeostatic mechanism allows plasma sodium concentrations to be maintained.

Several studies have reported that urine sodium concentrations rise with water deprivation in response to increasing plasma osmolarity. McKinley et al. (1983) showed that water deprivation over

a two day period caused an increase in urinary sodium excretion in fed sheep. This observed natriuresis was also found by Michell and Moss (1995) where they restricted water intake in sheep fed a low, moderate and high sodium diet over a three day period. Dehydration natriuresis was detected in sheep fed the higher sodium diets, suggesting that this may serve a homeostatic function to buffer against hypernatremia. Jacob et al. (2006c) reported similar findings, with increasing urinary concentrations of sodium with increasing duration of water restriction up to 48 hours. It is important however that although natriuresis occurs as a result of water deprivation, total urinary excretion of sodium per day will decrease with total urinary volume as dehydration ensues (Parker et al., 2004).

2.4.4   Dehydration

Although sheep cope reasonably well with water deprivation, recent studies in Australian abattoirs have shown that a large percentage of lambs at slaughter experience subclinical dehydration. Jacob et al. (2006c) estimated the losses to be approximately AU$22.5 million annually through carcass yield losses alone and therefore dehydration warrants further investigation.

Dehydration due to total water loss is known as hypertonic dehydration. This type of dehydration occurs as a result of water restriction in ruminants (DiBartola, 2006) and results in total water loss and an increase in osmolarity and tonicity in both the intracellular and extracellular space. Water may be lost from the body through urine, faeces, sweat and respiration; however, most water excretion and loss is through the kidneys (DiBartola, 2006).

Precise quantification of dehydration status in animals can be difficult and requires both subjective and objective methods (Stockham and Scott, 2013). Clinically, a variety of methods are used to assess dehydration; however, not all indicators used to measure dehydration are practical for use in an abattoir environment.

2.4.4.1   Physical signs of dehydration

Small deficits in percentage body water loss (1-4% body weight) do not normally result in clinical detection of dehydration, but as the volume of body water lost increases, so do the clinical signs associated with dehydration (Thrall et al., 2012). Clinical signs that are used to detect dehydration and percentage of body water lost result from changes in interstitial fluid volume changes; these include eye position (enopthalmus) or skin elasticity (skin tenting). Decreased peripheral blood flow leads to injected capillaries and increased capillary refill time (Thrall et al., 2012).

2.4.4.2   Urine volume and specific gravity

Urine output and urine specific gravity (USG) are other methods of assessing hydration status. In a study by Weeth et al. (1967), of water deprivation in Hereford heifers, urine weight was unchanged on the first day of water deprivation, but had decreased by 33% on the second day and by 72% on day four, representing a water conservation of approximately 5.6kg of water. In this study, urine osmotic pressure was unaffected by water deprivation until the third day off water. Osmotic pressure in the water deprived treatment group was 1196mOsm/kg compared to 780mOsm/kg in the control group on the same day, with a 53% increase in urine osmolarity. This finding was similar to that of an earlier study (Weeth and Lesperance, 1965) indicating that cattle are not able to excrete high osmolal urine compared to sheep (Macfarlane et al., 1961b).

Jacob et al. (2006c) assessed the hydration status of different consignments of Australian lambs at two commercial abattoirs over a 12 month period and found that average urine specific gravity was

higher than expected compared to values determined from previous studies in lambs (Jacob et al., 2006b). For sucker lambs the average urine specific gravity (USG) was 1.041 ± 0.002 compared with 1.037 ± 0.001 for carry over lamb consignments. Furthermore, it was found that there was a positive correlation between duration of on farm curfew and average urine specific gravity per consignment. These values are similar to those reported in yearling cattle, in which water deprivation for 36 hours resulted in an increase in USG from 1.015 to 1.032 (McLennan, 2005).

2.4.4.3 Packed cell volume

Packed cell volume is common objective measurement used in combination with total protein to measure the hydration status in animals. Increases in packed cell volume can also occur as a result of erythrocytes being released due to sympatho-adrenal stimulation due to stress and splenic contraction, which can also occur at exsanguination (Knowles et al., 1995), therefore limiting its use in abattoir environments.

2.4.4.4 Plasma sodium and total protein

Hypernatremia and hyperproteinemia are an indication of a relative water deficit and increase in plasma osmolarity caused by dehydration. Early work by Macfarlane et al. (1961a) showed a 5% increase in plasma sodium from 142-147mmol/L to 152-156mmol/L over a four day period of water deprivation. Jacob et al. (2006b) found plasma sodium concentrations increased with feed deprivation time in lambs with no access to water, but stayed constant when there was access to water. Thus sodium may be useful in quantifying the dehydration status in fasted lambs and therefore useful in an abattoir environment at slaughter. In the lambs deprived of water for 48 hours, plasma sodium concentrations exceeded normal ranges of 145-152mmol/L (Radostits et al., 2007), 139-152mmol/L (Kaneko et al., 2008) and 142-152 (Lepherd et al., 2009). Similarly, Parker et al. (2003) found that duration of water restriction had a significant impact on plasma sodium concentrations. Sheep deprived of water for 72 hours had significantly increased plasma sodium concentrations between 24 and 48 hours and overall higher plasma sodium concentrations compared to that in sheep that had access to water. In Bos indicus steers deprived of water for up to 90 hours, sodium concentrations were higher compared to animals with access to water; however, although this was statistically significant, changes were small and still within normal published reference ranges (Parker et al., 2004).

In ruminants total plasma protein concentration above 8g/dL can be expected with dehydration (Radostits et al., 2007). Knowles et al. (1996) attributed dehydration as a contributing factor to elevated total protein levels of approximately 7.6g/dL in lambs transported for 24 hours, which declined after lambs had access to water in lairage. In cattle, overnight lairage has been associated with increases in plasma total protein concentration for both cattle directly consigned from farm (7.6 ± 0.1g/dL to 7.9 ± 0.3g/dL) or through saleyards (7.8 ± 0.9 g/dL to 8.2 ± 0.8 dL) (Jarvis et al., 1996b), although there was no effect on plasma osmolarity. A study assessing the effects of water restriction in Awassi sheep showed that total protein concentrations were higher at four days of water restriction (80.26g/L) compared to two days (73.89g/L) of water restriction (Jaber et al., 2004) indicating reduced blood volume.

Seasonality is also likely to affect hydration status of animals, particularly during transport and other periods of water restriction. In a study assessing long distance road transport (19 hours) in cattle it was shown that total protein were highest during summer (61.7 ± 0.67g/L) compared to in winter (58.8 ± 0.67g/L), however no differences were reported for plasma sodium levels (Bernardini et al., 2012). Sheep may be more resilient to the effects of season as wool acts as an excellent insulator and prevents heat transference to the skin, minimising the panting required to maintain normal core body temperature and reducing evaporative water losses (Macfarlane et al., 1958).

2.4.5   Impact of dehydration on meat quality and yield

Water deprivation has been shown to impact on meat colour attributes in lamb. Jacob et al. (2006b) found that water deprivation for 48 hours caused significant changes in the lightness (L*) but no changes in the hue (type) or chroma (intensity) of meat colour. Dehydration has been shown to cause muscle shrinkage, demonstrated by reduced muscle diameters (Jacob et al., 2006b). Therefore, this may result in a tighter myofibrillar framework, which would result in less penetration and more reflectance of light, thus a higher L* value. This appears in agreement with Hopkins et al. (2006b) who observed that M. *semimembranous* (topside) weight and water content declined after 48 hours of water deprivation, with muscle becoming darker in colour.

While there has been some evidence that dehydration has a negative impact on meat quality, there appears to be little effect on eating quality attributes and in some cases may even improve the eating experience for the consumer. Jacob et al. (2006a) investigated the impact of dehydration on lamb eating quality. In this study it was found that a large proportion of lambs had high USG values (> 1.045) but found no differences in tenderness, juiciness, flavour and overall liking in the M. *longissimus lumborum* and M. *biceps femoris* of lambs with low and high USG. However, they did find that the number of consumers who rated the loin as unsatisfactory was higher for the low USG (less dehydrated) lambs. Similarly, Warner et al. (2002) found that water deprivation may actually improve tenderness of lamb meat.

Water deprivation has been shown to decrease live weight, carcass weight and lean meat yield (Jacob et al., 2006b; Greenwood et al., 2008). Jacob et al. (2006b) found that water deprivation increased muscle dry matter percentage and increased muscle supernatant osmolality as well as reduced muscle weights and muscle fibre cross sectional area, cooking loss and drip loss. In support of this, Gregory et al. (2000) found that urinary sodium was negatively correlated with meat stickiness in the M longissimus lumborum muscle of beef cattle, suggesting that animals were actively retaining sodium in order to conserve body water being drawn from muscle, affecting the physical properties of the muscle.

The effect of water deprivation may not be consistent across all muscles within a carcass. Jacob et al. (2006b) found that the M. *semitendinosus* (ST) had a lower dry matter percentage than the M. *semimembranosus* (SM) and the M. *longissimus lumborum* (LL), which may be due to type IIB fibres being more common in the ST than the SM or LL (Briand et al., 1981). Type IIB fibres within the LL were also larger in cross sectional area and may have contained more water than Type I or Type IIA fibre types (Jacob et al., 2006c). The effects on carcass weight and dressing percentage are less evident. This may be because the changes in muscle weight may have been relatively small in comparison to the weight of a full carcass, which also includes bone and adipose tissue (Jacob et al., 2006b), thus making the impact of dehydration on carcass yield difficult to determine.

2.5   General Aims and Hypotheses

Limited research has been conducted in prime lambs under Australian commercial conditions that has measured physiological and metabolic indicators of acute and chronic stress and assessed the impact on meat quality and carcass yield. Identifying physiological indicators in plasma that may be used to quantify acute and chronic stress at exsanguination and the associated impact of production and environmental factors may assist to make inferences regarding pre-slaughter management. Minimising stress during the pre-slaughter period is essential not only from an animal welfare point of view but also as it is established that stress is a cause of poor meat quality including dark cutting (Tarrant, 1989) and tenderness (Warner et al., 2007; Gruber et al., 2010). Chronic stress as a result of feed and water deprivation also negatively affects carcass yields (Thompson et al., 1987; Jacob et al.,

2006b). The challenge in measuring and evaluating pre-slaughter stress is that mechanisms underpinning both acute and chronic stress overlap and often confound one another.

This thesis examines the influence of acute and chronic stress on lamb meat quality and carcass yield. Utilising plasma indicators that reflect acute stress, muscle injury and feed and water deprivation, this thesis will assess whether indicators can relate to ultimate pH, shear force and carcass yield, which are investigated in the following chapters. The hypotheses for each chapter are outlined below.

Chapter 4: Levels of plasma indicators of acute and chronic stress at slaughter in Australian lamb

The objective of this chapter was to quantify levels of plasma indicators reflecting acute stress, muscle damage, feed deprivation and dehydration in lambs at slaughter. In addition, it describes the association of production and genetic factors such as site of production, kill group, birth year, sire type, and sex and dam age on plasma indicators of acute and chronic stress. The specific hypotheses tested were:

- Significant variation in plasma indicators will be present between sites and kill groups.

- Merino breed types will have a higher acute stress response than Terminal and Maternal breed types.

- Physiological indicators of dehydration, feed deprivation and acute stress will be higher in lambs at slaughter than basal levels reported in the literature.

- Magnesium levels and indicators of muscle damage in finished lambs, slaughtered under best practice management, will be within normal physiological range.

- Plasma indicators at slaughter will be related to carcass weight and carcass composition such as muscling and fatness.

Chapter 5: Association between indicators of acute and chronic stress and ultimate pH in Australian lamb

The objective of this study is to assess the association between plasma indicators reflecting acute and chronic stress and lamb ultimate pH. Several hypotheses were tested;

- Indicators of acute stress including plasma lactate, glucose, cortisol, CK and AST concentrations at slaughter will be positively associated with an increased ultimate pH at 24 hours post-slaughter.

- Feed deprivation reflected by increased NEFA and BHOB levels, and water deprivation reflected by increased sodium and total protein, will not be associated with ultimate pH.

- Increasing plasma magnesium concentration at slaughter will be associated with a decrease in ultimate pH.

Chapter 6: Lamb loin tenderness is not associated with plasma indicators of acute and chronic stress

The objective of this study was to examine if an association exists between lamb tenderness, as measured by Warner Bratzler Shear Force (WBSF) and plasma stress indicators. In addition, it was examined how selection for increased yield through the use of Australian Sheep Estimated breeding values (ASBVs) drives WBSF in lamb and whether it underpins the link between stress and tenderness. Several hypotheses were tested:

- Increasing plasma lactate, glucose, NEFA, cortisol, CK, AST, sodium, total protein and haptoglobin concentrations at slaughter will be associated with increased WBSF values in lamb loin.

- Increased plasma magnesium concentrations at slaughter will be associated with a reduction in loin WBSF.

Chapter 7: The metabolic response to feed deprivation in lambs genetically selected for high and low carcase yield characteristics

The objective of this chapter was to assess how selection for increased carcass yield through the use of ASBVs for post-weaning weight (PWT), post weaning fat depth (PFAT) and post weaning eye muscle (PEMD) influence the metabolic response to feed deprivation under resting conditions. Several hypotheses were tested:

- Terminal sired lambs will have decreased plasma glucose and increased plasma NEFA and BHOB concentrations in response to feed deprivation compared to Merino sired lambs.

- The metabolic changes due to feed deprivation would be greater in progeny of sires with greater

  growth (PWT), muscling (PEMD) and leanness (PFAT) breeding values.

Chapter 8: The metabolic response to commercial feed deprivation and the impact on lamb carcass yield and meat quality

The objective of this chapter was to build on the results from Chapter 7 and examine the metabolic response to feed deprivation under commercial conditions in lambs selected for low and high carcass yield. In addition, the impact of commercial feed deprivation on lamb meat quality and yield was be examined. Several hypotheses were tested:

- Under commercial feed deprivation and slaughter conditions, Terminal breed types will

  demonstrate higher NEFA and BHOB concentrations than Merinos due to greater adipose tissue

  responsiveness to acute stress (adrenaline). However, these effects will diminish under

  conditions of extended feed deprivation due to the greater metabolic response of Merinos.

- The progeny of sires with increased PEMD breeding values will have greater NEFA and BHOB at

  slaughter due to their greater adipose response to acute stress (adrenaline).

- Alternatively, the progeny of sires with lower PWT and PFAT breeding values will have higher

  NEFA and BHOB due to their greater response to feed deprivation and these differences will

  increase with the duration of feed deprivation.

Chapter 3 General materials and methods

3.1 Animals

The design of Meat and Livestock Australia Genetic Resource flock has been described previously (Fogarty et al., 2007; Van der Werf et al., 2010). Wether and female lambs (n = 2877, Table 3-1) were produced from artificial insemination of Merino, Border Leicester x Merino (BLM) and Commercial Maternal (CM) dams over a two year period (2013 and 2014) at the Katanning, Western Australia and Kirby, New South Wales research sites. The lambs were the progeny of 394 different sires, which comprised Terminal sire types (Ile De France, Poll Dorset, Suffolk, Texel, Charolais and White Suffolk), Maternal sire types (Booroola, Border Leicester, Coopworth, Dohne Merino and Prime SAMM) and Merino (Merino and Poll Merino) sires, representing the major production types in the Australian sheep industry. These sires were chosen to represent the full range of Australian Sheep Breeding Values (ASBVs) for key traits within each sire type. Semen from all three sire types was used to artificially inseminate Merino dams, while only semen from Maternal and Terminal sires was used to inseminate cross-bred (BLM and CM) ewes. Maternal lambs sent to slaughter comprised very few females (which were retained for breeding purposes), meaning effective comparisons between sexes could only be made within the Terminal and Merino sired lamb groups and Maternal sired lambs from Merino dams. The lambs were maintained on extensive pasture grazing, with grain, hay or feedlot pellets supplemented when pasture supply was limited.

For each site, lambs were assigned to smaller kill groups (n = 17) of between 48 and 307 lambs to be killed on the same day to enable carcass weight targets to be achieved. Given selection for slaughter was made based on weights, the average age of lambs in each kill group varied between 193 and 416 days old at slaughter, however within individual kill groups the age range was smaller, varying as little as 16 days and by up to 33 days of age.

Prior to slaughter, lambs were mustered, yarded, and taken off feed and water (from 2.5 to 18 hours) allowing lambs to reduce gut fill prior to being weighed to predict dressing percentage. Lambs were then transported to one of three commercial abattoirs, with transport times ranging from 0.5 hours (Katanning site) and 1.5 to 2.5 hours (Kirby site). Lambs were held overnight in lairage, with access to water, but no feed (15 – 26 hours) and slaughtered the following day after electrical stunning.

The use of lambs utilised in this thesis was approved by the Department of Agriculture Western Australia Animal Ethics Committee #2-13-07.

3.2 Blood sample collection and processing

At slaughter, lamb ear tag identification was recorded in order of lambs slaughtered (kill order).

Blood samples were collected from each lamb at slaughter, immediately following exsanguination. Blood samples were collected into 9mL lithium heparin Vacuette® tubes (Greiner bio-one, Austria) which were then capped and inverted gently several times. Tubes were immediately placed in ice for approximately 2-5 hours (due to distance between processing plant and laboratory) until centrifugation.

Blood samples were centrifuged using an Eppendorf 5702 centrifuge (Eppendorf, Germany) at 3000rpm for 15 minutes in a cold room at 3°C (Katanning site, 2013, 2014) or 3000rpm at 4°C using a Beckman J2-21M refrigerated centrifuge (Beckman, USA) at 2750rpm at 0°C for 15 minutes using an Eppendorf 5810R centrifuge (Eppendorf, Germany) (Kirby site 2013, 2014).

Following centrifugation, plasma samples were pipetted in two separate aliquots and stored in 2mL micro tubes at -80°C until processing. Prior to plasma analysis, frozen plasma samples were allowed to thaw at room temperature. This was carefully monitored to ensure that samples remained cool. If sample analysis was delayed, samples were kept refrigerated until analysis (< 2 hours). Once samples were thawed, each sample was gently inverted several times before a 100µL sample was pipetted into 1.7mL sample cups (Ref # 729101, Greiner Bio-one, Kremsmüster, Austria) for analysis on an Olympus AU400 automated chemistry analyser (Olympus Optical Co. Ltd, Melville, NY).

Plasma glucose, lactate, NEFA, Magnesium, CK, AST and total protein (TP) were analysed on aliquot 1. Samples were frozen and then thawed a second time for sodium determination. Cortisol was analysed on aliquot 2. The subset samples were re-frozen and then analysed for haptoglobin and BHOB. Otherwise subset samples only had 1 freeze thaw cycle.

Table 3-1 Number of lambs from which blood samples were collected at each site, within each year, sex, dam breed and sire type.

Site	Year		Sex Dam breed (Sire type)											
	2013	2014	F Merino (Mat.)	M Merino (Mat.)	M CM (Mat.)	M BLM (Mat.)	F Merino (Mer.)	M Merino (Mer.)	M Mer. (Ter.)	F Mer. (Ter.)	M CM (Ter.)	F CM (Ter.)	M BLM (Ter.)	F BLM (Ter.)
Kirby	1128	721	**37**	98	n/a	43	80	194	273	268	n/a	n/a	311	354
Katanning	524	504	2	118	17	5	34	199	156	215	78	109	8	19
Total	1652	1225	39	216	17	48	114	393	429	483	78	109	319	373

F: female lamb; M: male (wether) lamb
CM: Commercial Maternal dam breed; BLM: Border Leicester x Merino dam breed.
(Mat.): Maternal sire type; (Mer.): Merino sire type; (Ter.): Terminal sire type. n/a: not applicable

3.3   Plasma indicator measurements

Laboratory analyses of plasma were carried out as batch samples using the Olympus AU400 automated chemistry analyser (Olympus Optical Co. Ltd, Melville, NY) .

Plasma lactate, glucose, non-esterified fatty acid (NEFA), magnesium, creatine kinase (CK), aspartate aminotransferase (AST), total protein (TP) and Sodium (Na) were analysed in our laboratory at Murdoch University, Perth, WA. Plasma haptoglobin and BHOB were analysed by the Western Australian Department of Agriculture (DAFWA) Animal Health Laboratories. Plasma cortisol was analysed by Vetpath Veterinary Laboratories, Perth, WA. For analysis of each plasma indicator using commercial kits, the correlating control and calibration sera was used.

3.3.1   Lactate

The plasma L-Lac was analysed with a reagent kit (Olympus Diagnostics, Tokyo, Japan, Cat. No. OSR6193) which used an enzymatic method (Trinder, 1969; Barham and Trinder, 1972).

3.3.2   Glucose

Glucose analysis was carried out using the glucose reagent kit (Olympus Diagnostics, Tokyo, Japan, Cat. No. OSR6121) which utilises the enzymatic method of Barthelmai and Czok (1962).

3.3.3   Non-esterified fatty acid (NEFA)

Plasma NEFA concentration was analysed using a NEFA-C kit (C Kit Wako Pure Chemical Ind., Osaka, Japan; modified for the Olympus AU400 Automated Chemistry Analyser) which uses an enzymatic method based on the protocol of Itaya and Ui (1965) and Duncombe (1964).

3.3.4   β-hydroxybutyrate (BHOB)

The commercial kit used to analyse β-hydroxybutyrate (Randox Laboratories kit, County Antrim, UK, Ranbut, Cat. No. RB1007) used the automatic kinetic method described by McMurray et al. (1984).

3.3.5   Cortisol

Plasma cortisol levels were determined using chemiluminescent immunoassay performed on a subset of 500 samples using an Immulite® 2000 Immunoassay system (Siemens, Germany).

3.3.6   Magnesium

Magnesium analysis was carried out using the magnesium reagent kit (Olympus diagnostics, Tokyo, Japan, Cat. No. OSR6189) which used the spectrophotometric method (Mann and Yoe, 1956).

3.3.7 Sodium

Plasma sodium was determined using the commercial sodium kit (Randox Laboratories kit, County Antrim, UK, Cat. No. NA7167) which used the enzymatic method described by Berry et al. (1988)

3.3.8 Total Protein

Total protein was determined using a commercial reagent kit (Olympus diagnostics, Tokyo, Japan, Cat. No. OSR6132) by a photometric colour biuret test (Weichselbaum, 1946).

3.3.9 Creatine kinase and aspartate aminotransferase

Plasma creatine kinase and aspartate aminotransferase were determined using commercial kit (Olympus diagnostics, Tokyo, Japan, Cat. No. OSR6179, Cat. No. OSR6109) which utilises the International Federation of Clinical Chemistry approved catalytic method (Hørder et al., 1991).

3.3.10 Haptoglobin

Plasma haptoglobin was determined using the method described by Eckersall et al. (1999).

3.4 Carcass processing

Following slaughter, lambs were dressed according to AUS-MEAT standards and hot carcass weight (HCWT) was recorded. The average HCWT across all lambs was 23.2 kg (Std Dev = 2.94). All carcasses underwent medium voltage electrical stimulation to optimise pH and temperature decline to achieve a carcass loin temperature at pH6 between 18 - 25°C (Pearce et al., 2010), in order to prevent cold shortening. Carcasses were chilled overnight (3-4°C) before sampling.

3.4.1 Assessment of pH decline and ultimate pH

Standardised protocols have been developed by the Sheep CRC to determine pH decline and ultimate pH (Pearce, 2009; Pearce et al., 2010). Muscle pH was measured with an Orion 250A pH meter (cat. no. 0250A2, Orion Research Inc., Boston, MA USA) using a glass body, spear tipped probe (cat. no. 8163BN, Orion Research). Muscle temperature was measured using a stainless steel cylindrical probe attached to the pH meter. The pH meter was regularly calibrated using buffers with a known pH of 4 and 7. pH was measured on the left caudal section of the M. l*ongissimus lumborum* (loin) muscle at the lumbosacral junction, where a small 4cm incision was made to expose the caudal end of the loin muscle. A single pH measurement was collected from each carcass, taken as close as possible to target carcass temperatures of 35°C, 20°C and 12°C degrees. This data was then used to calculate the temperature at pH6 and pH at 18°C (pH18) to indicate whether the carcass meets the pH-temperature window. The rate of pH decline was assessed using pH18 (Pearce, 2009). The last pH reading was taken at 24 hours post slaughter for determination of ultimate pH (pH24LL).

From the carcass saddle region, the left short loin (AUS-MEAT 4480) (AUS-MEAT, 2005) up to the 12th rib was removed. From this, the M. *longissimus lumborum* was prepared by removing sub-cutaneous fat and connective tissue (epimysium) to produce the eye of shortloin (AUS-MEAT 5150).

3.4.2   Objective assessment of tenderness

Shear force was used to objectively describe tenderness. Approximately 65g sample of muscle was removed from the cranial aspect of the loin. The samples were vacuum-packed and chilled for 5 days at -1°C prior to freezing at -20°C until subsequent testing. Frozen samples were cooked in plastic bags in a water bath for 35 minutes at 71°C and cooled in running water for 30 minutes after cooking. Six core slices (approximately 3- 4cm long, 1cm^2 thick) from each loin sample were cut and Warner-Bratzler shear force (WBSF) was measured on each core slice using a Lloyd texture analyser (Model LRX, Lloyd Instruments, Hampshire, UK) with a Warner–Bratzler shear blade (Hopkins et al., 2010). Shear force was expressed in Newtons (N). Laboratory processing of loin samples and measurement of WBSF was performed at the University of New England Meat Science Department (Armidale, New South Wales, Australia).

3.5   General statistical analysis

Dependent variables were analysed using linear mixed effect models in SAS (SAS Version 9.1, SAS Institute, Cary, NC, USA). The base model included fixed effects for site, year, kill group within site by year, sire type, flock by year, sire type by flock, sire type by year, sire type by year by flock, sex by dam breed within sire type, age of dam and birth type. Sire identification and dam identification by year were included as random terms.

For analysis using the subset of animals with cortisol data, the base model included site (as above), kill group within site, sex and dam breed within sire type (as above), age of dam and birth type. Sire identification was included as a random effect.

When appropriate, covariates were included in the core model as a covariate along with first order and quadratic interactions. In all models, non-significant (*P*>0.05) terms were removed in a stepwise manner.

The following sections 3.6 and 3.7 outline experiments that were performed to validate blood collection and storage methodologies in Chapter 7.

3.6   Effect of sampling method and storage conditions on non-esterified fatty acid (NEFA) concentrations in sheep plasma

3.6.1   Introduction

In studies assessing stress sensitivity, plasma NEFA may be measured as an indicator of stress response (Martin et al., 2011). This applies for studies investigating pre-slaughter stress metabolism, when blood sampling is often carried out in the immediate post-mortem bleeding period. This sampling can be undertaken quite rapidly, as abattoir chain-speeds exceed 8 lambs per minute and the bleed-out time is within 8 seconds. However, storage of the samples may not always occur instantaneously, and this could influence analyte concentrations to be measured. For example, plasma NEFA concentrations have been shown to increase over storage time, particularly at higher temperatures (Stokol and Nydam, 2005).

The sample storage procedure itself may also impact blood analyte concentrations, with previous studies showing that NEFA concentrations are higher in blood samples collected in lithium heparin

(LH) tubes (Stokol and Nydam, 2005) compared to Potassium ethylenediaminetetraacetic acid (k-EDTA) at higher temperatures (McGann and Hodson, 1991). Thus, we expect that NEFA concentrations will be higher in plasma collected in LH tubes compared to k-EDTA tubes at both room temperature and under chilled conditions. Additionally we expect that NEFA concentrations will increase with storage time.

3.6.2   Materials and methods

3.6.2.1   Animals, experimental design and sample processing

Three sheep of mixed breed and age were used for this experiment. Sheep were housed in a shearing shed for one day prior to undertaking the experiment, fed ad lib chaff with free access to water overnight.

Two blood samples were collected from each lamb via jugular venepuncture into 8mL Lithium heparin Vacuette® tubes (Greiner bio-one, Austria) or potassium EDTA Vacuette® tubes (Greiner bio-one, Austria). Care was taken to minimise stress during blood collection. Samples were immediately placed on ice and stored under these conditions for 5 hours. This was performed to replicate sample handling performed at the abattoir.

Blood samples were centrifuged at 3000rpm for 15 minutes in a cold room (2°C). For samples to be stored under chilled conditions, plasma was pipetted into three 2mL tubes per EDTA and LH treatment and stored at 3.1°C. The remaining plasma was left in either the K-EDTA or LH tubes and stored at room temperature (23.5°C) before pipetting aliquots into separate tubes at time 0, 1 and 2 hours. Following pipetting at each time point, k-EDTA and LH plasma samples were stored at -80°C until processing. Samples were thawed and kept chilled prior to NEFA analysis.

Plasma NEFA concentration was analysed using a NEFA-C kit (C Kit Wako Pure Chemical Ind., Osaka, Japan; modified for the Olympus AU400 Automated Chemistry Analyser which uses an enzymatic method based on the protocol of Itaya and Ui (1965) and Duncombe (1964).

3.6.2.2   Statistical analysis

NEFA concentration data were analysed in SAS using linear mixed effect models. Fixed effects included sampling time, anticoagulant within temperature treatment and sampling time by anticoagulant within temperature treatment. Animal identification was included as a random term in the model.

3.6.3   Results

NEFA concentration varied between anticoagulants within a temperature treatment ((P<0.05; Table 3-2). In chilled samples NEFA concentration in k-EDTA tubes (0.077 ± 0.0012 mmol/L) was 7.8 % higher (P<0.05) than LH tubes (0.072 ± 0.0012 mmol/L). At room temperature NEFA concentrations in k-EDTA tubes (0.077 ± 0.0012 mmol/L) were 8.3% higher (P<0.05) compared to that in LH tubes 0.071 ± 0.0012 mmol/L). There was no difference (P>0.05) in NEFA concentrations between chilled or room temperature k-EDTA plasma samples or chilled and room temperature LH plasma samples.

Storage time had an impact on NEFA concentration; however this was only evident within LH tubes stored at room temperature (P<0.05, Table 3-3). NEFA increased (P<0.05) by 10% from 0.068

mmol/L at time 0 hours to 0.075mmo/L at time 2 hours. NEFA concentration did not change in chilled LH tubes or in EDTA tubes kept chilled or at room temperature.

Table 3-2 F values, P values and numerator and demoninator (NDF, DDF) of freedom for the effects of the base model for plasma non-esterified fatty acid (NEFA) concentrations.

Effect	NDF, DDF	F Value
Time	2, 22	2.79ns
Anticoagulant (storage temperature)	3, 22	19.35**
Time*anticoagulant (storage temperature)	6, 22	3.12*

* $P<0.05$; **$P<0.01$, ns: not significant ($P>0.05$)

Table 3-3 Predicted means ± standard error for non-esterified fatty acid (NEFA) concentrations (mmol/L) collected with Potassium ethylenediaminetetraacetic (k-EDTA) and lithium heparin (LH) anticoagulant stored at room and chilled temperatures for 0, 1 and 2 hours.

Anticoagulant (storage temperature)		Time		
		0h	1h	2h
k-EDTA	Chilled	0.079 ± 0.0016[e]	0.076 ± 0.0016[e]	0.077 ± 0.0016[e]
LH	Chilled	0.071 ± 0.0016[ab]	0.072 ± 0.0016[abc]	0.072 ± 0.0016[bcd]
k-EDTA	Room	0.075 ± 0.0016[cde]	0.077 ± 0.0016[e]	0.077 ± 0.0016[e]
LH	Room	0.068 ± 0.0016[a]	0.069 ± 0.0016[ab]	0.075 ± 0.0016[cde]

[a,b,c] Letters that differ between columns and rows are significantly different ($P<0.05$).

3.6.4   Discussion

Contrary to the hypothesis, NEFA concentration was lower in plasma collected in LH tubes compared to k-EDTA tubes, however this difference was small and unlikely to be of clinical significance. Previous work by Morris et al. (2002), found that there was no difference in NEFA concentrations between heparin and EDTA tubes. This may be because lipoprotein lipase is normally bound to capillary endothelial cells within tissues and only becomes blood-borne upon intravenous heparin administration (Yang et al., 1999). Thus, as there is no heparin entering the blood supply, plasma NEFA levels are not affected.

In partial support of our hypothesis, NEFA was higher in samples stored at room temperature; however this was only evident from plasma collected in LH tubes and higher at 2 hours of storage. Previous authors also show that NEFA concentration significantly increases by 22% with increasing storage time at 1 hour (Sampson and Hensley, 1975), 6 hours (Rogiers, 1978) and up to 100% at 24 hours (Howorth et al., 1966) when stored at room temperature. Increases in NEFA concentration in heparin tubes at higher temperatures may be due to lipase catalysed hydrolysis of esterified fats (McGann and Hodson, 1991)

There was no difference in NEFA concentration over time in either LH or EDTA plasma. This is in contrast to studies in sheep where NEFA concentration increased significantly over a 72 hour period when stored at 4°C Morris et al. (2002). However, Stokol and Nydam (2005) found that NEFA concentrations did not change over time up to 72 hours when stored at 4°C. However when stored at 24°C, NEFA increased sequentially over time up to 72 hours and this effect was greater in lithium heparin tubes compared to EDTA tubes. Within this study, storage times were significantly shorter than reported by Stokol and Nydam (2005), highlighting the importance of minimising storage time and keeping samples cool when analysing NEFA concentrations in plasma. The results of this study indicate that LH tubes are suitable for blood collection and determination of plasma NEFA concentrations in sheep. However, it is essential that temperature and sampling methods are optimised when analysing blood NEFA concentrations. This will ensure that NEFA concentrations will remain stable between sampling and analysis.

3.7 Administration of low and high doses of heparin causes changes in plasma non-esterified fatty acid (NEFA) concentration

3.7.1 Introduction

The anticoagulant properties of heparin have led to the routine use of heparin or heparinised saline flushes to prevent thrombus formation and prolong the patency of indwelling cannulas (Randolph et al., 1998). Intravenous administration of heparin results in the rapid appearance of two groups of triacylglycerol (TAG) lipases in plasma, including lipoprotein lipase (LPL). Lipolytic enzymes are released from the luminal surface of vascular endothelial cells from a number of tissues and cause hydrolysis of plasma lipoprotein TAG and the uptake of free fatty acids (FFA) for utilisation or storage (Tume et al., 1988; Kaneko et al., 2008). Much of the research in this area has centred around the risk of potential drug binding displacement as NEFA competes for binding sites on albumin (Desmond et al., 1980).

However, limited work exists on very low dose heparin used to retain cannulae patency for the purpose of repeated blood sampling for metabolic studies. Of particular interest is whether very low dose heparin will cause artificial increases in plasma NEFA concentration. This is most relevant in metabolic studies involving repeated sampling, as this may erroneously elevate the apparent plasma NEFA concentrations.

Desmond et al. (1980) showed in human patients that following administration of heparin at a dose rate of 100IU, FFA increased two fold within 10 minutes. Work in sheep has shown that bolus intravenous administration of heparin at 100IU/kg LW increased lipoprotein lipase activity and plasma free fatty acid concentrations from 0.04 to 0.12mmol/L, which returned to near pre-treatment levels within 60 minutes (Tume et al., 1988). The effect on maximum NEFA concentration also appears to be dose dependent, with greater NEFA responses seen with higher doses. Increasing heparin dose from 0.4IU/kg to 20IU/kg has also been shown to significantly increase the maximum FFA response in human subjects as well as result in a longer time to return to pre-treatment levels from approximately 30 to 120 minutes (Jaume et al., 1996).

Alternatively, in sheep Seoane et al. (1972) found that heparin administration at 250IU/kg had no impact on NEFA concentration 15 minutes after heparin administration, suggesting that even high doses of heparin do not affect blood NEFA concentration. Although fully fed ruminants have high levels of lipoprotein lipase activity, which increases on heparin administration, ruminants have very low levels of circulating low density lipoproteins (VLDL) and chylomicrons, the major transporters of

TAG in plasma (Tume et al., 1988). As such, heparin may not be particularly powerful in elevating NEFA in ruminants.

Whole body adiposity has also been shown to have an impact on LPL activity. Expression of the LPL gene in sheep is highly correlated with the size of adipocytes (Barber et al., 2000) and work in lambs has shown that increased adipose tissue deposition increases LPL activity (Haugebak et al., 1974). Given that Terminal breed types are selected for increased muscling and leanness compared to Merino bred lambs, resulting in a phenotype with lower proportion of adipose tissue and a higher percentage of lean (Anderson et al., 2015), it could be expected that they will have a lower NEFA response to heparin administration. Likewise, female lambs are proportionally fatter than male lambs (Anderson et al., 2015) and therefore may also higher LPL activity and thus a higher NEFA response to heparin administration.

This study tested the hypotheses that heparin administration at low doses of 250IU will not cause a change in plasma NEFA concentration. However, at high dose administration of 1000 IU, it is expected that heparin will induce an increase in plasma NEFA concentrations. In addition, we expect female lambs and Terminal sired lambs to have a lower overall NEFA response to heparin administration compared to male lambs and Merino sired lambs. Furthermore, we expect that NEFA levels will return to basal concentrations within 60 minutes of administration of heparin.

3.7.2 Materials and Methods

3.7.2.1 Animals and experimental design

Fourteen Merino (n = 7) and Terminal (n = 7) sired lambs, balanced for sex within sire type were selected from the Katanning, Western Australia site of the Meat and Livestock Australia genetic resource flock. Lambs had previously been used in a feed deprivation trial (group 3, Chapter 7) with a 2.5 day rest between experiments. Lambs were housed in individual pens and fed a commercial complete pelleted diet (EasyOne®, Milne Feeds™, Analysis on dry matter basis: Crude Protein (min) 14.5%, Metabolisable Energy 11.0 MJ/Kg, Crude Fibre (max) 20.0%, Urea (max) 1.8%, Vitamin E 60mg/kg, Selenium 300µg/kg, Lasalocid Acid 37.0ppm) once a day at 4% live weight per day to which they had been previously acclimatised. During the heparin challenges, lambs were fed a quarter of their total daily feed at 7.30am, another quarter at 11.30am and the remaining half of their daily feed requirements at 4pm.

3.7.2.1.1 Cannulation method

In-dwelling cannulas were placed 4 days prior to commencement of study. Prior to cannula placement, the neck region was clipped to expose both jugular veins. The lamb was quietly restrained by an experienced handler in a relaxed standing position. The jugular groove was cleaned and sterilised using chlorhexidine scrub and 70% ethanol solution. 1mL of lignocaine was injected subcutaneously around the jugular vein. A 14 gauge, 1.5 inch needle (Monoject™) was then used to puncture the skin into the jugular vein (towards the heart) and sterilised polythene tubing (Microtube extrusions Pty Ltd, Eastwood, New South Wales; internal diameter = 1.00mm, outer diameter = 1.50mm) was then inserted to through the needle into the vein to 20cm. The tubing was then flushed using heparinised saline (17IU/mL) and a three way tap was attached. The needle was then removed and the tubing secured in place using superglue to the skin adjacent to the vein. The cannula was then flushed again. To protect the cannula entry site, Elastoplast was wrapped around the neck.

To maintain cannula patency, cannulae were flushed with 1.8mg/mL EDTA saline during blood sampling and heparinised saline (17IU) after the last blood sample for the day. Cannulas were removed 1 day after completing experimental work.

3.7.2.1.2 Heparin and Saline challenges

Each lamb was subjected to 3 treatments; low heparin (0.25mL, 250IU), high heparin (1mL, 1000IU) or control (1mL of 0.9% NaCl saline). Each challenge was randomly allocated over 1.5 days, with all 14 lambs being challenged the afternoon of day 1 and the morning and afternoon of day 2.

3.7.2.1.3 Blood sampling procedure

Blood samples were collected at the following time points; -30, -15, -10, -5, 0, 2.5, 5, 10, 15, 20, 30, 45, 60, 90, 120, 125 and 130 minutes relative to the administration of the challenge, where time 0 is the treatment administration. Prior to blood sample collection, 2mL of blood was withdrawn and discarded. If the cannula was blocked, it was flushed with 1mL of EDTA saline and the process repeated. Blood samples were collected into S-Monovette Vacutainer® tubes (Sarstedt Australia Pty. Ltd, SA, Australia). Post sampling, cannulae were flushed with 5-10mL of EDTA saline. Blood tubes were immediately placed on ice. Within 30 minutes of sample collection, blood samples were centrifuged at 4°C for 15 minutes at 3000rpm. Plasma samples were pipetted into 2mL tubes and then stored at -80°C.

3.7.2.2 **Plasma sample analysis**

Frozen plasma samples were allowed to thaw at room temperature, gently inverted several times and after which 100µL was pipetted into sample cups for analysis. Plasma NEFA concentration was analysed using a NEFA-C kit (C Kit Wako Pure Chemical Ind., Osaka, Japan; modified for the Olympus AU400 Automated Chemistry Analyser) which uses an enzymatic method based on the protocol of Itaya and Ui (1965) and Duncombe (1964).

3.7.2.3 **Modelling of response curves**

Basal concentrations of NEFA were calculated as the mean of samples collected at -30, -15, -10, -5 and 0 minutes relative to the challenge. The concentrations of each substrate were then plotted against time for each challenge administered to each lamb and a derived function with multiple exponential components (Equation 2) was fitted to the raw data Figure 3-1).

Equation 2

$$y(t)=Int+(e[-\beta t]*[-\gamma/(\beta-\alpha)+\gamma/(\beta-\alpha-\Delta)-\varepsilon/\beta+\gamma/(\beta-\alpha)*e((\beta-\alpha)-\gamma/(\beta-\alpha-\Delta)*e((\beta-\alpha-\Delta)+\varepsilon/\beta*e(\beta t)])$$

- Where:
- y(t) - substrate concentration (mM) at any given point in time
- t- time (minutes)
- Int - basal substrate concentration (mM) prior to challenge
- γβαΔ - exponential constraints
- ε - the adjustment from basal substrate concentrations
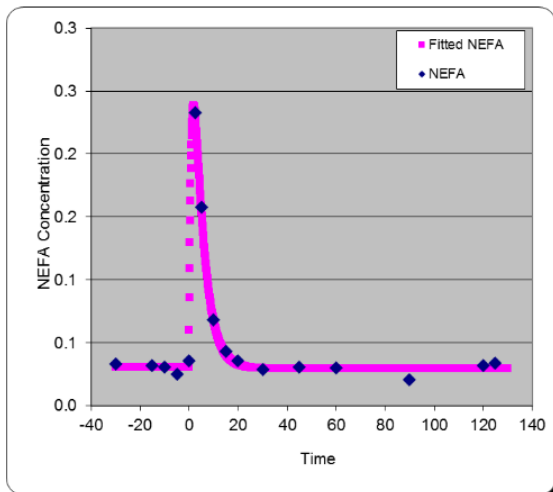
Figure 3-1 The response curve for non-esterified fatty acids over time following a challenge at time 0 (adjusted from x).

The use of this function allows the plasma concentration response curve to be modelled at different time points, pre and post challenge. This enables the qualification of the substrate response and out-putting components for analysis, including area under curve (AUC) up to any time point specified. The peak response was determined to have been reached at 10 minutes following challenge administration and was chosen for the AUC calculations because it best reflected time to reach the maximum NEFA response (AUC 10). The reason for doing this is because the change in plasma NEFA concentration up to this point is as a result of heparin administration. Incorporating the NEFA response in time following maximum concentration will expose differences in the capacity of each lamb to clear NEFA from circulation, returning NEFA levels to baseline levels.

3.7.2.4 Statistical analysis

NEFA AUC10, basal NEFA concentration, maximum NEFA concentration and time of maximum concentration were analysed using a linear mixed effects model in SAS (SAS Version 9.2, SAS Institute, Cary, NC, USA).

The core model for NEFA AUC10, maximum NEFA concentration and time of maximum concentration included fixed effects for sire type, sex and challenge (high heparin, low heparin, saline). Animal identification was included as a random term. The models for NEFA AUC10 and maximum NEFA concentration were also corrected for basal NEFA concentration. The base model for basal NEFA concentration included sex, sire type and their interactions, as well as animal identification as a random term.

Time to return to basal NEFA concentration was determined by the first time point following heparin challenge administration that was not different (P>0.05) from pre-challenge NEFA levels (time -30 to time 0). NEFA concentration from animals treated with low and high heparin was analysed using a linear mixed effects model and included fixed effects for time, challenge, sire type and sex and their interactions, as well as animal identification as a random term.

All relevant first order interactions between fixed effects were tested and non-significant (P>0.05) terms were removed in a stepwise manner.

3.7.3 Results

The effects of challenge and basal NEFA on NEFA AUC10 and maximum NEFA concentration are described in Table 3-4.

Table 3-4 F values, P values and numerator and denominator (NDF, DDF) degrees of freedom for the effects of challenge and basal non-esterified fatty acid (NEFA) on NEFA AUC10 and maximum concentration

Effect	NEFA AUC10		NEFA maximum concentration		NEFA maximum concentration with basal NEFA	
	NDF, DDF	F-Value	NDF, DDF	F-Value	NDF, DDF	F-Value
Challenge	2, 26	43.96**	2,26	8.55**	2, 25	59.27***
Basal NEFA					1, 25	819.23***

* $P<0.05$; **$P<0.01$

3.7.3.1 Effect of challenge, sex and sire type on the non-esterified fatty acid (NEFA) response to challenge

Heparin challenge dose had a significant impact on NEFA AUC10 ($P<0.05$, Table 3-4). The high heparin challenge was approximately 25% higher ($P<0.05$) than for the low heparin challenge (Figure 3-2). The low dose heparin and high dose heparin were 0.73 units and 0.99 units higher than the saline treatment ($P<0.05$). Sex, sire type and basal NEFA concentrations had no impact on NEFA AUC10 ($P>0.05$).
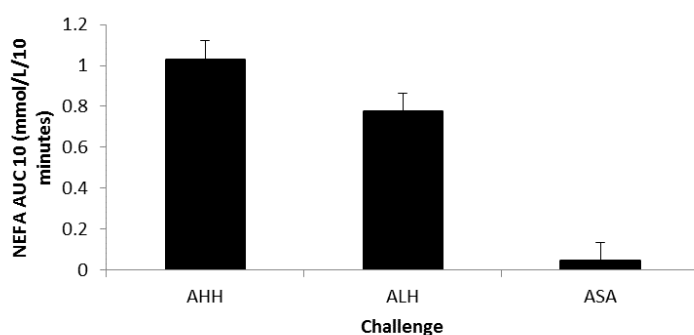
Figure 3-2 NEFA AUC10 for the high heparin (AHH), low heparin (ALH) and saline (ASA) challenges

3.7.3.2 Effect of sex, sire type and challenge on maximum NEFA concentration

Challenge type had a significant impact on maximum NEFA concentration ($P<0.05$, Table 3-4). The high heparin challenge (0.32 ± 0.056 mmol/L) had 20% higher maximum NEFA concentrations than the low heparin (0.26 ± 0.056 mmol/L) challenge, while these two were 72% and 36% higher than the

saline (0.19 ± 0.056 mmol/L) challenge. Sex and sire type had no impact on maximum NEFA concentration (P>0.05).

There was a strong positive association (P<0.05, Table 3-4) between maximum response and basal NEFA concentration. As basal NEFA increased from 0.05mmol/L to 0.06mmol/L, there was an associated increase in maximum concentration of NEFA from 0.12mmol/L to 0.70mmol/L (Figure 3-3). The magnitude of effect of challenge on maximum NEFA concentration remained unchanged when the model was corrected for basal NEFA concentration. There was no impact of sex, sire type or challenge on time of maximum NEFA concentration (P>0.05).
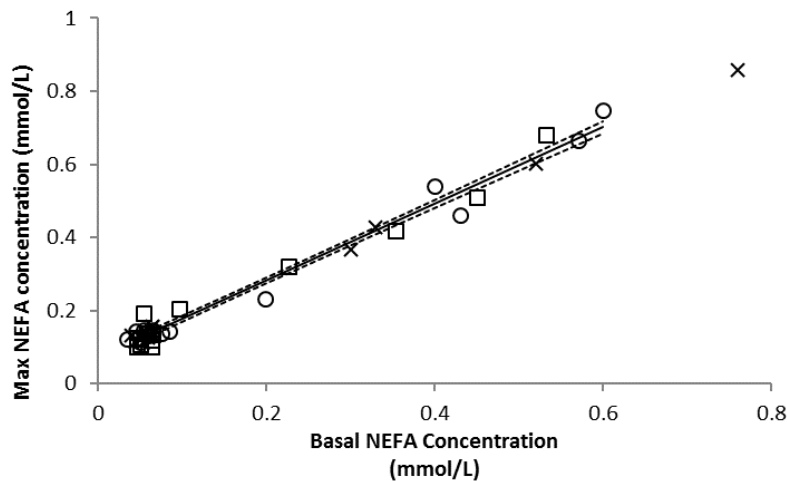
Figure 3-3 Association between maximum non-esterfied fatty acid (NEFA) concentration and basal NEFA concentration. Line represents predicted mean ± standard error. Icons denote high heparin (○) low heparin (□) and saline (x) challenge residuals from response surface.

3.7.3.3   Time to return to pre-treatment NEFA concentrations

Time to return to basal was determined by the first time point following challenge administration that was not different (P>0.05) from pre-challenge NEFA levels. There was no difference in pre-treatment NEFA levels from time -30 to time 0 (P>0.05). Time to return to basal NEFA concentration was the same for both low and high heparin treatment groups (Figure 3-4), with NEFA levels returning to basal levels by 30 minutes post heparin administration. NEFA concentration did not change (P>0.05) over time in the saline treatment.
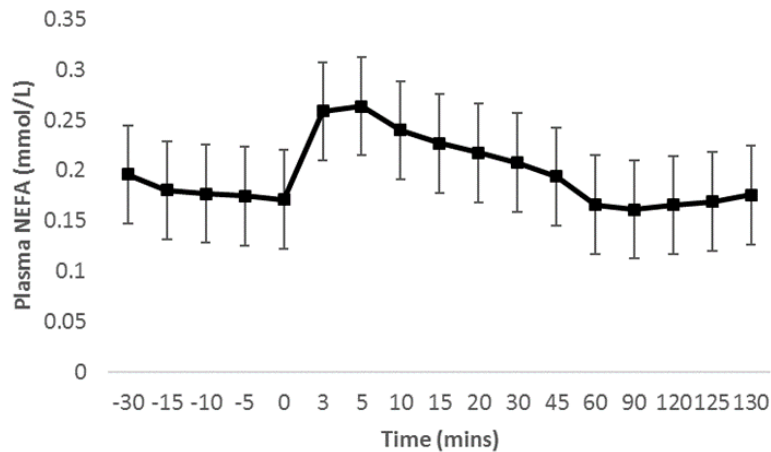
Figure 3-4 Association between non-esterified fatty acid (NEFA) concentration (mmol/L) and time (minutes).

3.7.4   Discussion

3.7.4.1   Effect of heparin on NEFA response

In partial support of the hypothesis, there was an increase in NEFA response (measured by NEFA AUC10 and maximum concentration) following administration of heparin at the high dose. This is in line with previous work which has shown a dose dependent response in NEFA concentrations to heparin administration (Jaume et al., 1996). However, the high heparin dose used in this study was relatively conservative compared to other studies and it indicates that even at these levels, NEFA concentrations will be moderately increased. In contrast to our hypothesis, the low dose heparin also elevated plasma NEFA response. Whilst there is conflicting results between authors (Seoane et al., 1972; Desmond et al., 1980) on the effect of low dose heparin, it does indicate that this level of heparin would also affect repeated sampling of blood samples (as conducted in this experiment) for NEFA analysis within a short (<1 hour) time period.

3.7.4.2   Effect of sex and sire type on NEFA response to heparin challenge

Contrary to the hypothesis, there was no effect of sex or sire type on NEFA response to heparin administration. This was unexpected as lipoprotein lipase activity is strongly related to the size of the adipose tissue depot (Haugebak et al., 1974; Barber et al., 2000). It is possible that in the current study is that the animals used were not divergent enough in their muscling and leanness attributes. In addition, this was a small study and larger numbers of animals may be required to study the impact of sex and genotype on NEFA responses to heparin, particularly when using conservative heparin doses and looking for very small differences in the magnitude of NEFA response.

Physiological maturity may also have been a reason differences were not detected in NEFA response between Terminal and Merino sire types. Terminal sired lambs are bred to grow rapidly and are typically leaner and more muscular than merinos (Anderson et al., 2015). Therefore, at the same age, terminal sired lambs are physiologically more mature than the Merinos (Butterfield, 1988) and may have been similar in whole body carcass fatness compared to the Merinos. As lipoprotein lipase levels are higher with increased adipose tissue deposition (Singh et al., 1988), it may have resulted in softening of the expected difference in NEFA response between merinos and terminals sired lambs.

Furthermore, the lambs used in this study were around 1 year old, thus growth rates may have slowed, also reducing lipoprotein lipase activity and therefore NEFA response to heparin administration (Haugebak et al., 1974).

3.7.4.3 Time to return to pre-treatment NEFA concentrations

Supporting the hypothesis, NEFA levels returned to baseline levels within 30 minutes for both the low heparin and high heparin challenge. This is comparable to previous findings in sheep (Tume et al., 1988), although this study significantly lower heparin doses were used in the current experiment. Overall the results indicate that collection of a blood sample and flushing with heparinised saline to maintain cannula patency would be unlikely to affect NEFA concentrations if sampling was performed at greater than 1 hour time intervals.

3.7.5 Conclusion

This study has evaluated the impacts of a low and high dose heparin administration on plasma NEFA response. Results indicate that heparin as an anticoagulant should be avoided where frequent blood samples are required within intervals less than 1 hour. However, for repeated sampling at intervals greater than 1 hour, judicious flushing with heparinised saline is unlikely to have an impact on NEFA levels.

Chapter 4 Physiological indicators of acute and chronic stress in lambs at slaughter

4.1 Introduction

Currently, there is limited data on physiological levels of plasma indicators of stress undergoing commercial slaughter under Australian conditions. Identifying indicators in plasma that may be used to quantify acute and chronic stress at exsanguination and what production and environmental factors influence them may assist in making inferences regarding pre-slaughter management. Minimising stress during the pre-slaughter period is essential not only from an animal welfare point of view but it is also well established in the literature that stress can negatively affect meat quality and carcass weight (Ferguson and Warner, 2008). These indicators can be classified across various categories including acute stress, muscle damage and feed and water deprivation.

During the immediate pre-slaughter period sheep may be subjected to various potential stressors leading up to the slaughter process including unfamiliar environments, increased handling and human contact, and changes in social structure, which can cause acute stress (Ferguson and Warner 2008). The acute stress response elicits two central mechanisms, a sympatho-adrenal component of the autonomic response and a hypothalamic-pituitary-adrenal (HPA) response. The sympatho-adrenal component of the autonomic response is mediated by adrenaline and results in significant changes in energy metabolism including lipolysis, glycogenolysis in muscle and gluconeogenesis (Kuchel, 1991), leading to increased plasma lactate, non-esterified fatty acids (NEFA) and glucose above resting basal levels (Pethick et al., 2005; Radostits et al., 2007; Bórnez et al., 2009; Lepherd et al., 2009).

Activation of the HPA axis causes cortisol secretion which also potentiates changes in energy and fat metabolism, especially characterised by elevated glucose concentrations via gluconeogenesis (Chrousos, 1998). Many studies has shown that cortisol increases above normal levels (40 – 80nmmmol/L) (Radostits et al., 2007) in response to acute pre-slaughter stress (Grandin, 1997; Liste et al., 2011; Sutherland et al., 2016) and as such has been used extensively as a marker of acute stress in ruminants.

Acute stressors have also been shown to increase levels of acute phase proteins, such as haptoglobin, which are normally present at low levels (<0.1g/L) in plasma (Lepherd et al., 2009). Haptoglobin is released by the liver as part of the innate immune response activated by harmful stimuli such as inflammation, tissue damage, infection and bacterial components (Baumann and Gauldie, 1994). However, increases in haptoglobin above normal have been observed as a result of transportation, mixing of animal (Lomborg et al., 2008; Cray et al., 2009; Piccione et al., 2012) and increased cortisol levels (Alsemgeest et al., 1996) and therefore may represent a useful indicator of pre-slaughter stress.

The acute stress response may be modulated by intrinsic animal factors such as genetics, sex and nutrition. For example, the merino breed has been previously identified as more sensitive to stress (Gardner et al., 1999), while differences in adipose tissue and glycogen metabolism have been identified in sheep and cattle that are selected for increased carcass muscling (Martin et al., 2011; McGilchrist et al., 2011). Female lambs have been reported as more stress susceptible compared to wethers (Hernandez et al., 2010), which may be related to their greater behavioural reactivity (Dodd et al., 2014). Research has shown that magnesium supplementation may reduce the effects of stress in animals by reducing catecholamine secretion (D'Souza et al., 1998; Gardner et al., 2001a). As levels

in plasma are reflective of gastrointestinal absorption and tissue uptake (Martens and Schweigel, 2000; Pye et al., 2015), normal plasma magnesium concentrations (0.9 – 1.26mmol/L) (Radostits et al., 2007) would be expected under best practice pre-slaughter management.

In contrast, chronic stress may occur as a result of pre-slaughter management practices that impose longer term physiological effects within the week or days prior to slaughter. Factors such as mustering, yarding and transport can lead to fatigue and bruising, while feed and water deprivation cause significant mobilisation of energy reserves and disturbances to fluid homeostasis.

Muscle damage and fatigue associated with pre-slaughter mustering, transport and yarding has shown to cause increases in indicators of muscle damage such as creatine kinase (CK) and aspartate aminotransferase (AST) (Russell and Roussel, 2007; Liste et al., 2011). This can be seen with unaccustomed exercise and bruising (Tollersrud et al., 1971; Tarrant, 1990) which may occur during transport and yarding during the pre-slaughter period. However, it has also been shown that while levels of CK and AST may increase in response to routine management, these effects may be minimal and transient (Cockram et al., 1996; Pettiford et al., 2008) under good husbandry practices.

Under routine pre-slaughter protocols lambs may undergo a period of feed deprivation of up to 48 hours or longer (Jacob et al., 2005b). Feed deprivation leads to increased adipose tissue mobilisation and a shift towards β-oxidation of long chain fatty acids, with increases in plasma non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHOB), as well as progressive depletion of liver glycogen and a decrease in plasma glucose (Heitmann et al., 1987). Although water should be available in the pre-slaughter period, work by Jacob et al. (2006c) indicated that a large percentage of lambs are dehydrated in lairage. Water deprivation may cause hypertonic dehydration indicated by progressive hypernatremia and hyperproteinemia (Parker et al., 2003; Jaber et al., 2004; DiBartola, 2006; Jacob et al., 2006b) indicated by elevated plasma sodium (>152mmol/L) and total protein (> 7.9g/dL) concentrations (Radostits et al., 2007).

This paper describes the association of production and genetic factors such as site and kill group, birth year, breed, sex, dam age and birth type on acute and chronic plasma indicators of stress. Due to the physiological effects of feed and water deprivation and acute stress during the pre-slaughter period, it is hypothesised that physiological indicators of dehydration, feed deprivation and acute stress will be higher in lambs at slaughter compared to basal levels. Alternatively, it is expected that magnesium levels and indicators of muscle damage in finished lambs slaughtered under best practice management will be within the normal physiological range.

4.2   Materials and Methods

4.2.1   Experimental design and pre-slaughter management

The design of Meat and Livestock Australia Genetic Resource flock has been described previously (Fogarty et al., 2007; Van der Werf et al., 2010). Wether and female lambs (n = 2877) were produced from artificial insemination of Merino, Border Leicester x Merino (BLM) and Commercial Maternal (CM) dams over a two year period (2013 and 2014) at the Katanning, WA and Kirby, NSW research sites, to represent a broad cross section of Australian production systems. Details of animals used in the experiment are provided in Table 4-1. The lambs were the progeny of 394 different sires, which comprised terminal sire types (Ile De France, Poll Dorset, Suffolk, Texel, Charolais and White Suffolk), maternal sire types (Booroola, Border Leicester, Coopworth, Dohne Merino and Prime SAMM) and Merino (Merino and Poll Merino) sires, representing the major production types in the Australian sheep industry. These sires were chosen to represent the full range of Australian Sheep Breeding Values (ASBVs) for key traits within each sire type. Semen from all three sire types was used to

artificially inseminate Merino dams, while only semen from Maternal and terminal sires was used to inseminate cross-bred (BLM and CM) ewes. Maternal lambs sent to slaughter comprised very few females (which were retained for breeding purposes), meaning effective statistical comparisons between sexes could only be made within the terminal and Merino sired lamb groups and maternal sired lambs from Merino dams. The lambs were maintained on extensive pasture grazing, with grain, hay or feedlot pellets supplemented when pasture supply was limited.

For each site, lambs were assigned to smaller kill groups (n = 17) of between 48 and 307 lambs to be killed on the same day to enable carcass weight targets to be achieved. Given selection for slaughter was made based on weights, the average age of lambs in each kill group varied between 193 and 416 days old at slaughter, however within individual kill groups the age range was smaller, varying as little as 16 days and by up to 33 days of age. Lambs were yarded on farm the day before slaughter and were taken off feed and water for between 5 and 18 hours. Lambs were then weighed and transported by truck to one of three commercial abattoirs (1 in WA and 2 in NSW). At the Katanning site transportation lasted for 0.5 hours compared to 1.5 to 2.5 hours for the Kirby site. Lambs were held overnight in lairage with free access to water and slaughtered the following day after electrical stunning.

Table 4-1 Number of lambs from which blood samples were collected at each site, within each year, sex, dam breed and sire type.

Site	Year		Sex Dam breed (Sire type)											
	2013	2014	F Merino (Mat.)	M Merino (Mat.)	M CM (Mat.)	M BLM (Mat.)	F Merino (Mer.)	M Merino (Mer.)	M Mer. (Ter.)	F Mer. (Ter.)	M CM (Ter.)	F CM (Ter.)	M BLM (Ter.)	F BLM (Ter.)
Kirby	1128	721	37	98	n/a	43	80	194	273	268	n/a	n/a	311	354
Katanning	524	504	2	118	17	5	34	199	156	215	78	109	8	19
Total	1652	1225	39	216	17	48	114	393	429	483	78	109	319	373

F: female lamb; M: male (wether) lamb
CM: Commercial Maternal dam breed; BLM: Border Leicester x Merino dam breed.
(Mat.): Maternal sire type; (Mer.): Merino sire type; (Ter.): Terminal sire type.
n/a: not applicable

4.2.2 Blood collection and sample processing

Blood samples were collected into 9mL lithium heparin Vacuette® tubes (Greiner bio-one, Austria) from each lamb at slaughter, immediately following exsanguination. Tubes were immediately placed in ice for between 2-5 hours until centrifugation at 2500 - 3000rpm for 15 minutes. Following centrifugation, plasma samples were pipetted in two separate aliquots and stored in 2mL tubes at -80°C until processing.

Laboratory analyses of plasma were carried out as a batch samples. Plasma lactate, glucose, NEFA, magnesium, total protein, CK and AST were analysed using commercial available reagent kits (Olympus Diagnostics, Tokyo, Japan). Plasma sodium was analysed using a commercially available sodium kit (Randox Laboratories kit, County Antrim, UK). Samples were analysed using the Olympus AU400 automated chemistry analyser (Olympus Optical Co. Ltd, Melville, NY). For each commercial kit, the correlating control and calibration sera was used.

Plasma haptoglobin and BHOB were analysed by the Western Australian Department of Agriculture (DAFWA) Animal Health Laboratories, South Perth. Plasma β-hydroxybutyrate was analysed using the commercial reagent kit (Randox Laboratories kit, County Antrim, UK). Plasma haptoglobin was determined using an in-house method, based on the method described by (Eckersall et al., 1999). Plasma Cortisol levels were determined using chemiluminescent immunoassay performed on a subset of 500 samples from the 2013 cohort using an Immulite® 2000 Immunoassay system (Siemens, Germany) at Vetpath Veterinary services, (Perth, Australia).

4.2.3 Carcass measurements

Following slaughter, lambs were dressed according to AUS-MEAT standards and hot carcass weight (HCWT) was recorded at an average of 23.2 kg (Std Dev = 2.94). All carcasses underwent medium voltage electrical stimulation to optimise pH decline such that the carcass loin temperature at pH6 lies between 18 - 25°C (Pearce et al., 2010) and were chilled overnight (3-4°C) before sampling.

GR fat depth (mm) was measured using a GR knife on the external fat of the carcass 110mm from the midline and over the 12th rib. C-site fat depth (mm) was measured adjacent to the exposed loin eye.

At 24 hours post slaughter the shortloin (M. *longissimus lumborum*) was removed from the carcass saddle region caudal to the middle of 12th/13th rib (Anonymous, 2005). From this, the subcutaneous shortloin fat was removed and the entire trimmed shortloin weighed (LLWT).

4.2.4 Statistical analysis

Each plasma indicator was analysed using linear mixed effect models in SAS (SAS Version 9.1, SAS Institute, Cary, NC, USA). The base model included fixed effects for site, year, kill group within site by year, sire type, sex by dam breed within sire type, age of dam and birth type. Sire identification and dam identification by year were included as random terms.

For the cortisol analysis only plasma samples from 2013 were analysed, hence the base model included site (as above), kill group within site, sex and dam breed within sire type (as above), age of dam and birth type. Sire identification was included as a random effect.

In separate models, kill order was included in the base model as a covariate along with relevant interactions. To test the association of plasma indicators with carcass weight, carcass fatness and leanness, in separate models, carcass weight (Hot carcass weight) and composition traits (hot carcass weight and loin weight, GR-tissue depth and c-site fat depth) were included simultaneously in the base model as a covariate along with relevant interactions. In all models, non-significant (P>0.05) terms were removed in a stepwise manner.

Partial correlations between indicators were calculated in a multivariate analysis of variance (PROC MANOVA, SAS Version 9.1, SAS Institute, Cary, NC, USA). These models contained all significant fixed effects and their interactions. Simple Pearson correlations were calculated using the PROC CORR function in SAS (SAS Version 9.1, SAS Institute, Cary, NC, USA).

4.3   Results

Outcomes of the base models for each indicator are presented in Table 4-2. The mean, standard deviation and minimum and maximum values for plasma indicators have been summarised in Table 4-3, which also includes published reference ranges and normal values for each indicator (where available).

4.3.1   Association between production and environmental factors and plasma stress indicators

Overall site (which reflects a combination of farm and slaughter location) had the largest impact (P<0.01, Table 4-2) on all plasma indicators. On average, indicators were higher (P<0.05, Table 4-5) at the Kirby site compared to Katanning. The only exception was for NEFA and BHOB, which were higher (P<0.05, Table 4-5) at Katanning. In addition, the effect of site also differed across years for all indicators, except haptoglobin and cortisol (P<0.05, Table 4-2).

For most indicators, kill group explained a large amount of variation (P<0.01, Table 4-2). Within any site and year group, these differences between kill groups were as large as 6.92 ± 0.291 mmol/L and as little as 2.02 ± 0.177 mmol/L for lactate, 5.77 ± 0.06 mmol/L and 3.71 ± 0.059 mmol/L for glucose, and 1.10 ± 0.010 mmol/L to 0.78 ± 0.009 mmol/L for magnesium. Plasma NEFA varied between kill groups (P<0.05, Table 4-2) ranging between 0.80 ± 0.037 mmol/L and 1.72 ± 0.04 mmol/L. Levels of BHOB varied between kill groups from 0.295 ± 0.010 mmol/L and 0.60 ± 0.009 mmol/L. Kill group explained the greatest degree of variation in plasma sodium levels (P<0.05, Table 4-2) ranging from as low as 146.79 ± 0.243 mmol/L to as high as 152.0 ± 0.409 mmol/L. Plasma total protein levels varied between kill groups (P<0.05, Table 4-2) and when comparing extremes, ranged between 75.50 ± 0.742 g/L and 62.86 ± 0.468 g/L. Within any site of production in any year, the mean kill group AST was as high as 202.25 ± 4.38 IU/L and as low as 122.0 ± 3.278 IU/L. Creatine kinase varied between as low as 237.24 ± 21.843 IU/L to as high as 750.38 ± 36.066 IU/L. Haptoglobin concentration varied between kill groups (P<0.05, Table 4-2), with no consistent trend and ranged between a 0.50 ± 0.029 mg/mL and 0.31 ± 0.029 mg/mL. Cortisol levels varied between kill groups (P<0.05, Table 4-2) and when comparing extremes ranged between 110.60 ± 6.393nmol/L and 211.04 ± 6.246 nmol/L.

Phenotypic partial correlation coefficients and simple correlation coefficients are presented in Table 4-4. Generally the correlations between the metabolic indicators glucose, lactate, NEFA and BHOB were higher than for the other plasma analytes measured. Correlations were positive between lactate and glucose and between NEFA and BHOB. Correlations were negative between lactate and both NEFA and BHOB and glucose and NEFA and BHOB. Magnesium had moderate correlations with these metabolic indicators, while most other plasma measurements demonstrated very low

correlations. The only exception to this was for the correlations between TP and glucose, TP and magnesium, TP and AST and CK and AST, yet these were still relatively low.

Table 4-2 F-values and Numerator and Denominator (NDF, DDF) degrees of freedom for the fixed effects for plasma indicators measured

Effect	NDF, DDF	Lactate	NDF, DDF	Glucose	NDF, DDF	NEFA	NDF, DDF	Magnesium	NDF, DDF	BHOB	NDF, DDF	Cortisol
Site	1,360	211.54**	1, 362	672.85**	1, 358	858.13**	1, 362	225.24**	1, 333	195.96**	1,358	51.48**
Year	1, 1752	210.66**	1, 1752	131.25**	1, 1738	4.21*	1, 1737	92.33**	1, 1700	6.31*		
Kill group (site*year)#	13,360	20.95**	13,362	45.1**	13,358	40.57**	14,362	38.02**	13,333	83.47**	5,358	18.13**
Sire type	2,360	3.21*			2,358	13.01**			2,333	12.53**		
Sex dam breed (Sire type)	9,360	3.24**	11,362	2.93**	9,358	4.44**						
Birth type												
Site*year	1,360	49.11**	1,362	45.52**	1,558	14.66**			1,333	153.05**		

NEFA: non-esterified fatty acids; BHOB: β-hydroxybutyrate; AST: Aspartate Aminotransferase; n/a: non-applicable *: $P< 0.05$, **: $P<0.01$, ***: $P<0.001$. # cortisol fixed effect model describe kill group(site)

Table 4-2 *continued* **F-values and Numerator and Denominator (NDF, DDF) degrees of freedom for the fixed effects for plasma indicators measured**

Effect	NDF, DDF	Sodium	NDF, DDF	Total protein	NDF, DDF	AST	NDF, DDF	Creatine Kinase	NDF, DDF	Haptoglobin
Site	1, 336	29.85**	1, 354	552.58**	1, 362	130.47**	1,347	61.07**	1,325	18.55**
Year	1, 1695	10.82**	1, 1724	68.60**	1,1746	9.3**	1,1730	27.04**		
Kill group (site*year)#	13,336	36.17**	13,354	20.98**	14,362	11.46**	13,347	11.01**	15,325	2.93**
Sire type			2,354	9.45**				8.64**		
Sex dam breed (Sire type)			9,354	4.17**	11,362	4.47**				
Birth type			2,354	5.63**						
Site*year	1,336	8.66*	1,354	8.84**			1,347	86.66**		

NEFA: non-esterified fatty acids; BHOB: β-hydroxybutyrate; AST: Aspartate Aminotransferase; n/a: non-applicable *: P< 0.05, **: P<0.01, # cortisol fixed effect model describe kill group(site)

Table 4-3 Descriptive statistics of plasma indicators and carcase variables analysed, including total number, mean, standard deviation (SD) and minimum and maximum values and published normal levels

Variable (units)	N	Mean ± SD	Min	Max	Published normal levels
NEFA (mmol/L)	2761	1.19 ± 0.54	0.17	3.26	0.1 – 0.3[c]; 0.07 – 0.08[d]
BHOB (mmol/L)	2684	0.43 ± 0.15	0.11	1.04	0.47 – 0.63[a]; 0.2 – 0.7[b]
Glucose (mmol/L)	2780	4.67 ± 0.94	1.47	10.50	2.8 - 4.4[a]; 2.7 – 4.8[b]
Lactate (mmol/L)	2779	3.48 ± 2.29	0.47	16.43	1.0 – 1.3[a]
Magnesium (mmol/L)	2751	0.90 ± 0.14	0.36	1.63	0.90 – 1.26[a]; 0.90 – 1.18[b]
Creatine Kinase (IU/L)	2732	463.29 ± 341.57	83.07	3731.80	35 – 280[a]; 180 - 454[b]
Aspartate aminotransferase (IU/L)	2775	152.63 ± 42.79	61.82	613.73	60-280[a]; 83 – 140[b]
Sodium (mmol/L)	2681	148.92 ± 3.40	130.08	161.32	145 - 152[a,b]
Total Protein(mg/L)	2788	68.65 ± 6.21	30.15	93.32	60 – 79[a] 51-64[b]
Haptoglobin (mg/mL)	2701	0.42 ± 0.38	0.01	4.82	0.06 – 0.12[b]
Cortisol (nmol/L)	471	152.27 ± 62.57	5.50	395.00	42 - 82[a]

[a] Radostits et al. (2007); [b] Lepherd et al. (2009); [c] Annison (1960); [d] Pethick et al. (1987)

Table 4-4 Phenotypic partial correlation coefficients (below diagonal) and simple correlations (above diagonal) for the plasma measures analysed

Plasma indicators	Lactate	Glucose	NEFA	BHOB	Mg	TP	Sodium	AST	CK	Hp
Lactate		**0.56**	**-0.46**	**-0.39**	**0.20**	**0.19**	0.01	**0.12**	**0.15**	-0.01
Glucose	**0.41**		**-0.47**	**-0.36**	**0.27**	**0.35**	-0.02	**0.20**	**0.17**	0.00
NEFA	**-0.32**	**-0.14**		**0.60**	**-0.34**	**-0.18**	**0.09**	**-0.12**	**-0.15**	**-0.06**
BHOB	**-0.27**	**-0.18**	**0.44**		**-0.24**	**-0.06**	**0.11**	**-0.02**	-0.06	**0.10**
Magnesium	**0.17**	**0.14**	**-0.23**	**-0.19**		**0.34**	**-0.16**	**0.12**	**0.09**	0.03
TP	**0.11**	**0.23**	**0.08**	**0.04**	**0.18**		-0.01	**0.33**	**0.11**	**0.11**
Sodium	**0.08**	0.00	**0.08**	0.00	**-0.12**	**-0.05**		**0.07**	**-0.05**	-0.03
AST	0.03	0.04	**0.05**	-0.01	**0.10**	**0.24**	0.03		**0.28**	0.00
CK	0.01	-0.02	0.02	**0.06**	**0.05**	0.01	-0.04	**0.20**		0.03
Hp	-0.03	**-0.05**	-0.02	**0.12**	-0.02	**0.11**	**-0.05**	-0.04	0.00	

NEFA: Non-esterified fatty acid; BHOB: β-hydroxybutyrate; TP: Total protein; Mg: Magnesium; Hp: Haptoglobin. Bolded values are significant ($P<0.05$).

Table 4-5 Predicted Least Squared Means and standard errors (in brackets) for fixed effects of site, year and site by year for plasma indicators analysed

Effect	Category	AST IU/L	BHOB (mmol/L)	CK IU/L	Glucose (mmol/L)	Haptoglobin mg/mL	Magnesium (mmol/L)	Sodium (mmol/L)	NEFA (mmol/L)	TP g/L	Lactate (mmol/L)	Cortisol# (nmol/L)
Site	Kirby	159.93a (1.721)	0.43ab (0.005)	483.91d (13.526)	4.94a (0.031)	0.46a (0.011)	0.94a (0.004)	149.37bc (0.101)	1.03a (0.018)	72.12a (0.317)	4.04c (0.086)	162.51a (3.586)
	Katanning	136.59b (1.858)	0.51e (0.006)	372.28ab (14.699)	3.99b (0.033)	0.38b (0.013)	0.85b (0.005)	148.60a (0.113)	1.64e (0.019)	65.54b (0.321)	2.50ab (0.093)	124.42b (4.245)
Year	2013	151.75a (1.789)	0.48d (0.005)	461.31cd (13.785)	4.65c (0.032)		0.87a (0.004)	148.74a (0.106)	1.36c (0.019)	67.71a (0.314)	3.94c (0.089)	
	2014	144.77b (1.779)	0.46c (0.005)	394.88ab (13.349)	4.27d (0.031)		0.93b (0.005)	149.23b (0.120)	1.31b (0.018)	69.95b (0.317)	2.60ab (0.087)	
Site*year	Kirby 2013		0.41a (0.006)	584.04e (16.105)	5.24e (0.037)			148.92ab (0.128)	1.02a (0.022)	71.35a (0.349)	5.02d (0.103)	
	Kirby 2014		0.45bc (0.007)	383.77ab (17.191)	4.64f (0.039)			149.82c (0.156)	1.04a (0.023)	72.88b (0.370)	3.05b (0.110)	
	Katanning 2013		0.55f (0.007)	338.59a (18.388)	4.07g (0.041)			148.57a (0.154)	1.70f (0.024)	64.07c (0.367)	2.86ab (0.117)	
	Katanning 2014		0.46cd (0.007)	405.98bc (18.069)	3.90h (0.041)			148.63a (0.164)	1.58d (0.024)	67.01d (0.372)	2.15c (0.116)	

AST: Aspartate aminotransferase; BHOB: β-hydroxybutyrate; CK: Creatine Kinase; NEFA: non-esterified fatty acids; TP: Total protein #: cortisol subset of data from 2013 year only

4.3.2 Association between animal factors and plasma stress indicators

4.3.2.1 Effect of sire type

Sire type had a small but significant effect on NEFA, BHOB, CK and total protein concentrations (P<0.05, Table 4-2). On average, NEFA levels were approximately 10% lower in terminal sired lambs (1.24 ± 0.015 mmol/L) compared to Merino (1.39 ± 0.026 mmol/L) and maternal (1.37 ± 0.036 mmol/L) sired lambs. There was no difference (P>0.05) in NEFA levels between Merino and maternal sired lambs.

Similarly, BHOB levels were lowest in terminal sired lambs (0.45 ± 0.004 mmol/L) compared to Merino (0.47 ± 0.006 mmol/L) and maternal (0.49 ± 0.008 mmol/L) sired lambs.

On average, CK levels were highest (P<0.05) in terminal sired lambs (462.76 ± 9.178 IU/L) compared to Merino (385.89 16.018 IU/L) and maternal (420.51 ± 18.615 IU/L) sired lambs. There was no difference (P >0.05) in CK levels between Merino and maternal sired lambs.

There was a small but significant effect of sire type on plasma total protein concentration (P<0.05). Total protein was lowest (P<0.05) in terminal sired lambs (67.87 ± 0.27 g/L) compared to Merino (69.55 ± 0.40 g/L) and maternal (69.055 ± 0.507 g/L) sired lambs.

4.3.2.2 Effect of dam breed

The effect of dam breed can only be compared among the progeny of terminal sired lambs and male lambs by maternal sires. Where dam breed was significant, female lambs with Merino dams had the highest metabolite concentration.

The effect of dam breed on glucose was small and only observed in female terminal sired lambs, where lambs from Merino (4.55 ± 0.034 mmol/L) dams had higher (P<0.05) glucose than lambs from Border Leicester x Merino (4.43 ± 0.043 mmol/L) dams.

Dam breed had a significant effect on plasma NEFA (P<0.05, Table 4-2) however this was only evident in terminal sired lambs. Female lambs from Merino dams (1.18 ± 0.020 mmol/L) had lower NEFA compared to Border Leicester x Merino dams (1.26 ± 0.025 mmol/L). Similarly, male lambs from Merino dams (1.22 ± 0.022 mmol/L) had lower NEFA compared to Border Leicester x Merino dams (1.29 ± 0.027 mmol/L).

The only effect of dam breed on total protein was in terminal sired female lambs, where TP in lambs from Merino dams (68.89 ± 0.34 g/L) was higher than in lambs from Border Leicester x Merino dams (67.93 ± 0.38 g/L).

The only effect of dam breed on lactate was in terminal sired female lambs, where lactate in lambs from Merino dams (3.52 ± 0.097 mmol/L) was higher (P<0.05) than in lambs from Border Leicester x Merino dams (3.05 ± 0.121 mmol/L).

4.3.2.3 Effect of sex

The effect of sex could only be compared among the progeny of Merino and terminal sired lambs and within maternal sired lambs from Merino dams (Table 4-1). Overall the effect of sex was small

(P<0.05, Table 4-2) with differences mainly being observed in Merino sired lambs or lambs with Merino dams.

The majority of plasma indicators were higher in female lambs, the only exception being plasma NEFA, where it was lower (P<0.05) in females (1.278 ± 0.04 mmol/L) compared to males (1.49 ± 0.02 mmol/L) in Merino sired lambs. In Merino sired lambs, plasma AST was higher in females (154.9 ± 4.11 IU/L) compared to males (140.7 ± 2.19 IU/L). Similarly, in terminal sired lambs, female lambs from Merino dams (155.5 ± 1.93 IU/L) had higher (P<0.05) AST levels than male lambs (147.2 ± 2.10 IU/L). Additionally, in terminal sired BLM lambs, female lambs (152.59 ± 2.38 IU/L) had higher AST than male lambs (143.27 ± 2.63 IU/L). Glucose was higher (P<0.05) in female lambs compared to male lambs by Merino sires (4.65 ± 0.074 mmol/L vs. 4.41 ± 0.039 mmol/L) and terminal sires with Merino dams (4.55 ± 0.034 vs. 4.44 ± 0.037 mmol/L). Merino sired female lambs (70.38 ± 0.61 g/L) had higher (P<0.05) plasma total protein levels than male lambs (68.72 g/L). Similarly, in terminal sired lambs from Merino dams, females (68.89 ± 0.33 g/L) had higher total protein levels than male lambs (67.55 ± 0.36 g/L). In terminal sired lambs from commercial maternal dams, females (68.86 ± 0.57 g/L) had higher TP than male lambs (66.68 ± 0.64 g/L). Plasma lactate was higher (P<0.05) in Female lambs compared males lambs, however this difference was small and only evident in lambs with Merino sires (3.87 ± 0.21 mmol/L vs. 3.2 ± 0.11 mmol/L) and terminal sired lambs with Merino dams (3.52 ± 0.1 mmol/L vs. 3.09 ± 0.11mmol/L).

4.3.2.4 Effect of birth type

Birth type had a small but significant effect on total protein concentration. The only difference observed was between single born lambs (68.3 ± 0.23 g/L) which had a lower (P<0.05) total protein compared to twin born lambs (69.0 ± 0.23 g/L).

4.3.3 Effect of kill order on plasma indicators

There was a linear association between plasma glucose and kill order (P<0.05, Figure 4-1). As kill order increased from 0 to 300, there was an increase in plasma glucose concentration by 6%, from 4.37 mmol/L to 4.62 mmol/L.

There was an overall positive association between plasma lactate and kill order (P<0.01, Figure 4-1) and as kill order increased from 0 to 300, plasma lactate increased by 25% from 3.02mmol/L up to 3.8mmol/L. This effect was not consistent between sites and years, with the overall association driven by significant positive associations at the Kirby site in 2013 and Katanning site in 2014. At the Kirby site as kill order increased from 0 to 200, plasma lactate increased by 1.29mmol/L (4.59 ± 0.138 mmol/L to 5.83 ± 0.192mmol/L). At the Katanning site as kill order increased from 0 to 300, plasma lactate increased by 1.99mmol/L (1.33 ± 0.259mmol/L to 3.32 ± 0.352mmol/L). Alternatively, at the Katanning site in 2013, there was negative association between plasma lactate and kill order (P = 0.0504) and as kill order increased from 0 to 300, plasma lactate decreased by 0.70mmol/L (3.13 ± 0.196mmol/L to 2.43 ± 0.224mmol/L). There was no effect (P>0.05) of kill order on plasma lactate at the Kirby site in 2013.

There was a curvilinear association between plasma NEFA and kill order (P<0.05, Figure 4-1). As kill order increased from 0 to 100, there was a small decrease (P<0.05) in plasma NEFA from 1.42mmol/L to 1.31mmol/L after which a plateau was reached. This effect was inconsistent across sites and years (P<0.05). At the Kirby site in 2013 as kill order increased from 0 to 50, plasma NEFA decreased (P<0.05) by 0.06mmol/L (1.08 ± 0.0292mmol/L to 1.022 ± 0.022mmol/L) after which a plateau was reached. Similarly in the Kirby site in 2014, as kill order increased from 0 to 150, plasma NEFA decreased (P<0.05) by 0.09mmol/L (1.10 ± 0.034mmol/L to 1.01 ± 0.027mmol/L). At the

Katanning site in 2014 plasma NEFA demonstrated a negative curvilinear association with kill order. As kill order increased from 0 to 300, plasma NEFA decreased by 0.84mmol/L (2.01 ± 0.054 mmol/L to 1.17 ± 0.074mmol/L). There was no association (P>0.05) between plasma NEFA and kill order at the Katanning site in 2013.

There was a curvilinear association between plasma BHOB and kill order (P<0.05, Figure 4-1). As kill order increased from 0 to 200 there was a small decrease (P<0.05) in plasma BHOB from 0.52mmol/L to 0.44mmol/L after which a plateau was reached. This effect was inconsistent across sites and years (P<0.05). At the Kirby site the association was consistent and demonstrated a negative curvilinear association in both 2013 and 2014. In 2013, as kill order increased from 0 to 150, plasma BHOB decreased (P<0.05) by 0.059mmol/L (0.45 ± 0.009mmol/L to 0.38 ± 0.009mmol/L) after which a plateau was reached until a kill order of 200. In 2014, as kill order increased from 0 to 150, plasma BHOB decreased (P<0.05) by 0.06mmol/L (0.49 ± 0.010mmol/L to 0.43 ± 0.008mmol/L) after which a plateau was reached until a kill order of 250. At the Katanning site the curvilinear association between plasma BHOB and kill order was inconsistent. In 2014, as kill order increased from 0 to 300 plasma BHOB decreased (P<0.05) by 0.33mmol/L (0.63 ± 0.016mmol/L to 0.30 ± 0.022mmol/L). There was no association (P>0.05) between plasma BHOB and kill order in 2013.

There was a curvilinear association between CK and kill order (P<0.05, Figure 4-1). Overall, as kill order increased from 0 to 200, CK decreased from 468.58IU/L to 393.85IU/L after which a plateau was reached. This effect also differed between sites (P<0.05) and years (P<0.05). There was a negative curvilinear association between plasma CK and kill order at the Kirby site, where as kill order increased from 0 to 200 plasma CK decreased by 139.04IU/L (552.43 ± 18.746IU/L to 413.39 ± 21.088IU/L) after which a plateau was reached from kill order of 200 to 250. There was no association (P>0.05) between CK and kill order at the Katanning site. There was a negative curvilinear association between plasma CK and kill order in 2013 where as kill order increased from 0 to 250, plasma CK decreased by 118.42IU/L (523.28 ± 22.458IU/L to 404.86 ± 28.192IU/L). There was no association (P>0.05) between plasma CK and kill order at the Katanning site or in 2014.

There was a trend (P = 0.0852) for an association between plasma cortisol and kill order at the Katanning site in 2013. As kill order increased from 0 to 300, plasma cortisol increased by 24.5nmol/L from 135.7 ± 4.73nmol/L to 160.3 ± 8.64nmol/L (Figure 4-1).
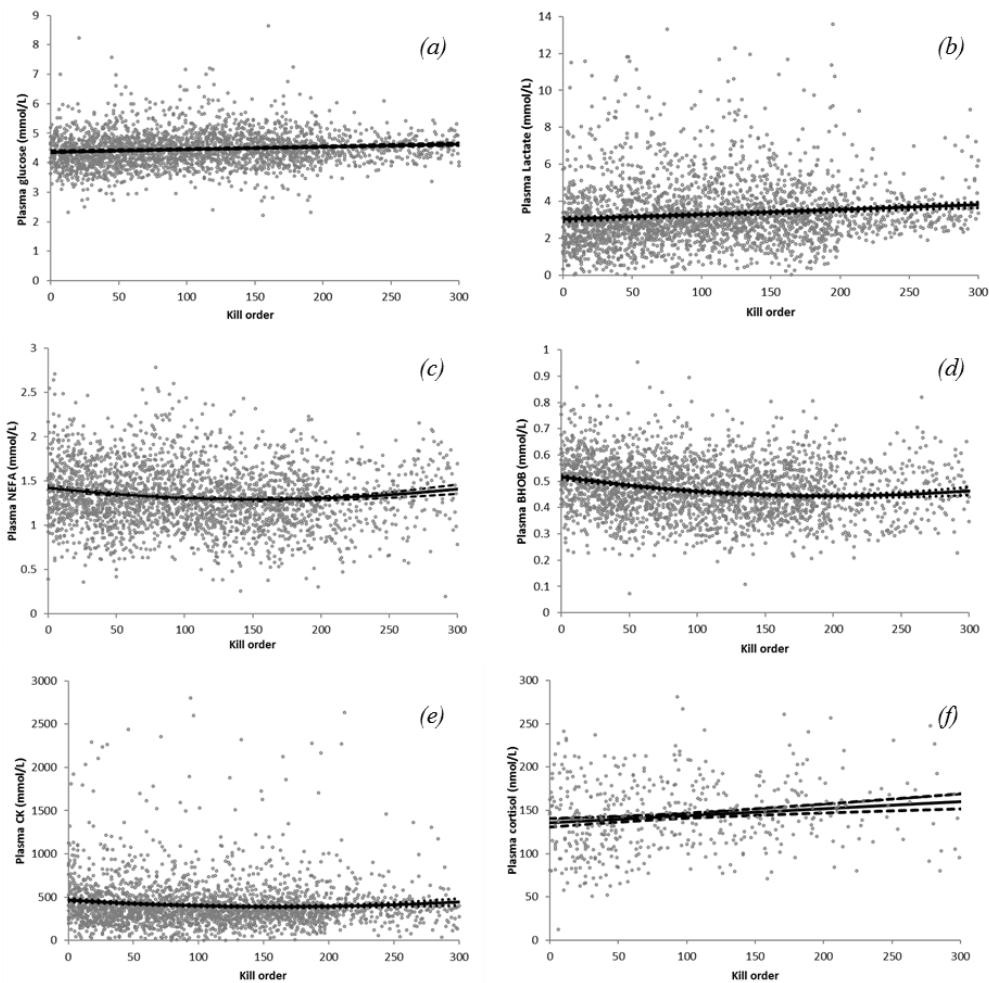
Figure 4-1 Association between plasma (a) glucose (mmol/L), (b) lactate, (c) non-esterified fatty acid (NEFA), (d) β-hydroxybutyrate (BHOB), (e) Creatine Kinase (CK), (f) cortisol and and kill order. Lines represent predicted least squared means ± standard error. Icons (○) denote residuals from response surface

4.3.4 Effect of carcass weight and composition on plasma indicators at slaughter

4.3.4.1 Hot carcass weight

Increasing HCWT was associated with a decrease in NEFA concentration (P<0.05); an effect which also varied between sites and years. As HCWT increased from 15 to 30kg, NEFA decreased by 0.31mmol/L at the Kirby site in 2013 (1.12 ± 0.05 to 0.87 ± 0.043mmol/L) and by 0.19mmol/L in 2014 (1.15 ±0.058 to 0.96 ±0.043mmol/L). The largest magnitude of effect was observed at the Katanning site in 2013. As HCWT increased from 15 to 30kg, NEFA decreased by 0.94mmol/L from 2.17 ± 0.066 to 1.23 ± 0.066mmol/L. There was no association (P>0.05) between HCWT and NEFA at the Katanning site in 2014.

Similarly, increasing HCWT was associated with a decrease in BHOB concentration (P<0.05); an effect which also varied between sites and years. At the Kirby site in 2013 as HCWT increased from 15 to 35kg BHOB decreased from 0.45 ± 0.017 mmol/L to 0.35 ± 0.024 mmol/L. Similarly, at the Katanning

site in 2013 as HCWT increased from 15 to 35kg, BHOB decreased from 0.71 ± 0.02 mmol/L to 0.29 ± 0.032 mmol/L. There was no association (P>0.05) between HCWT and BHOB at the Kirby or Katanning sites in 2014.

There was an effect of HCWT on plasma lactate concentration, however this effect differed across years and between sire types (P<0.05). In 2013 increasing HCWT from 15 to 30kg was associated with an increase in plasma lactate from 3.07 ± 0.240 mmol/L to 4.58 ± 0.235 mmol/L. In Merino sired lambs, increasing HCWT from 15 to 30kg was associated with a decline in plasma lactate from 3.72 ± 0.259 mmol/L to 2.93 ± 0.586 mmol/l. There was no association (P>0.05) between lactate and HCWT in 2014 or in maternal or terminal sired lambs.

Increasing HCWT from 15 to 30kg was associated with an increase (P<0.05) in plasma cortisol from 127.0 ± 9.63 nmol/L to 171.7 ± 14.98 nmol/L.

There was no effect (P>0.05) of HCWT on haptoglobin, glucose, AST, Sodium, CK, Magnesium or total protein.

4.3.4.2 GR tissue depth and c-site fat depth

Increasing GR tissue depth from 1 to 30mm was associated with a 0.24mmol/L decrease (P<0.05) in NEFA from 1.43 ± 0.03mmol/L to 1.19 ± 0.04mmol/L.

BHOB and GR tissue depth demonstrated an opposing association between sire types (P<0.01). As GR tissue depth increased from 1 to 30mm, BHOB decreased by 0.08mmol/L in maternal sired lambs (0.53 ± 0.019 mmol/L to 0.44-± 0.025mmol/L) and by 0.07mmol/L in Terminal sired lambs (0.48-± 0.012 to 0.41 ± 0.016mmol/L). Alternatively, in Merino sired lambs, as GR depth increased from 1 to 25mm, BHOB increased by 0.12mmol/L (0.41 ± 0.016 mmol/L to 0.56 ± 0.027mmol/L).

BHOB and GR tissue depth also demonstrated an association in five kill groups (P<0.05). In four kill groups this association was negative. The greatest decrease in BHOB was 0.13mmol/L (0.66 ± 0.036 to 0.53 ± 0.035mmol/L), as GR tissue depth increased from 1 to 25mm. Alternatively, in one kill group, as GR tissue depth increased from 1 to 15mm, BHOB increased by 0.14mmol/L (0.41 ± 0.026 to 0.55 ± 0.021mnmol/L).

There was a positive association between plasma glucose and GR tissue depth (P<0.01). As GR tissue depth increased from 1 to 30mm, there was an associated increase in plasma glucose of 30% from 4.10 ± 0.058 mmol/L to 4.98 ± 0.070 mmol/L.

There was a positive association between plasma lactate and GR tissue depth. As GR tissue depth increased from 1 to 30mm, plasma lactate increased from 2.44 ± 0.170 mmol/L to 4.24 ± 0.207mmol/L.

There was a positive association between AST and GR-tissue depth (P<0.01). As GR tissue depth increased from 1 to 30mm, plasma AST increased by 12% from 140.4 ± 3.19 IU/L to 158.5 ± 3.88 IU/L.

Increasing GR tissue depth from 1-30 mm was associated with an increase (P<0.05) in total protein from 67.5 ± 0.44 g/L to 70.2 ± 0.53 g/L. Sodium and GR tissue depth demonstrated an opposing association between years (P<0.05). As GR tissue depth increased from 1 to 30mm, Sodium increased by 1.6mmol/L (148.1 ± 0.29 to 149.7 ± 0.36mmol/L) in 2013, and decreased by 1.4mmol/L (149.8 ± 0.36 to 148.4 ± 0.47mmol/L) in 2014.

BHOB and c-site fat depth demonstrated an opposing association between sire types (P<0.05). In Merino sired lambs, as c-site fat depth increased from 1 to 10mm, BHOB increased by 0.13mmol/L

(0.42 ± 0.01 mmol/L to 0.55 ± 0.02mmol/L). Alternatively, in terminal sired lambs, as c-site fat depth increased from 1 to 10mm, BHOB decreased by 0.07mmol/L (0.47 ± 0.01 mmol/L to 0.40 ± 0.01mmol/L). There was no association (P>0.05) between BHOB and GR tissue depth in maternal sired lambs.

There was no effect (P>0.05) of GR tissue depth on CK, haptoglobin, cortisol, magnesium. There was no effect (P>0.05) of c-site fat depth on haptoglobin, lactate, AST, CK glucose, Magnesium, total protein, cortisol, sodium, NEFA.

4.3.4.3   **Loin weight**

NEFA and LLWT demonstrated a negative association, however this was only observed at the Katanning site (P<0.05). As LLWT increased from 200 to 500g, NEFA decreased by 0.22mmol/L from 1.71 ± 0.044 to 1.49 ± 0.043mmol/L. There was a small but significant association between plasma sodium and LLWT (P<0.05). As LLWT increased from 170 to 600g, plasma sodium decreased from 149.72 ± 0.26 mmol/L to 148.48 ± 0.36 mmol/L. There was no effect (P>0.05) of LLWT on AST, CK, BHOB, glucose, lactate, haptoglobin, magnesium, total protein or cortisol.

4.4   Discussion

4.4.1   Environmental factors influencing plasma indicators

Overall, environmental factors had the largest impact on all stress indicators. Differences in indicators were explained by production site, year and kill group differences in pre-slaughter management, reflecting on-farm nutrition, feed and water deprivation and acute stress. Adjusting for this complex array of effects during statistical analysis of plasma data is of paramount importance in order to make higher level conclusions regarding other factors influencing pre-slaughter stress.

Supporting the hypothesis, NEFA was elevated at slaughter compared to published normal levels; indeed levels were significantly higher than previously reported in sheep (Pethick et al., 1993). NEFA levels varied between sites and kill groups, which is likely to reflect differences in pre-slaughter feed deprivation (Pethick et al., 2005) as a result of on-farm curfews and time in lairage. In addition to the effects of feed deprivation, acute stress immediately pre-slaughter is likely to have contributed to the high levels of NEFA at slaughter. Ruminant adipose tissue is highly sensitive to adrenaline (Martin et al., 2011; McGilchrist et al., 2011) with high intensity handling shown to increase the NEFA response above the more chronic response associated with effects of transport and lairage (Sutherland et al., 2016). Consequently the elevated levels of NEFA observed in this study are to be expected and due to a combination of feed deprivation and acute stress stimulating lipolysis (Pethick et al., 2005).

Contrary to the hypothesis, BHOB and glucose levels were mostly within normal physiological range (Heitmann et al., 1987; Radostits et al., 2007; Lepherd et al., 2009). Previous work tends to indicate that feed deprivation up to 48 hours has only a small effect on BHOB concentrations in sheep as the substrate changes from rumen derived butyrate (fed) to mobilised NEFA during fasting (Warriss et al., 1989; Knowles et al., 1995; Fisher et al., 2010) and supports the moderate correlation observed between NEFA and BHOB. Increasing time off feed is associated with utilisation of hepatic glycogen (Jacob et al., 2009) and a progressive decline in plasma glucose concentration. The moderate negative correlation between glucose and NEFA concentration tends to support this assertion. However, it is likely that the confounding physiological effects of feed deprivation and pre-slaughter stress led to the normal glucose levels observed. Other than the extreme NEFA response evident at slaughter, the

results tend to indicate a normal homeostatic response to feed deprivation in lambs under commercial feed deprivation.

Supporting the hypothesis, plasma lactate levels were elevated above normal (Radostits et al., 2007) in this study, however they were significantly lower than previously reported in cattle at slaughter (Coombes et al., 2014) and comparable to levels reported in a recent study by Sutherland et al. (2016). A possible explanation is that peak lactate response to acute stress and associated adrenaline release (Martin et al., 2011; McGilchrist et al., 2011) was not captured by a single blood sample collected at slaughter, thus reducing its accuracy in measuring acute stress. In addition, plasma lactate can increase as a result of emotional stress and stimulation of the "flight or fight" response or alternatively be the result of exercise due to pre-slaughter yard movements. This causes a contraction-linked increase in the rate of muscle glycogenolysis (Gardner et al., 2001b) which will vary due to differences in yard design and facilities (Hemsworth et al., 2011) as well as handling practices (Cockram and Corley, 1991; Warner et al., 2007). Rates of glycogenolysis within muscle have been shown to be higher with increased muscle glycogen concentrations (Daly et al., 2006) which fluctuates between consignments (Jacob et al., 2005a). Thus, the lower lactate responses and environmental variation found in this study may reflect more consistent nutritional management in the months leading up to slaughter (Gardner et al., 2014). Overall the levels of lactate found in this study are largely representative of animals undergoing normal adaptation to physical exertion and systemic stress. In addition, a moderate negative correlation was found between lactate and NEFA. High levels of circulating NEFA due to fasting may have depressed muscle glycolytic activity, reducing the ability to dispose of mobilised muscle glycogen. This is due to conversion of NEFA into aerobic intermediate citrate which causes feedback inhibition of phosphofructokinase 1, sparing glucose utilisation (Randle et al., 1963).

Plasma cortisol levels in this study were markedly elevated above basal levels (Radostits et al., 2007), comparable to those previously reported for sheep during routine pre-slaughter procedures and probably contributed to significant changes in energy and fat metabolism as discussed above. Previous work has shown increases in cortisol with unloading and loading (Broom et al., 1996) therefore levels reported here may reflect stress associated with immediate pre-slaughter handling. Although lairage may allow animals to rest and acclimatise after transport (Liste et al., 2011), exposure to novel environments and feed deprivation (Ward et al., 1992) may have contributed to the high cortisol levels found in this study. Previous work by Sutherland et al. (2016) and Ekiz et al. (2012) showed similar results, with cortisol elevated in lambs post-transport and overnight lairage. In addition, time of blood sampling might have also influenced plasma cortisol concentration (Nazifi et al., 2005), due to the secretion of glucocorticoids in response to circadian rhythm. Levels reported in this study are likely to be within the expected range of lambs slaughtered under commercial practice.

As expected, plasma magnesium was within normal physiological range. However, there was considerable variation between sites, years and kill groups, most likely reflecting differences in feed availability and supplementation. Stress causes an increase in circulating levels of catecholamine's (adrenaline) which stimulates lipolysis and has been shown to cause decreases in plasma magnesium concentrations (Rayssiguier, 1977). This association may have contributed to the moderate negative correlation between magnesium and NEFA found in this study. Likewise, supplementation with magnesium has been shown to attenuate the stress response (D'Souza et al., 1998) by altering the release of stress hormones (Classen et al., 1986) and reducing neuromuscular stimulation as well as the secretion of acetylcholine by motor nerve impulses (Hubbard, 1973).

In line with the hypothesis average haptoglobin levels reported within this study are higher than published reference ranges (Lepherd et al., 2009). Circulating levels are normally negligible (Murata et al., 2004) with increases observed in response to inflammation, infection, trauma or stress and used in a clinical setting as a diagnostic tool for monitoring health and disease (Ceciliani et al., 2012). The

levels reported in this study are similar to levels reported in a study by Piccione et al. (2012) which assessed transport stress in ewes and complex stress in cattle (Lomborg et al., 2008). Alternatively, Arthington et al. (2003), showed that mixing had no impact on haptoglobin, highlighting the complex nature of the acute phase protein response to stress. The mechanism behind the stress-induced acute phase protein response is not known, but may be related to activation of the hypothalamic-pituitary-adrenal axis resulting in increased production of glucocorticoids (Alsemgeest et al., 1995). Feed deprivation and dexamethasone administration has also been shown to increase circulating haptoglobin levels (Yoshino et al., 1993). Thus, levels reported in this study are likely to reflect normal acute phase protein response to pre-slaughter management processes.

Rejecting the hypothesis, plasma sodium and total protein concentrations were mostly within normal physiological ranges in lambs at slaughter, indicating that lambs were largely not dehydrated at slaughter. Under best practice pre-slaughter pathways, water is available in lairage and ruminants tend to cope with long periods of water deprivation (Fisher et al., 2009). However, the effectiveness of supplying water to rehydrate lambs prior to slaughter is not well known (Jacob et al., 2006b) and not all lambs will drink water in unfamiliar environments (Knowles et al., 1993; Ferguson and Warner, 2008; Jongman et al., 2008) which may explain the variation/range in indicators observed. Moreover, plasma sodium concentrations were at the upper limits for reference ranges with 17% of lambs having plasma sodium concentrations indicative of dehydration (Radostits et al., 2007). While this is significantly less than the 50% reported under commercial conditions by Jacob et al. (2006c), it does indicate that further work is required to understand hydration status in lambs during the pre-slaughter period. The hydration status of lambs was less clear when evaluating TP concentrations with the proportion of lambs with elevated levels depending on which "normal" published values are used. The proportion of lambs with elevated TP ranged between 5% (Radostits et al., 2007) and 80% (Lepherd et al., 2009). Although, in this study total protein was also found to be also influenced by breed and sex (see below), so may be affected by other physiological states.

Partially supporting the hypothesis, plasma AST levels in lambs were within published reference ranges for sheep (Radostits et al., 2007) indicating no long term muscle damage had occurred (Kaneko et al., 2008). However, a large proportion of lambs had CK levels above normal levels indicating that some degree of tissue damage had occurred in the pre-slaughter period (Boyd, 1988). Approximately 72% of lambs had CK levels above 280 IU/L (Radostits et al., 2007) and 36% above 454 IU/L (Lepherd et al., 2009). Elevations in CK are widely considered specific and sensitive measures indicators of acute muscle damage in ruminants (Russell and Roussel, 2007) due to their high concentration and activity in skeletal muscle. Differences in CK between sites and kill groups is likely to reflect differences in handling, muscular exertion (Berg and Haralambie, 1978), transport (Warriss et al., 1995; Fisher et al., 2010) and/or bruising (Tarrant, 1990). However, rapid elevations in CK have also been observed with pre-slaughter handling (Hemsworth et al., 2011) and adrenergic stimulation (McVeigh and Tarrant, 1981), thus the acute stress response may explain the large proportion of lambs with elevated CK levels found in this study.

4.4.2   Effect of breed and sex on plasma indicators

Breed differences in stress susceptibility (Gardner et al., 1999a) and whole body fatness (Chilliard et al., 2000) are likely to have contributed to the higher NEFA response in Merino sired lambs. Moreover, the lower BHOB levels in Terminal sired lambs are likely to reflect lower fat mobilisation under nutritional restriction. In addition, Merino sired lambs and female lambs had higher plasma lactate concentrations than castrate males, reflecting higher stress susceptibility (Gardner et al., 1999a) in these cohorts. Differences in adipose tissue and glycogen metabolism have been identified in sheep and cattle that are selected for increased carcass muscling (Martin et al., 2011; McGilchrist

et al., 2011), while female lambs have been reported as more stress susceptible compared to wethers (Hernandez et al., 2010).

Total protein concentrations were lower in Terminal sired lambs, Border-Leicester dams, wethers and single born lambs. Although this effect was small, previous work has shown that total protein concentrations may be affected by protein degradation (Oddy and Neutze, 1991). Richardson and Herd (2004) showed that more efficient cattle with lower residual feed intake had lower plasma total protein indicating lower rates of protein catabolism. This may explain the lower total protein in male lambs and in high growth, muscular and lean breeds such as Terminal and Maternal breed types compared to Merinos.

The sire type differences in CK concentration may reflect greater muscle mass or a greater susceptibility to physical damage of muscle tissue during the pre-slaughter slaughter handling and yarding in Terminal sired lambs. However, smaller framed lambs may be more susceptible to bruising during the pre-slaughter pathway, due to hierarchical differences (Warriss et al., 1990; Sutherland et al., 2009). This may explain the small increase in AST in female compared to male lambs. Alternatively, higher rates of protein catabolism could have also contributed to the higher AST concentrations in females (Richardson and Herd, 2004).

4.4.3 Effect of carcass weight and composition on plasma indicators at slaughter

Slaughter levels of the metabolites glucose, NEFA, BHOB and lactate were affected by HCWT, GR tissue depth and c-site fat depth. It would be expected that as size of the adipose depot increases proportionally with carcass weight, so would the lipolytic response (Chilliard et al., 2000). However, in this study a mainly negative association between NEFA and BHOB and carcass weight and fatness traits was found. This suggests that other mechanisms may regulate pre-slaughter fat turnover. Ruminant adipose tissue is highly sensitive to adrenaline and previous work by Gregory et al. (1986) showed that the adipose tissue of lean sheep has greater levels of vascularisation, which may potentiate the rate of adrenaline-induced lipolysis. This may underpin the negative association between NEFA and BHOB with GR-tissue depth. In contrast, in Merino sired lambs, NEFA and BHOB demonstrated a positive association with GR-tissue depth, suggesting that fat turnover at slaughter may respond differently in this breed type.

Plasma lactate and glucose was higher in lambs with higher HCWT and GR tissue depth also suggesting that muscle glycogen turnover may be higher in heavier and fatter lamb phenotypes (Martin et al., 2011; McGilchrist et al., 2011).

Increasing LLWT (corrected for HCWT) was associated with a decline in NEFA concentration, an effect that was observed at the Katanning site only. This indicates that more muscular phenotypes may have a reduced NEFA response at slaughter. This contradicts previous work that has shown that selection for increased muscling increases the adipose tissue response to adrenaline (Martin et al., 2011; McGilchrist et al., 2011). However, levels of NEFA at slaughter are likely to reflect the combined effects of adrenergic stimulation and feed deprivation. Plasma NEFA was significantly higher at the Katanning site, suggesting that at higher levels of stress and/or feed deprivation, fat turnover may be greater in lighter and leaner lambs as shown by Thompson et al. (1987).

Plasma cortisol demonstrated a positive association with HCWT. Previous work has found that proportionately fatter sheep had a greater cortisol response to ACTH administration (Knott et al., 2010). Whilst an association with carcass fatness was not demonstrated in this study, it indicates that

higher cortisol levels at slaughter are modulated by intrinsic animal factors other than just stress, such as body composition and feed efficiency.

The negative association between plasma sodium and LLWT was small, however this could reflect the impact that dehydration has on muscle weight. Jacob et al. (2006b) found that water deprivation reduced muscle weights and muscle fibre cross sectional area in lambs undergoing water deprivation. The associations found between total protein and sodium with GR tissue depth in the current study were unexpected. Dehydration may stimulate fat breakdown to increase the availability of metabolic water (Chedid et al., 2014), however given the duration of water deprivation in the current study and marginal dehydration evident it is unlikely that it would have caused an effect.

4.4.4   Association between plasma indicators and kill order

A new finding in this study was the association between kill order and plasma glucose concentration. The kill-order term within our model describes the order that lambs were slaughtered within each kill group, with chain speeds running at approximately 8-10 lambs per minute. Under this system a kill group with 200 lambs would be processed in approximately 20 - 30 minutes. Although the effect of kill order on plasma glucose and lactate was numerically small it may reflect the duration of exposure to immediate pre-slaughter processes during the lead up to the stunning restrainer, which are likely to cause high levels of stress. Hemsworth et al. (2011) suggested that animals that take longer to be moved from lairage to stunning may have higher stress levels because they may be difficult to handle, receive more handling or may be fearful of humans or the facilities. In addition, animals with lower hierarchy are likely to be more stressed under close confinement (Eldridge et al., 1988; Warriss et al., 1990). Therefore, lambs killed later within a kill group may have a greater exposure to stress underpinning the link between kill order and acute stress.

Alternatively, as kill order increased, there was a decrease in plasma BHOB and CK concentration, although this association was inconsistent across sites and years. The mechanistic reason for this is unclear, but as kill order may act as a proxy for time and as NEFA concentrations are very high, the negative association between BHOB and kill order may reflect rate of clearance. Previous work has shown that adrenergic stimulation increases plasma CK (McVeigh and Tarrant, 1981) and has previously been shown to be positively correlated with time spent within a race (Hemsworth et al., 2011). Although this was not observed in the current study, elevations in CK may also be transient (Grigor et al., 1997; Pettiford et al., 2008).

4.5   Conclusion

This is the first large scale study that has examined physiological indicators of acute and chronic stress in Australian lamb indicators under commercial conditions at slaughter. Concentrations of metabolic indicators in this study were as expected with levels reflecting a combination of feed deprivation and acute stress. More sensitive indicators such as cortisol and haptoglobin levels were similar to those reported in previous studies at slaughter. Overall lambs were largely not dehydrated with 17% having above normal plasma sodium levels. However, it is likely that this would be higher on a more commercial scale. Large percentages of lambs within the current study had elevated CK levels and while this is likely to reflect a combination of both muscle damage and exertion, acute stress may also have influenced the result and warrants further investigation. The significant elevation in plasma NEFA at slaughter also requires further investigation into the combined effects of feed deprivation and acute stress at slaughter in prime lambs. In addition, female lambs and the Merino breed appear to be more sensitive to acute stress and feed and water deprivation as indicated by overall higher levels of plasma measures of stress at slaughter. Plasma indicators at slaughter were also affected by carcass weight and body composition at slaughter. Variation in indicators exists as a result of

environmental factors and likely to reflect differences in pre-slaughter management, which cannot be understood from this study. However, it is important to be able to account for the variation due to these factors in order to assess if associations exist between pre-slaughter stress and meat quality and yield. As such, the association between loin ultimate and tenderness with plasma indicators of stress will be investigated in chapter 5 and 6.

Chapter 5   Association between loin ultimate pH and plasma indicators of pre-slaughter stressors in Australian lamb

5.1   Abstract

The purpose of this study was to test if associations exist between plasma indicators of acute and chronic stress and lamb ultimate pH. Blood was collected at exsanguination from 2877 lambs from the Meat and Livestock Australia Genetic Research flock with a suite of indicators analysed. Ultimate pH was measured in the loin (M. *longissimus lumborum*) at 24 h post-slaughter. There was a positive association ($P<0.05$) between ultimate pH and plasma glucose and lactate concentrations, which indicates that opportunities exist to reduce variation in ultimate pH by reducing stress in the pre-slaughter period. These effects were small by comparison to production factors, however further understanding of how to best manage lambs in the pre-slaughter period is required in order to minimise stress and maximise lamb wellbeing and meat quality.

5.2   Introduction

Previous work has demonstrated that pre-slaughter stress has a negative impact on ultimate pH and is frequently attributed to an increased incidence of high pH (> 5.7) meat (Tarrant, 1989; Warriss, 1990). Meat from carcasses with an ultimate pH above 5.7 has a dark colour, variable tenderness, poor flavour and cooking attributes (Mendenhall, 1989; Cox et al., 1994) and reduced shelf life (Ferguson et al., 2001; Thompson, 2002).

Whilst best practice pathways under the Meat Standards Australia (MSA) grading systems promote reduced stress during the pre-slaughter period, it is inevitable that stress can still occur (Ferguson and Warner, 2008), therefore further understanding of the link between stress and meat quality is required.

Pre-slaughter stress is multifactorial as lambs are exposed to many different processes from farm to slaughter. Increased handling, novel environments and changes to social structure may cause acute and chronic psychological stress. Lambs also undergo periods of feed and water deprivation and may experience periods of muscular exertion during mustering or transport (McVeigh et al., 1982; Tarrant, 1989; Harman and Pethick, 1994; Apple et al., 2005; Warner et al., 2005; Mach et al., 2008; Jacob et al., 2009). These stress events are associated with a multitude of physiological and metabolite changes in plasma, however limited studies have been conducted in lamb (Sutherland et al., 2016) attempting to relate plasma indicators of stress with ultimate pH.

Acute stress results in the secretion of adrenaline which accelerates pre-slaughter muscle glycogen turnover (Gardner et al., 2014) through its activation of glycogen phosphorylase (Franch et

al., 1999) and inhibition of glycogen synthase (Roach, 1990), resulting in elevated plasma lactate concentration. Adrenaline also causes increased rates of adipose tissue lipolysis, liver glycogenolysis and liver gluconeogenesis (Kuchel, 1991), leading to increased circulating glucose and non-esterified fatty acid concentrations. Acute stress also activates the hypothalamic pituitary axis leading to cortisol release. Many studies have shown elevated cortisol levels in response to pre-slaughter factors such as transport and handling (Warriss, 1990).

The acute phase response is an innate response activated by harmful stimuli such as inflammation, tissue damage and infection (Baumann and Gauldie, 1994; Cray et al., 2009; Piccione et al., 2012). During the acute phase response, cytokines released from macrophages and other cell types are transported to the liver, where they induce synthesis of acute phase proteins, such as haptoglobin in hepatocytes, which then act to remove inflammatory stimuli, promote healing and restore homeostasis (Baumann and Gauldie, 1994). Acute phase proteins such as haptoglobin have been used mainly as indicators of disease and inflammation in livestock, however there is evidence that haptoglobin may be a useful biomarker of stress with previous work showing that transportation and mixing of animals (Arthington et al., 2003; Lomborg et al., 2008; Piccione et al., 2012) cause elevations in blood haptoglobin levels.

Lambs may experience increased physical demands during the pre-slaughter period due to mustering, handling and transport. Increases in circulating creatine kinase (CK) and aspartate aminotransferase (AST) can be seen with unaccustomed exercise, transport handling stress and low-level trauma or bruising (Tollersrud et al., 1971; Tarrant, 1990; Pettiford et al., 2008; Sutherland et al., 2009; Fisher et al., 2010) and have previously been associated with increased ultimate pH (Warriss, 1984). In support of this notion, Warner et al. (2005) showed that exercise in the immediate pre-slaughter period caused higher ultimate pH in lamb.

Feed deprivation causes significant increases in circulating non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHOB), indicative of increased lipolysis, yet research tends to show that feed deprivation *per se* has minimal impact on muscle glycogen and ultimate pH (Daly et al., 2006). Similarly, Lowe et al. (2002b) showed that water deprivation even under heat stress, had no effect on ultimate pH or meat colour in lamb, while Jacob et al. (2006b) showed it slightly improved lamb ultimate pH. Ruminants have been shown to be relatively resilient to periods of water restriction, with minimal changes in indicators of dehydration such as plasma sodium and total protein (Parrott et al., 1996; Jacob et al., 2006c; Fisher et al., 2010).

Magnesium has been shown to attenuate the stress response (Hubbard, 1973) by reducing catecholamine and glucocorticoid secretion (Kietzmann and Jablonski, 1985; Classen et al., 1986). Circulating levels of plasma magnesium reflect the nutritional intake of an animal with supplementation shown to reduce stress-mediated glycogen losses in the pre-slaughter period and prevent high ultimate pH (D'Souza et al., 1998; Gardner et al., 2001a).

The objective of this study was to assess the association between plasma indicators that reflect acute and chronic stress and lamb ultimate pH. Several hypotheses were tested: 1. Indicators of acute stress including plasma lactate, glucose, cortisol, CK and AST concentrations at slaughter will be positively associated with an increased ultimate pH at 24 hours post-slaughter; 2. Feed deprivation reflected by increased NEFA and BHOB levels, and water deprivation reflected by increased sodium and total protein, will not be associated with ultimate pH; 3. Increasing plasma magnesium concentration at slaughter will be associated with a decrease in ultimate pH.

5.3   Materials and Methods

This study was approved by the Department of Agriculture Western Australia Animal Ethics Committee #2-13-07

5.3.1   Experimental design, animals and pre-slaughter management

The design of the Sheep CRC Information Nucleus Flock (INF), now referred to as the Meat and Livestock Australia Genetic Resource flock has been described previously (Fogarty et al., 2007; Van der Werf et al., 2010).

Wether and female lambs (n = 2877) were produced from artificial insemination of Merino, Border Leicester x Merino (BLM) and commercial maternal (CM) dams over a two year period (2013 and 2014) at the Katanning, Western Australia (WA) and Kirby, New South Wales (NSW) research sites. The lambs were the progeny of 394 different sires, which comprised terminal sire types (Ile De France, Poll Dorset, Suffolk, Texel, Charolais and White Suffolk), maternal sire types (Booroola, Border Leicester, Coopworth, Dohne Merino and Prime SAMM) and Merino (Merino and Poll Merino) sires, representing the major production types in the Australian sheep industry. Semen from all three sire types was used to artificially inseminate Merino dams, while only semen from maternal and terminal sires was used to inseminate cross-bred (BLM and CM) ewes. Maternal lambs sent to slaughter comprised very few females (which were mostly retained for breeding purposes), meaning effective comparisons between sexes could only be made within the terminal and Merino sired lamb groups and maternal sired lambs from Merino dams (Table 5-1). Dam breed comparisons could only be made within maternal sired male lambs and in terminal sired male and female lambs (Table 5-1). The lambs were maintained on extensive pasture grazing, with grain, hay or feedlot pellets supplemented when pasture supply was limited.

At both the Katanning and Kirby flocks, lambs were consigned to 17 different kill groups and slaughtered at a target carcass weight of 21-22kg. Given selection for slaughter was made based on weight, the average age of lambs in each kill group varied between 193 and 416 days old at slaughter, however within individual kill groups the age range was smaller varying between 16 and 33 days.

Prior to slaughter, lambs were yarded on farm, taken off feed and water for between 5 and 18 hours before being transported to one of three commercial abattoirs (1 in WA and 2 in NSW). For the Katanning flock transportation lasted for 0.5 hours compared to 1.5 – 2.5 hours for the Kirby flock. Lambs were held overnight in lairage, with free access to water and slaughtered the following day.

Table 5-1 Number of lambs measured for M. *longissimus lumborum* (loin) pH at 24 hours post slaughter (pH24LL) at each flock, within each year, sex, dam breed & sire type

Flock	Year		Sex Dam breed (Sire type)											
	2013	2014	F Merino (Mat.)	M Merino (Mat.)	M CM (Mat.)	M BLM (Mat.)	F Merino (Mer.)	M Merino (Mer.)	M Mer. (Ter.)	F Mer. (Ter.)	M CM (Ter.)	F CM (Ter.)	M BLM (Ter.)	F BLM (Ter.)
Kirby	1128	721	37	98	n/a	43	80	194	273	268	n/a	n/a	311	354
Katanning	524	504	2	118	17	5	34	199	156	215	78	109	8	19
Total	1652	1225	39	216	17	48	114	393	429	483	78	109	319	373

F: female lamb; M: male (wether) lamb
CM: Commercial Maternal dam breed; BLM: Border Leicester x Merino dam breed.
(Mat.): Maternal sire type; (Mer.): Merino sire type; (Ter.): Terminal sire type. n/a: not applicable

5.3.2 Blood collection and processing

Blood samples were collected into 9 mL lithium heparin Vacuette® tubes (Greiner bio-one, Austria) from each lamb at slaughter, immediately following exsanguination. Tubes were immediately placed in ice for between 2-5 hours until centrifugation at 3000 rpm for 15 minutes. Following centrifugation, plasma samples were pipetted in two separate aliquots and stored in 2mL tubes at -80°C until processing.

Once aliquot samples were thawed, they were gently inverted several times before a sub-sample (approximately 100 µL) was pipetted into 1.7 mL sample cups (Greiner Bio-one, Kremsmüster, Austria). Plasma lactate, glucose, NEFA, magnesium, total protein, CK, AST and sodium were analysed on aliquot one at Murdoch University, Perth, WA, performed using an Olympus AU400 automated chemistry analyser (Olympus Optical Co. Ltd, Melville, NY). Commercially available reagent kits were used to analyse plasma lactate, glucose, magnesium, total protein, CK, AST (Olympus Diagnostics, Tokyo, Japan), Sodium (Randox Laboratories kit, County Antrim, UK) and NEFA (C Kit Wako Pure Chemical Ind., Osaka, Japan). Aliquot two was used to analyse plasma haptoglobin, BHOB and cortisol. Plasma haptoglobin and BHOB were analysed at the Western Australian Department of Agriculture (DAFWA) Animal Health Laboratories, South Perth, Australia. Plasma β-hydroxybutyrate was analysed using the commercial reagent kit (Randox Laboratories kit, County Antrim, UK). Plasma haptoglobin was determined using the method described by Eckersall et al. (1999). Analyses were performed using an Olympus AU400 automated chemistry analyser (Olympus Optical Co. Ltd, Melville, NY). Plasma cortisol levels were determined on a subset of samples (n = 471) using chemiluminescent immunoassay performed using an Immulite® 2000 Immunoassay system (Siemens, Germany) at Vetpath Veterinary services, (Perth, Australia).

5.3.3 Carcass processing

Following slaughter, lambs were dressed according to AUS-MEAT standards (AUS-MEAT, 2005) and hot carcass weight (HCWT) was recorded at an average of 23.2 kg (Std Dev = 2.94). All carcasses underwent medium voltage electrical stimulation to optimize pH decline such that the carcass loin temperature at pH6 was between 18 - 25°C (Pearce et al., 2010) and were chilled overnight (3 - 4°C) before sampling.

At 24 hours post-mortem M. longissimus lumborum (loin) pH (pH24LL) was measured as described by Pearce et al. (2010) on the left caudal section of the muscle at the lumbar-sacral junction, where a small 4 cm incision was made to identify the caudal end of the loin muscle. Muscle pH was measured using an Orion 250A pH meter (cat. no. 0250A2, Orion Research Inc., Boston, MA, USA) fitted with a glass body, spear tipped probe (cat. no. 8163BN, Orion Research Inc., Boston, MA, USA). The pH meter was regularly calibrated using buffers with known pH of 4 and 7.

5.3.4 Statistical analysis

Loin ultimate pH (pH24LL) data were analysed using linear mixed effect models in SAS (SAS Version 9.1, SAS Institute, Cary, NC, USA). The base model included fixed effects for flock, year, kill group within flock by year, sire type, age of dam, sire type by flock, sire type by year, sex and dam breed within sire type and sire type by flock by year. Sire identification and dam identification by year were included as random terms. All relevant interactions between fixed effects were tested and non-significant terms (P>0.05) were removed in a stepwise manner. Plasma indicators and kill order were included separately within the base model as covariate terms along with relevant interactions with other terms in the base model. A subset of animals

(n = 471) from one year (2013) had plasma cortisol analysed. This covariate was also tested as described above, however in this case the base model for pH24LL did not include the fixed effect for year, kill group was tested within flock only and the only random effect fitted was sire identification. All relevant interactions between covariates and fixed effects were tested and non-significant terms (P>0.05) were removed in a stepwise manner.

5.4   Results

Raw means and standard deviations for pH24LL and covariates analysed are presented in Table 5-2. Outcomes of the base model are presented in Table 3. The average pH24LL was 5.63 and the proportion of variance in pH24LL described by the base model (Table 5-3) was 26%. Predicted least square means (± SE) for the significant effects of sex by dam breed within sire type for pH24LL are presented in Table 5-4.

Flocks differed in their pH24LL values although this varied each year (P<0.01, Table 3). There was greatest variation in the Katanning flock which for the 2013 year had an average pH24LL of 5.72 ± 0.007, compared to 5.63 ± 0.007 for the 2014 year. There was less variation in pH24LL at the Kirby flock between years, which differed by 0.03 pH units from 5.64 ± 0.008 and 5.61± 0.006 for the lambs killed in 2013 and 2014 respectively.

Within each year at each flock, there was also variation in pH24LL between kill groups, which differed by as much as 0.15 pH units. The pH24LL for kill groups ranged from as high as 5.81 ± 0.010 in Katanning in 2013 to as low as 5.56 ± 0.013 in Kirby in 2013.

Table 5-2 Descriptive statistics including mean, standard deviation and range of raw pH24LL and plasma indicator data analysed.

Variable (units)	Mean	SD	Range
pH24LL	5.63	0.13	5.34-6.88
Covariates (units)			
Non-esterified fatty acid (mmol/L)	1.19	0.54	0.165-3.26
Glucose (mmol/L)	4.67	0.94	1.978-10.503
Lactate (mmol/L)	3.48	2.29	0.467-16.427
Magnesium (mmol/L)	0.90	0.14	0.356-1.627
Creatine Kinase (IU)	463.29	341.57	83.07-3731.8
Aspartate Aminotransferase (IU)	152.63	42.79	61.82-613.73
Total Protein (g/L)	68.65	6.21	30.15-93.32
Haptoglobin (mg/mL)	0.42	0.38	0.01-4.82
β-hydroxybutyrate (mmol/L)	0.43	0.15	0.11-1.04
Cortisol (nmol/L)	152.27	62.57	5.5-395

Table 5-3 F values, P values and numerator and denominator degrees of freedom (NDF, DDF) for the effects of the base linear mixed effects model of pH24LL of the M. *longissimus lumborum* of lambs.

Effect	NDF,DDF	F-values
Flock	1, 387	82.01**
Year	1, 1789	80.09**
Kill group (flock*Year)	1, 387	37.23**
Sire type		12.29**
Sex dam breed (Sire type)		2.24*
Flock*Year		41.65**
Sire type*Year		3.6*

* $P<0.05$; ** $P<0.01$

Sire type had a small effect on pH24LL, however this effect differed across years with pH24LL being highest in 2013 across all sire types. In 2013 terminal sired lambs had the lowest ($P<0.05$) pH24LL value of 5.65 ± 0.005 compared to maternal (5.70 ± 0.014) and Merino sired lambs (5.71 ± 0.011). Similarly, in 2014 pH24LL was lowest ($P<0.05$) in terminal sired lambs (5.61 ± 0.007) compared to Merino sired lambs (5.64 ± 0.009). However there was no difference ($P>0.05$) between terminal and

maternal (5.61 ± 0.013) sired lambs. In both 2013 and 2014 there was no difference (P>0.05) in pH24LL between Merino and maternal sired lambs.

Table 5-4 Predicted means and standard error (SE) for pH24LL for the fixed effects for sex and dam breed within sire type

Category	Mean ± (SE)
F Merino (Maternal)	5.69 (0.020) [a]
M Merino (Maternal)	5.63 (0.008) [bc]
M CM (Maternal)	5.63 (0.029) [bc]
M BLM (Maternal)	5.66 (0.018) [bc]
F Merino (Merino)	5.67 (0.012) [d]
M Merino (Merino)	5.68 (0.007) [d]
F Merino (Terminal)	5.63 (0.006) [eg]
F CM (Terminal)	5.62 (0.012) [e]
F BLM (Terminal)	5.61 (0.007) [eh]
M Merino (Terminal)	5.63 (0.006) [fg]
M CM (Terminal)	5.65 (0.014) [f]
M BLM (Terminal)	5.63 (0.008) [fh]

a,b,c,d,e : letters that differ between rows within siretype are different (P<0.05).
F: female lamb; M: male (wether) lamb
CM: Commercial Maternal dam breed; BLM: Border Leicester x Merino dam breed

The only impact of sex on loin pH24LL was observed in maternal sired lambs with Merino dams and terminal sired lambs with CM dams (P<0.05, Table 3). In the maternal sired lambs, female lambs had pH24LL values 0.055 units higher than male lambs. The opposite was observed in terminal sired lambs where the pH24LL of female lambs was 0.04 pH units lower than male lambs. There was no effect of dam breed on pH24LL (P>0.05).

An association was observed between pH24LL and kill order, however this was inconsistent, varying across flocks (P<0.05). At the Katanning flock, as kill order increased from 0 to 250 there was a 0.06 pH unit decrease from 5.72 ± 0.011 to 5.66 ± 0.009, after which a plateau was reached up to a kill order of 300. The opposite was observed at the Kirby flock as kill order increased from 0 to 100 there was no change in pH24LL (5.61 ± 0.007 to 5.62 ± 0.006), however from a kill order of 100 to 250 there was a 0.05 pH unit increase from 5.62 ± 0.006 to 5.67 ± 0.013.

5.4.1   Association between plasma indicators and pH24LL

There was a significant positive association between pH24LL and glucose concentration (P<0.01, Figure 5-1). As plasma glucose concentration increased from 2mmol/L to 10mmol/L, pH24LL increased by 0.16 pH units from 5.60 to 5.76.
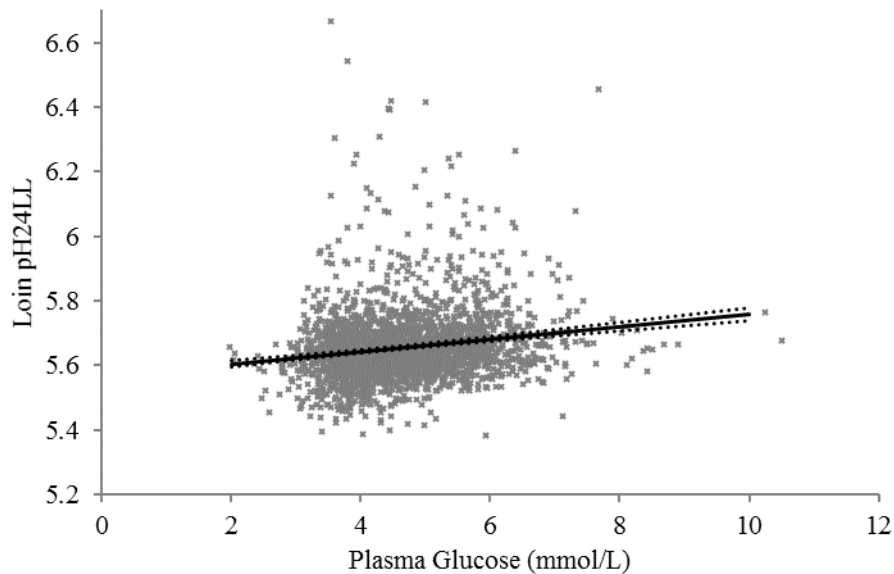
Figure 5-1 Association between plasma glucose at slaughter (mmol/L) and loin pH24LL. Lines represent ls means ± standard error. Icons (×) denote lamb residuals from the response surface.

On average, increasing plasma lactate was associated with an increase in loin pH24LL by 0.09 units across the range of lactate from 1mmol/L to 15mmol/L. The magnitude of this effect across the range of plasma lactate varied across flocks and years (P<0.05, Figure 5-2), ranging from no association in Katanning 2013, to an increase of 0.11 units across the 14 mmol/L range of lactate in Kirby 2014.

There was a curvilinear association between pH24LL and haptoglobin, however this was only observed in 2013 (P<0.05). As haptoglobin increased from 0.1mg/mL to 1.0mg/mL there was a there was a 0.06 unit increase in pH24LL from 5.60 ± 0.009 to 5.65 ± 0.010 after which there was a plateau.

The association between pH24LL and magnesium was inconsistent and differed between flocks and years (P<0.05, Figure 5-2). At the Kirby flock in 2013 and Katanning flock in 2014 there was a positive association between pH24LL and plasma magnesium. The opposite association was observed at the Katanning flock in 2013 and there was no association (P>0.05) between pH24LL and plasma magnesium at Kirby in 2014.

The association between pH24LL and plasma sodium was only observed at the Katanning flock and varied between years (P<0.05, Figure 5-2).

The association between pH24LL and BHOB was significant (P<0.05) but inconsistent, varying across flocks (P<0.05). At the Katanning flock, as BHOB increased from 0.1mmol/L to 0.7mmol/L there was a 0.09 pH unit increase in pH24LL from 5.61 ± 0.020 to 5.69 ± 0.009 after which a plateau was reached from 0.7mmol/L to 1.0mm/L. The opposite was observed at the Kirby flock as BHOB increased from 0.1mmol/L to 0.6mmol/L, pH24LL decreased from 5.68 ± 0.015 to 5.62 ± 0.007. From 0.6mmol/L to 0.8mmol/L there was a small increase in pH24LL up to 5.65 ± 0.020 mmol/L

There was an association between pH24LL and AST, however this was only observed at the Katanning flock (P<0.05). As AST increased from 70IU to 300IU there was a 0.06 pH unit decrease in pH24LL from 5.68 ± 0.011 to 5.63 ± 0.023.

There was a trend (P=0.062) for an association between pH24LL and plasma cortisol, however this was only observed at the Kirby flock. As plasma cortisol increased from 50nmol/L to 300nmol/L there was an increase in pH24LL from 5.62 ± 0.018 to 5.68 ± 0.021.

There was no association between plasma NEFA, CK, TP and concentration with pH24LL.
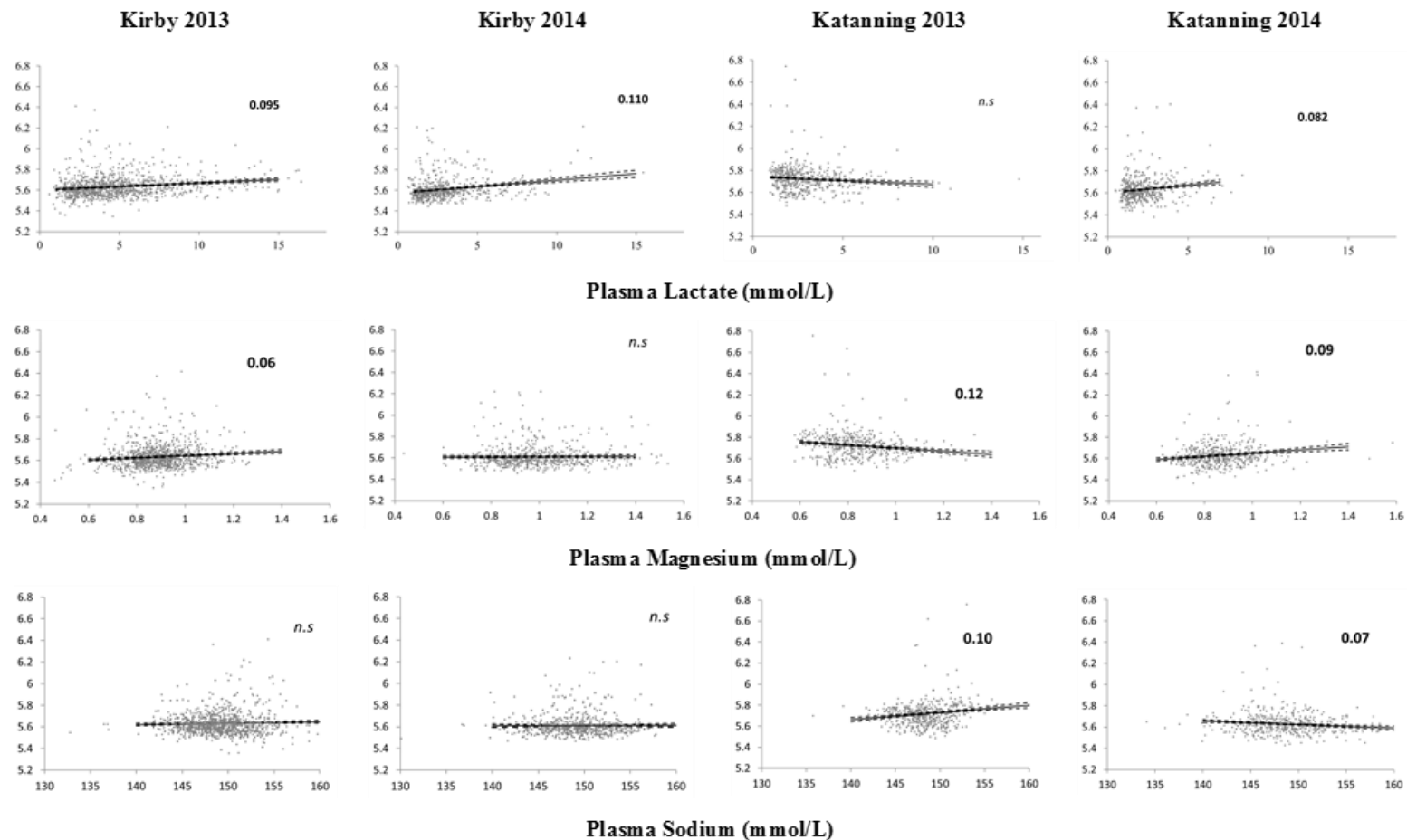
Figure 5-2 Table of figures showing association between pH24LL (y axis) and covariate effects: plasma lactate, magnesium and sodium at slaughter (mmol/L) at the Kirby and Katanning flocks in 2013 and 2014. Solid lines within figures represents the predicted least squared means, while dashed lines represent the standard error. Icons (×) denote lamb residuals from the response surface. The number in bold above each figure represents the unit change of pH24LL across the listed range in each covariate. n.s denotes non-significance.

5.5 Discussion

5.5.1 Association between plasma indicators of stress and pH24LL

In line with the hypothesis, increasing plasma lactate and glucose at slaughter were associated with increased loin ultimate pH in lamb, supporting the well-established link between acute stress and ultimate pH (Tarrant, 1981). Muscle glycogenolysis and hepatic glycogenolysis and gluconeogenesis are stimulated by adrenaline (Kuchel, 1991) explaining the associated increases in blood lactate and glucose concentrations. Flock differences in lactate and glucose response are likely to reflect a combination of interacting pre-slaughter animal factors. For example, variation in levels of acute stress and level of muscular activity pre-slaughter (Pethick et al., 1991; Gardner et al., 1999b) are likely to influence the levels of these metabolites in the live animal. The total amount of stored glycogen may also have affected the plasma lactate response. Previous work by Daly et al. (2006) demonstrated an association between increased glycogen storage and more rapid rates of pH decline post-mortem, which is indicative of increased rates of glycogen mobilisation and conversation to lactic acid. In a live animal this would be evident as increased lactate entry into plasma (Daly et al., 2006).

Contrary to the hypothesis, there was a significant association between pH24LL and BHOB. However, the magnitude of change in pH24LL across the range of BHOB was small and differed between flocks, suggesting that multiple mechanisms may be driving levels of BHOB as well as the association with ultimate pH. At the Katanning site, the positive association between pH24LL and BHOB may reflect greater lipolysis due to exposure to stress (adrenaline) as a result of elevated NEFA levels (Bassett, 1970). Several authors have reported an association between longer lairage times and incidence of dark cutting (Jacob et al., 2005a; Toohey and Hopkins, 2006), due to greater exposure to stress events prior to slaughter. This would increase the mobilisation of muscle glycogen (Tarrant, 1989) further elevating pH24LL. At the Kirby flock, there was a weak negative association between pH24LL and BHOB. Circulating levels of BHOB in non-fasted animals also reflect rumen derived butyrate (Bergman, 1990b) indicating a more positive energy balance. As such, these lambs may have also had more muscle glycogen, thus exceeding the minimum threshold required to reach an acceptable pH at 24 hours post-slaughter. Overall, the multiple confounding factors that could contribute to BHOB concentrations in the pre-slaughter period limit its use as an indicator of ultimate pH in lamb.

The small and inconsistent associations observed between plasma sodium, magnesium, AST, haptoglobin and cortisol with ultimate pH at slaughter suggests that they have limited use as indicators of stress response at slaughter. The levels of sodium found in this study indicate that some animals were dehydrated (Radostits et al., 2007), thus to maximise animal welfare water should be made more readily available. Higher stress in lairage can reduce drinking (Parrott et al., 1987) due to unfamiliarity of the environment or limited access to watering facilities (Thompson et al., 1987; Knowles et al., 1993; Ferguson and Warner, 2008). Further controlled studies are required in this area to fully understand the role of dehydration on lamb meat quality.

The small and inconsistent association between ultimate pH and magnesium may reflect the interaction between on-farm nutrition, mineral supplementation status and stress at slaughter (Gardner et al., 2014). However, as this experiment was not designed to investigate the impact of nutrition it is difficult to draw conclusions about the specific effects of nutritional regimes. Yet, the negative association observed at the Katanning flock in 2012 suggests that in at-risk groups, higher plasma magnesium levels may have a protective effect via reducing the impact of stress mediated glycogen losses (Gardner et al., 2001a).

In this study there was a negative association between pH24LL and AST, although only at the Katanning flock. This was unexpected as previous work has shown that elevations in AST are associated with exercise and muscle damage (Kaneko et al., 2008), which would tend to indicate a higher level of pre-slaughter stress. However, plasma AST levels in lambs were within published reference ranges for sheep (Radostits et al., 2007), which suggests that muscle stress may not have been high enough to cause significant turnover of muscle glycogen.

In line with the hypothesis there was a positive association between ph24LL and haptoglobin however, this effect was small and only observed in 2013. Levels of haptoglobin are normally negligible in plasma but increases similar to the current study have been observed as a result of transport, restraint and isolation (Lomborg et al., 2008; Pascual-Alonso et al., 2016). As haptoglobin is a highly sensitive indicator of stress, changes in concentration are likely to occur at a lower stress threshold compared to muscle glycogen turnover and may explain the limited association with pH in this study.

Partially supporting the hypothesis, there was a trend for increasing cortisol to be associated with higher ultimate pH. Elevated cortisol levels are associated with acute stress and have been found to be elevated in response to transport and handling (Shaw and Tume, 1992; Knowles et al., 1995). However, natural fluctuations in cortisol concentration due to its circadian rhythm (Chrousos, 1998) as well as individual variation between animals (Moberg, 1987) may have limited its accuracy as an indicator of stress.

The results of this study show that there was an association between plasma indicators of pre-slaughter stress and ultimate pH in lamb. It is well established that in order to maximise the concentration of muscle glycogen at slaughter and reduce the risk of dark cutting, pre-slaughter stress must be minimised (Tarrant, 1981). Adequate muscle glycogen concentrations are also essential to buffer the effects of pre-slaughter stress (Pethick et al., 2000; Gardner et al., 2014).

Despite relatively elevated levels found for most of the indicators measured, the association between plasma indicators and pH24LL is relatively small in comparison to the production and environmental factors reported on in this study. To demonstrate this further, the plasma indicators only described 10% of the variation in pH24LL (R^2 = 0.098, RMSE = 0.127).

Therefore, it is unlikely that plasma indicators could be used to predict high ultimate pH for individual carcasses. Importantly, a combination of stressors and physiological mechanisms contribute to levels of plasma indicators at slaughter. Thus, the stress response can be non-specific and highly variable (Bray et al., 1989; Broom et al., 1996) and may have contributed to the small effects seen in this study. Moreover, ultimate pH does not increase above 5.5 until glycogen levels in muscle pre-slaughter fall below about 0.6-0.7%. Given that the level of glycogen in the muscle of lamb can be as high as 2% (Pethick and Rowe, 1996) this suggests that ultimate pH does not change even in the face of substantial mobilisation of glycogen from muscle. The practical outcome of this study was to highlight that stress may play a role in determining ultimate pH in lamb on an industry level, with further work required to understand and mitigate the effects of pre-slaughter stress.

5.5.2   Association between environmental factors and pH24LL

Production traits including lamb kill group and flock (site of production) and year had the greatest effect on the ultimate pH of lamb loin. These effects remained unchanged when the model was corrected for the plasma indicators of stress, not surprising given the small and variable associations shown between these plasma indicators and pH24LL. Although this study shows there is evidence that lambs experience stress at slaughter, the multiple confounding factors that impact on these metabolites during the pre-slaughter period may have reduced their accuracy as indicators of

immediate pre-slaughter stress. Moreover, it is likely a multitude of interacting factors including animal genetics, production factors and environmental conditions are impacting glycogen turnover and therefore ultimate pH.

Site and year had the largest effect on pH24LL. The Kirby and Katanning flocks in this study were up to 3800km apart, meaning substantial climatic variation and differences in pasture types, availability and the type and provision of supplementary feed provided to lambs between different flocks at different times of year. Pre-slaughter factors, including handling, time off feed and transport distances to the abattoir also varied substantially between flocks. In addition, there were also differences in carcass processing between flocks, although these should be relatively standardised. Year of production also had a large impact on loin pH24LL. This is likely to reflect seasonal variation between years, particularly related to feeding regimes as well as differences in processing between years.

The slaughter group effect captures variation in slaughter day conditions, including differences in transport and lairage conditions, abattoir processing factors, as well as differences in nutrition during the finishing phase as these groups of lambs were slaughtered at different times across each year. Fluctuations in feed supply, environmental conditions and stress are likely to play a large role in determining ultimate pH through variation in muscle glycogen levels (Pethick et al., 1995).

The impact of lamb breed and sex on pH24LL was small compared to other production factors. Merino and Maternal sired lambs had the highest average pH24LL values compared to terminal sired lambs. Although this effect was small, it is in line with previous work by Gardner et al. (2006) that shows that Merinos have a propensity to produce meat with a higher ultimate pH, due to higher stress responsiveness, although we did not find a breed type by stress interaction in this study. In addition, genotype has also been shown to influence muscle glycogen turnover. The muscle tissue of more muscular genotypes has been shown to be less responsive to adrenaline. This has been shown across three different studies, one in sheep using lambs sired by high or low muscling potential rams (Martin et al., 2011) and two in cattle with the first comparing the progeny of highly muscled Piedmontese with the progeny of Angus and Wagyu sires (Gardner et al., 2009) and the second comparing the extremes in an Angus selection line diverged for muscling (McGilchrist et al., 2011). As Terminal genotypes have higher muscling characteristics and a greater proportion of lean in the carcass compared to Merino genotypes (Ponnampalam et al., 2008; Anderson et al., 2015), it may explain why terminal sired lambs had lower pH24LL in this study.

There were small differences between male and female lambs, but they were variable and differed between breed types. In terminal sired lambs, females had marginally lower pH24LL values than male lambs. The opposite result occurred in maternal sired lambs out of Merino dams, with female lambs having slightly higher pH24LL values. These results were unexpected as females have been shown to have more excitable temperaments (Voisinet et al., 1997) and a greater incidence of high ultimate pH (Scanga et al., 1998). This indicates that sex does not play a large role in determining ultimate pH under commercial conditions.

An association was observed between pH24LL and kill order, which differed between flocks. The kill-order term within our model describes the order that lambs were slaughtered within each kill group. This association was positive in the Kirby flock, indicating that those animals further back had a higher pH24LL. Lambs further back in the kill order may experience greater amounts of intensive handling, which has been shown to cause higher ultimate pH and slower pH declines in lamb (Sutherland et al., 2016). Alternatively, this association may reflect differences in temperament, with those lambs gravitating towards the back of the mob and further back in the kill order possibly being more flighty or of lower hierarchy, which can cause stress (Eldridge et al., 1988; Warriss, 1990; Hemsworth et al., 2011). Therefore, lambs killed later within a kill group may have greater depletion

of muscle glycogen stores prior to slaughter underpinning the link between kill order and ultimate pH. It is unclear why an opposite association was observed in the Katanning flock, however this could simply reflect the small impact of kill order in comparison to other production and environmental factors influencing glycogen turnover. Overall, the effect of kill order on pH24LL was small and unlikely to be greatly affecting ultimate pH on a commercial scale.

Approximately 18% of lambs in this study had a pH24LL greater than 5.71. This is significantly higher than 5% non-compliance rate reported for the Meat Standards Australian (MSA) grading system in beef (McGilchrist et al., 2014), which specifies a cut off for ultimate pH of 5.7. Currently, there is no such cut off for ultimate pH in Australian lamb. Further work is therefore required to understand glycogen metabolism in lamb as well as the production and environmental factors, including stress driving ultimate pH and how this affects lamb eating quality.

5.6   Conclusion

The associations found between plasma glucose and lactate and pH24LL indicate that opportunities exist to reduce variation in ultimate pH by reducing stress in the pre-slaughter period. Although these effects were small by comparison to production factors, further understanding of how to best manage lambs in the pre-slaughter period is required in order to minimise stress and maximise lamb wellbeing and meat quality.

Chapter 6 Lamb loin tenderness is not associated with plasma indicators of pre-slaughter stress

6.1 Abstract

The purpose of this study was to test if associations exist between plasma indicators of acute and chronic stress and lamb loin Warner Bratzler Shear Force (WBSF). Blood was collected at exsanguination from 2877 lambs from the Meat and Livestock Genetic Research flock with a suite of indicators analysed. Loin (M. *Longissimus lumborum*) WBSF was measured after 5 days aging. Plasma indicators of stress did not relate to WBSF, however a positive association was found between WBSF and kill order, indicating that immediate pre-slaughter factors may be causing reduced tenderness in lamb. In addition, selection for decreased fat depth (PFAT) was associated with increased loin WSBF, indicating that genetic selection for increased carcass leanness is negatively affecting lamb loin tenderness.

6.2 Introduction

Tenderness is a key driver of consumer acceptance of lamb due to its impact on eating quality (Pannier et al., 2014), yet even the higher quality cuts such as the loin (M. *longissimus lumborum*) are known to vary markedly in tenderness. This was demonstrated in a study by Pannier et al. (2014) whom showed that approximately 33% of lamb loins rated as good every day (3 star) and 7% as unsatisfactory (2 star) by untrained consumers under the Meat Standards Australia grading system.

Previous research in beef has indicated that stress prior to slaughter is linked with a reduction in tenderness (Warner et al., 2007). Warner et al. (2007) simulated acute stress by subjecting cattle to electric prodders immediately prior to slaughter, resulting in decreased consumer tenderness scores for the grilled loin. Gruber et al. (2010) also found that cattle with more excitable temperaments and higher flight scores were correlated with higher Warner Bratzler shear force (WBSF) values. Similarly, Pighin et al. (2015) found that loin hardness was greater in cattle under conventional immediate pre-slaughter handling compared to reduced stress handling.

Plasma indicators reflecting stress are well established, but the association between stress and tenderness has yet to be fully explored in lamb. Most recently work by Stewart et al. (2014) showed that lambs have elevated plasma glucose, lactate and non-esterified fatty acid (NEFA) concentrations at slaughter, reflecting adrenergic stress resulting in increased glycogenolysis and lipolysis (Martin et al., 2011). Cortisol is one of the most common stress indicators measured (Shaw and Tume, 1992) and has been shown to be elevated above basal at slaughter (Probst et al., 2014; Pighin et al., 2015). Increases in circulating creatine kinase (CK) and aspartate aminotransferase (AST) can be seen with unaccustomed exercise, transport handling stress and low-level trauma or bruising (Tollersrud et al., 1971; Tarrant, 1990; Pettiford et al., 2008; Sutherland et al., 2009; Fisher et al., 2010). Stress may

prevent animals from drinking in lairage (Hogan et al., 2007), which can result in dehydration and elevated plasma total protein and sodium concentrations (Jacob et al., 2006b; Radostits et al., 2007). Haptoglobin, an acute phase protein is normally present in very low levels in healthy animals (Ceciliani et al., 2012) but increases in response to infection, inflammation, tissue damage (Cray et al., 2009). More recently, haptoglobin has been used as a marker of stress in livestock (Lomborg et al., 2008; Salamano et al., 2008). Conversely, high plasma magnesium levels have been shown to attenuate the stress response (Hubbard, 1973) by reducing catecholamine and glucocorticoid secretion (Kietzmann and Jablonski, 1985; Classen et al., 1986).

The objective of this study was to examine if an association exists between lamb tenderness, as measured by Warner Bratzler Shear Force (WBSF) and plasma stress indicators. It was hypothesised that increasing plasma lactate, glucose, NEFA, cortisol, CK, AST, sodium, total protein and haptoglobin concentrations at slaughter will be associated with increased WBSF values in lamb loin. In addition, it was hypothesised that increased plasma magnesium concentrations at slaughter will be associated with a reduction in loin WBSF.

6.3   Materials and Methods

This study was approved by the Department of Agriculture Western Australia Animal Ethics Committee #2-13-07.

6.3.1   Experimental design and slaughter details

The design of Meat and Livestock Australia Genetic Resource flock has been described previously (Fogarty et al., 2007; Van der Werf et al., 2010). Wether and female lambs (n = 2877) were produced from artificial insemination of Merino, Border Leicester x Merino (BLM) and Commercial Maternal (CM) dams over a two year period (2013 and 2014) at the Katanning, WA and Kirby, NSW research sites. The lambs were the progeny of 394 different sires, which comprised Terminal sire types (Ile De France, Poll Dorset, Suffolk, Texel, Charolais and White Suffolk), Maternal sire types (Booroola, Border Leicester, Coopworth, Dohne Merino and Prime SAMM) and Merino (Merino and Poll Merino) sires, representing the major production types in the Australian sheep industry. These sires were chosen to represent the full range of Australian Sheep Breeding Values (ASBVs) for key traits within each sire type. Semen from all three sire types was used to artificially inseminate Merino dams, while only semen from Maternal and Terminal sires was used to inseminate cross-bred (BLM and CM) ewes. Maternal lambs sent to slaughter comprised very few females (which were retained for breeding purposes), meaning effective comparisons between sexes could only be made within the Terminal and Merino sired lamb groups and Maternal sired lambs from Merino dams. The lambs were maintained on extensive pasture grazing, with grain, hay or feedlot pellets supplemented when pasture supply was limited.

For each site, lambs were assigned to smaller kill groups (n = 17) of between 48 and 307 lambs to be killed on the same day to enable carcass weight targets to be achieved. Given selection for slaughter was made based on weights, the average age of lambs in each kill group varied between 193 and 416 days old at slaughter, however within individual kill groups the age range was smaller, varying as little as 16 days and by up to 33 days of age. Lambs were yarded on farm the day before slaughter and were taken off feed and water for between 5 and 18 hours. Lambs were then weighed and transported by truck to one of three commercial abattoirs (1 in WA and 2 in NSW). At the Katanning site transportation lasted for 0.5 hours compared to 1.5 to 2.5 hours for the Kirby site. Lambs were held overnight in lairage with free access to water and slaughtered the following day after electrical stunning.

6.3.2   Blood collection

Blood samples were collected into 9mL lithium heparin Vacuette® tubes (Greiner bio-one, Austria) from each lamb at slaughter, immediately following exsanguination. Tubes were immediately placed in ice for between 2-5 hours until centrifugation at 3000rpm for 15 minutes. Following centrifugation, plasma samples were pipetted in two separate aliquots and stored in 2mL tubes at -80°C until processing.

Once samples were thawed, each sample was gently inverted several times before a 100µL sample was pipetted into 1.7mL sample cups (Greiner Bio-one, Kremsmüster, Austria). Laboratory analyses of plasma were carried out as a batch samples using the Olympus AU400 automated chemistry analyser (Olympus Optical Co. Ltd, Melville, NY) and commercially available reagent kits at Murdoch University, Perth, WA or otherwise stated. For each commercial kit, the correlating control and calibration sera was used.

Laboratory analyses of plasma were carried out as a batch samples. Plasma lactate, glucose, NEFA, magnesium, total protein, CK and AST were analysed using commercial available reagent kits (Olympus Diagnostics, Tokyo, Japan). Plasma sodium was analysed using commercial available sodium kit (Randox Laboratories kit, County Antrim, UK). Samples were analysed using the Olympus AU400 automated chemistry analyser (Olympus Optical Co. Ltd, Melville, NY). For each commercial kit, the correlating control and calibration sera was used.

Plasma haptoglobin and BHOB were analysed by the Western Australian Department of Agriculture (DAFWA) Animal Health Laboratories, South Perth. Plasma β-hydroxybutyrate was analysed using the commercial reagent kit (Randox Laboratories kit, County Antrim, UK) Plasma haptoglobin was determined using an in-house method, based on the method described by Eckersall et al. (1999). Plasma Cortisol levels were determined from a subset of 500 lambs in 2013 using chemiluminescent immunoassay performed using an Immulite® 2000 Immunoassay system (Siemens, Germany) at Vetpath Veterinary services, (Perth, Australia).

6.3.3   Carcass measurements

Following slaughter, lambs were dressed according to AUS-MEAT standards and hot carcass weight (HCWT) was recorded at an average of 23.2 kg (Std Dev = 2.94). All carcasses underwent medium voltage electrical stimulation to optimise pH decline such that the carcass loin temperature at pH6 lies between 18 - 25°C (Pearce et al., 2010) and were chilled overnight (3-4°C) before sampling.

At 24 hours post-mortem M. longissimus lumborum (loin) pH (pH24LL) was measured as described by Pearce et al. (2010) on the left caudal section of the muscle at the lumbar-sacral junction, where a small 4cm incision was made to identify the caudal end of the loin muscle. Muscle pH was measured using an Orion 250A pH meter (cat. no. 0250A2, Orion Research Inc., Boston, MA USA) fitted with a glass body, spear tipped probe (cat. no. 8163BN, Orion Research). The pH meter was regularly calibrated using buffers with known pH of 4 and 7.

From the carcass saddle region the left short loin (AUS-MEAT 4480) (AUS-MEAT, 2005) up to the 12th rib was removed. From this, the M. longissimus lumborum (loin) was prepared by removing sub-cutaneous fat and connective tissue (epimysium). Loin samples of approximately 65g were collected from the cranial aspect of the loin muscle. Samples were vacuum packed, aged for 5 days at 1ºC and then frozen at −20 °C until subsequent testing. Packaged frozen samples were cooked in a water bath at 71°C for 35 minutes and then cooled in running water for 30 minutes after cooking. Six cores (approximately 3- 4cm long, 1cm2) from each loin sample were cut and Warner-Bratzler shear force

(WBSF) was measured on each core sample using a Lloyd texture analyser with a Warner–Bratzler shear blade fitted (Hopkins et al., 2010). Laboratory processing of loin samples and measurement of WBSF was performed at the University of New England Meat Science Department (Armidale, New South Wales, Australia).

6.3.4   Data analysed

Data from lambs (n = 2877) from the Kirby site in 2013 (n = 1128) and 2014 (n = 721) and Katanning sites in 2013 (n = 524) and 2014 (n = 504) were used in the analysis. Details of sex, dam breed and sire type across each year are shown in Table 6-1. Of the total lambs with WBSF data available, the base model used 2609 lambs that had all production data available. Table 6-2 shows the raw data means, standard deviation, minimum and maximum values for WBSF and plasma indicators analysed. Haptoglobin data had a skewed distribution and was log transformed to normalise the data.

Table 6-1 Number of lambs measured for M. *longissimus lumborum* (loin) Warner-Bratzler Shear Force (WBSF) for sex and dam breed for each sire type category within each site and year

Site	Year		F Merino (Mat.)	M Merino (Mat.)	M CM (Mat.)	M BLM (Mat.)	F Merino (Mer.)	M Merino (Mer.)	M Mer. (Ter.)	F Mer. (Ter.)	M CM (Ter.)	F CM (Ter.)	M BLM (Ter.)	F BLM (Ter.)
	2013	2014												
Kirby	1128	721	37	98	n/a	43	80	194	273	268	n/a	n/a	311	354
Katanning	524	504	2	118	17	5	34	199	156	215	78	109	8	19
Total	1652	1225	39	216	17	48	114	393	429	483	78	109	319	373

Sex Dam breed (Sire type)

F: female lamb; M: male (wether) lamb
CM: Commercial Maternal dam breed; BLM: Border Leicester x Merino dam breed.
(Mat.): Maternal sire type; (Mer.): Merino sire type; (Ter.): Terminal sire type.
n/a: not applicable

Table 6-2 Descriptive statistics of Warner Bratzler shear force (WBSF) and plasma indicator and M. *longissimus lumborum* (loin) pH at 24 hours (pH24LL) covariates analysed.

Variable	Mean	SD	Range
Warner-Bratzler Shear Force (N)	35.53	12.47	16.6 - 104.81
Covariates (units)			
Lactate (mmol/L)	3.48	2.29	0.467 - 16.427
Glucose (mmol/L)	4.67	0.94	1.978- 10.503
Non-esterified fatty acid (mmol/L)	1.19	0.54	0.165 - 3.26
β-hydroxybutyrate (mmol/L)	0.43	0.15	0.11 - 1.04
Magnesium (mmol/L)	0.90	0.14	0.356 - 1.627
Creatine Kinase (IU/L)	463.29	341.57	83.07 - 3731.8
Aspartate aminotransferase (IU/L)	152.63	42.79	61.82-613.73
Haptoglobin (g/L)	0.42	0.38	0.01 - 4.82
Cortisol (nmol/L)	152.27	62.57	5.5 - 395
pH24LL	5.63	0.13	5.34 - 6.88

Of the 394 sires used within this study, 59 Maternal, 138 Merino and 197 Terminal sires had Australian Sheep Breeding Values (ASBV) for Post Weaning Weight (PWT), Post Weaning Eye Muscle Depth (PEMD) and Post Weaning Fat Depth (PFAT). The breeding values for PEMD and PFAT are based upon live animal ultrasound measurement and PWT is based on live weight all measured post weaning (approximately 240 days of age). The ranges for these ASBVs varied within sire types as shown in Table 6-3.

Table 6-3 Number of sires, number of lambs and mean (min, max) of Australian Sheep Breeding Values for each sire type

Sire type	No. of Sires	No. of lambs	PEMD (mm)	PFAT (mm)	PWT (kg)
Maternal	59	335	0.33 (-1.34, 2.79)	-0.25 (-1.98, 1.52)	6.66 (-0.27, 14.19)
Merino	138	528	0.21 (-2.14, 3.16)	0.04 (-2.10, 1.75)	3.21 (-3.08, 9.25)
Terminal	197	1838	1.58 (-3.60, 5.15)	-0.59 (-2.08, 1.24)	12.98 (1.37, 18.05)

PEMD: Post-weaning eye muscle depth; PFAT: Post weaning fat depth; PWT: Post weaning weight.

6.3.5   Statistical analysis

Loin WBSF data was analysed using linear mixed effect models in SAS (SAS Version 9.1, SAS Institute, Cary, NC, USA). The base model included fixed effects for site, year, kill group within site by

year, sire type, sex by dam breed within sire type and age of dam (Table 4) sire identification and dam identification by year were included as random terms. Plasma indicators were included separately within the base model as covariate terms along with relevant interactions with other terms in the base model. Because cortisol was only measured in one year, the analysis of its association with WBSF differed. For cortisol, the base model for the analysis of WBSF included site (as above), kill group within site, sex and dam breed within sire type (as above) and age of dam. Random effects included sire identification. Cortisol was included as a covariate along with its relevant interactions.

In a separate model, sire Australian Sheep Breeding Values for PWT, PFAT and PEMD were tested for their associations with WBSF. Initially, all three breeding values were included simultaneously in the base model as both linear and quadratic covariates, as well as their first order interactions with other fixed terms. Non-significant (P>0.05) terms were removed in a stepwise manner. Due to the correlations that exist between the ASBV's in this dataset (PWT vs. PEMD = 0.5; PWT vs. PFAT = -0.3; PFAT vs. PEMD = 0.3) this process was repeated with each ASBV included one at a time to test the independence of their effects. Finally, to test whether plasma stress indicators partially or fully describe the ASBV effect, the 3 ASBV model was also tested with each plasma stress indicator included one at a time as linear and quadratic covariate.

In all models ultimate pH was included in the base model to ensure that any effects were not purely driven by ultimate pH. Non-significant terms (P>0.05) were removed in a stepwise manner.

6.4　Results

Outcomes of the base model are presented in Table 6-4.The average WBSF for all lambs over two years was 35.53 N and the base model described 45% of the variance in WBSF.

Sites varied significantly in their WBSF values between each year. This difference in WBSF was highest for the Kirby site, which in 2014 was 29.65 ± 0.67 N, 23% lower than WBSF in 2013 (38.69 ± 0.61 N). There was significantly less variation between years for the Katanning flock. In 2013 the WBSF was 33.67 ± 0.66N, with WBSF in 2014 on only 1.73 N lower (P<0.05) than this.

Table 6-4 F values, P values and numerator and denominator degrees of freedom for the effects of the base model for Warner-Bratzler shear force (WBSF) in the M. *longissiumus lumborum* (loin) of lambs.

Effect	NDF,DDF	Base model
Site	1, 389	5.64*
Year	1,1803	90.09**
Kill group (site*year)	1, 389	22.19**
Sex dam breed (Sire type)		4.69**
Site*year		55.56**

* *P*<0.05; ** *P*<0.01

Within each year at each site there was also large variation in WBSF between kill groups (P<0.001, Table 6-4), which varied by as much as 20 N between kill groups. The average kill group WBSF ranged from as high as 46.48 ± 0.94 N in Kirby in 2012 to as low as 26.30 ± 1.42N in Kirby in 2013.

The only impact of sire type on loin WBSF was observed in male lambs out of Merino dams, where Terminal sired lambs had WBSF 2.5 N and 1.9 N higher (P<0.05, Table 6-5) than Maternal sired lambs and Merino sired lambs respectively.

The only impact of sex on loin WBSF value was observed in Terminal sired lambs from Merino dams, where male lambs had WBSF values approximately 7% higher than female lambs. There were differences between dam breeds (P<0.01, Table 6-5), with lambs from the Border Leicester-merino (BLM) dams having 10% higher WSBF values than either of the other two dam breeds. This was the case for both male and female Terminal sired lambs and male Maternal sired lambs.

Table 6-5 Predicted means and standard error (SE) for the fixed effects of sex and dambreed within siretype for M. *longissimus lumborum* (loin) Warner-Bratzler shear force (WBSF)

Sex dam breed (sire type)	WBSF (N) ± SE
F Merino (Maternal)	32.1 ± 1.91[ab]
M Merino (Maternal)	32.1 ± 0.87[ab]
M Commercial Maternal (Maternal)	31.1 ± 2.69[a]
M Border-Leicester Merino (Maternal)	36.7 ± 1.63[fg]
F Merino (Merino)	32.9 ± 1.17[abc]
M Merino (Merino)	32.7 ± 0.64[ab]
F Merino (Terminal)	32.2 ± 0.56[ab]
F Commercial Maternal (Terminal)	33.3 ± 1.10b[cd]

F Border-Leicester Merino (Terminal)	35.4 ± 0.67[efg]
M Merino (Terminal)	34.6 ± 0.61[cde]
M Commercial Maternal (Terminal)	35.2 ± 1.28[def]
M Border-Leicester Merino (Terminal)	37.3 ± 0.74[g]

F: female; M: male; 50: Merino dam; CM: Commercial Maternal dam; BLM: Border Leicester Merino dam. Letters that differ between rows are significantly different (P<0.05).

6.4.1 Association between plasma indicators and kill order with WBSF

Glucose and creatine kinase (CK) demonstrated an association with WBSF, however overall these effects were small. As glucose increased from 2mmol/L to 9mmol/L there was a small increase (P = 0.061) in WBSF from 32.1 ± 1.01 N to 36.8 ± 2.04 N. Glucose and WBSF also demonstrated an opposing association in three kill groups (P < 0.05). In two kill groups as glucose concentration increased from 4mmol/L to 7mmol/L, WBSF decreased by 8N (40.0 ± 2.10 to 31.5 ± 2.62) and 12N (50.7 ± 1.64 N to 38.2 ± 2.86 N). Alternatively, in one kill group an increase in glucose from 4mmol/L to 7mmol/L associated with a 12.8 N increase in WBSF (31.2 ± 2.71 N to 44.0 ± 2.98 N). Creatine kinase demonstrated an association with WBSF however this was only evident in the 2013 year. As CK increased from 100 IU/L to 1300 IU/L, there was a small but significant linear decrease in WSBF by 7% from 37.3 N to 34.6 N. When the models for glucose and creatine kinase were corrected for pH24LL, the associations with WBSF were no longer significant (P>0.05). There was no association (P>0.05) between WBSF and lactate, NEFA, Magnesium, AST, sodium, total protein, haptoglobin or cortisol.

A positive linear association was observed between WBSF and kill-order. Increasing kill-order from 0 to 300 led to a 12% increase (P<0.01) in WBSF from 31.78 to 35.69 N (Figure 6-1). This overall effect remained significant (P<0.05) when pH24LL was included in the base model but the magnitude of effect was reduced by approximately 25%. In addition, the effect of kill-order was only significant (P<0.05) in the 2013 year.

Figure 6-1 Association between WBSF (N) and Kill order. Line represents least squared means ± standard error. × denotes residuals from the response surface.

Across the range of pH24LL values from 5.4 to 6.2, WBSF increased by 37% from 29.9 N to 42.7 N (P<0.05, Figure 6-2). In addition, the effect of pH24LL on WBSF also differed across kill groups (P<0.05). WBSF and pH24LL demonstrated a positive association (P<0.05) in nine kill groups, although the

magnitude of effect varied. In one kill group, WBSF and pH24LL demonstrated a negative association (P<0.05) and in seven kill groups there was no association (P>0.05) between WBSF and pH24LL.
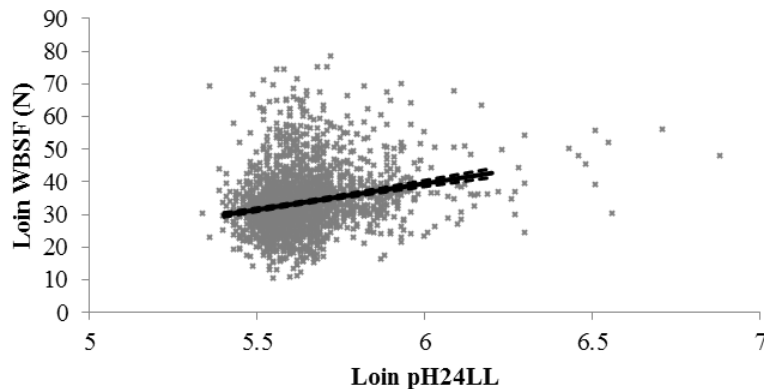
Figure 6-2 Association between M. _longissimus lumborum_ (loin) Warner Bratzler Shear Force (N) (WBSF) and loin pH at 24 hours post slaughter (pH24LL). Line represents least squared means ± standard error. × denotes residuals from the response surface.

6.4.2   Association between shear force and sire breeding values

When the sire ASBVs for PWWT, PEMD and PFAT were included simultaneously as covariates in the base linear mixed effects models, the PEMD and PFAT ASBV demonstrated some association with WBSF (P<0.05). However, when ASBVs was included in core model separately, there was no effect (P>0.05) of PEMD on WBSF. There was no association between PWT and WBSF.

There was a negative curvilinear association between PFAT and WBSF (P<0.05), reducing WBSF by 5.2N as PFAT increased from -2.0mm to 0.5mm. WBSF appears to plateau between PFAT of 0.5mm and 1.75mm (Figure 6-3). In addition, the association between WBSF and PFAT also varied (P<0.01) between sites. At the Kirby site there was an 8 N decrease in WSBF across the range of PFAT but there was no effect (P>0.05) of PFAT at the Katanning site. The association between WBSF and PFAT also varied (P<0.01) between years. In 2013, as PFAT increased from -2.0mm to 0.0mm, there was a decrease in WBSF by 8.7N. Increasing PFAT from 0 mm to 1.75mm was associated with an increase in WBSF by 4.7N. In 2014, increasing PFAT from -2.0mm to -0.5mm was associated with a plateau in WBSF. Increasing PFAT from -0.5mm to 1.75mm was associated with a 5.6N decrease in WBSF. When PFAT was included in the base model without the other ASBVs, magnitudes of the overall PFAT effect were halved.
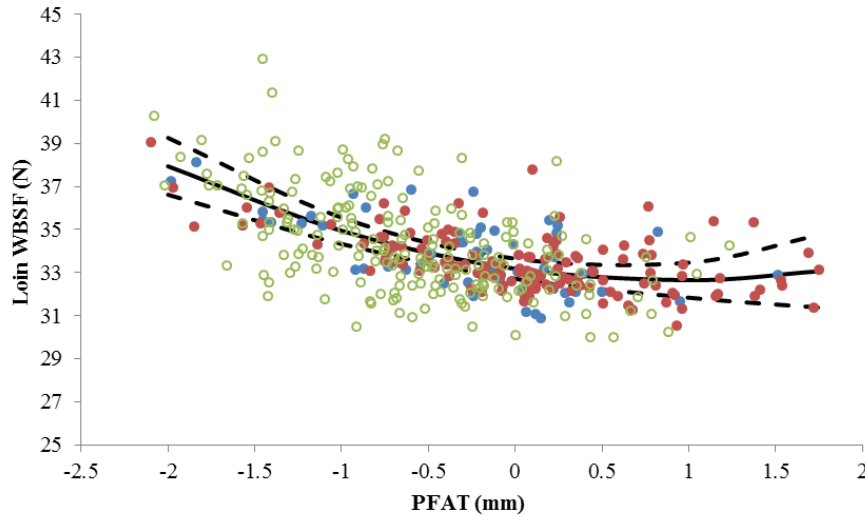
Figure 6-3 Association between sire estimates for loin Warner Bratzler Shear Force (WBSF) and Post-weaning Fat Depth (PFAT). Icons represent sire estimates for Terminal (green circles), Maternal (blue circles) and Merino (red circles) sires. Lines represent least squared means ± standard error.

6.5   Discussion

6.5.1   Association between plasma indicators and WBSF

Contrary to the hypothesis, largely there was no association between WBSF and plasma indicators of stress. This was unexpected, as the slaughters involved adequate variation in pre-slaughter stress, indicated by concentrations for plasma stress indicators that were above basal levels and had a large range in concentration. In addition, the base model explained less than 50% of variance in WBSF values, which indicates that there is a considerable degree of variation not explained by the production variables, which has not been captured by plasma indicators.

This may suggest that there is no association between stress and tenderness in lambs, contradicting the earlier study of Warner et al. (2007) and Gruber et al. (2010) in beef cattle. Alternatively, the plasma indicators themselves may be poor indicators of stress under commercial conditions. This is because multiple confounding factors can affect these metabolites during the pre-slaughter period, thus reducing their accuracy as indicators of immediate pre-slaughter stress. For example, plasma lactate concentrations can be elevated due to exercise which causes a contraction-linked increase in the rate of muscle glycogenolysis (Gardner et al., 2001b). This is likely to have occurred immediately pre-slaughter as lambs were drafted from the yards to the stunning restrainer. Likewise, feed deprivation causes elevated plasma NEFA concentrations due to a glucagon induced increase in the rate of adipose tissue lipolysis (Brockman and Bergman, 1975), thus factors other than just acute stress may have elevated these NEFA and lactate levels. Feed deprivation also lowers plasma glucose concentrations (due to reduced feed intake), but pre-slaughter stressors which induce adrenaline and the resultant glycogenolysis in the liver increase plasma glucose. Thus, these contradicting mechanisms at the time of slaughter may have also lead to the small and inconsistent associations observed between WBSF and glucose between kill groups.

In this study there was no association detected between WBSF and AST and a trend for increased CK to be associated with reduced WBSF. This was unexpected, as previous work has shown that elevations in AST and CK are associated with exercise and muscle damage (Russell and Roussel, 2007)

which would tend to indicate a higher level of pre-slaughter stress. However, pre-slaughter exercise has been shown to increase the rate of proteolysis (Bond et al., 2004), which may have led to the observed association between CK and WBSF.

The cortisol response to stress has been shown to vary between individuals as well as fluctuate during pre-slaughter handling (Fisher et al., 2010) and may have reduced its accuracy as an indicator of acute stress. There was no association between haptoglobin, magnesium, sodium or total protein with WBSF, indicating that oxidative stress, mineral status and dehydration have little to no impact on lamb tenderness.

One unexpected finding was the association between WBSF and kill-order. The kill-order term within this experiment describes the order that lambs were slaughtered within each kill group and may reflect the duration of exposure to immediate pre-slaughter processes (increased handling, human contact, novel environments) during the lead up to the stunning restrainer, which may cause stress (Ferguson and Warner, 2008). Therefore, lambs killed later within a kill group may have a greater exposure to stress underpinning the link between kill-order, acute stress and the resulting impact on tenderness. This suggests that kill-order may more accurately reflect the acute stress response at slaughter in contrast to plasma indicators, which are influenced by multiple other factors.

6.5.2   Production and genetic effects on WBSF

Overall, production and environmental factors had the greatest impact on loin WBSF in lamb. Importantly, the average WBSF values found in this study were higher than 27 N, which is associated with a 10% failure rate for lamb eating quality (Hopkins et al., 2006a).

The difference in loin WBSF varied significantly between the two research sites and years. Lambs from the Katanning site had the most consistent WBSF values with lambs from the Kirby site having 25% higher shear force values in 2013 compared to 2014, contributing to the large effect of year in the model. These differences may be related to differences in environmental factors and carcass and sample processing (Warner et al., 2010b) as they were not fully accounted for by ultimate pH.

There was considerable variation in WBSF between kill groups, varying by as much as 20N between kill groups. In addition, the kill group effect would also reflect other environmental factors such as nutritional background, transport and environmental conditions and abattoir effects such as processing variation, all of which are beyond the control of this study. Low planes of nutrition have been shown to cause higher shear force values in lamb (Hopkins et al., 2005). Likewise, processing conditions can cause variation in shear force values between consignments (Johnston et al., 2001).

Terminal sired lambs had overall higher WBSF values compared to Maternal and Merino sired lambs out of Merino dams. Although this effect was small, previous work shows that terminal sired lambs have higher WBSF (Hopkins et al., 2007) and are rated lower for tenderness by consumers (Pannier et al., 2014). This may reflect selection within this line for more muscular and leaner animals. Lambs from Merino dams had the lowest WBSF values. Although this effect was small, it is in line with previous studies (Hopkins et al., 2007; Okeudo and Moss, 2008) Female lambs had 6% lower WBSF values than male lambs, however this was only evident in Terminal sired lambs.

The association between pH24LL and WBSF highlights that any reduction in pH towards 5.5 is likely to have a positive impact on WBSF in lamb and improve eating quality consistency (Hopkins et al., 2006a).

6.5.3　Association between sire breeding values and WBSF values

Decreasing PFAT was associated with increased loin WBSF values; however this effect was small in comparison to the production factors previously discussed. These results demonstrated a 5.6 N decrease in WBSF across the PFAT range; however this effect decreased when PFAT was included in the model alone, highlighting that PEMD and PFAT co-explain the negative impact on WBSF. Recent work by Pannier et al. (2014) showed that increased selection for leanness resulted in a reduction in lamb sensory tenderness, an effect which was largely independent of WBSF. These results highlight that genetic selection for increased leanness has a direct and negative impact on objective measures of tenderness in lamb.

6.6　Conclusion

Production factors had the greatest impact on loin WBSF in lamb, however considerable variation was not accounted for. Although in our data set and under the commercial conditions of this study, there was no association found between some plasma indicators of the stress response and lamb loin shear force, as expected many plasma indicators had concentrations above basal levels with a large range in concentration, indicating that there was a degree of acute pre-slaughter stress. Further work under more controlled experimental conditions is required to better understand the immediate pre-slaughter factors, reflected by kill-order that are impacting on WBSF. In addition, further research is required to understand how the selection for leaner and more muscular carcasses is affecting WBSF, as this may provide some insight into how to improve tenderness of Australian lamb.

Chapter 7 Feed deprivation in Merino and Terminal sired lambs: (1) The metabolic response under resting conditions

7.1   Abstract

The aim of this study was to examine the metabolic response to feed deprivation up to 48 hours in low and high yielding lamb genotypes. It was hypothesised that Terminal sired lambs would have decreased plasma glucose and increased plasma non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHOB) concentrations in response to feed deprivation compared to Merino sired lambs. In addition, it was hypothesised that the metabolic changes due to feed deprivation would also be greater in progeny of sires with breeding values for greater growth, muscling, and leanness. Eighty nine lambs (45 ewes, 44 wethers) from Merino dams with Merino or Terminal sires with a range in Australian Sheep Breeding Values (ASBVs) for post-weaning weight (PWT), post-weaning eye muscle depth (PEMD) and post-weaning fat depth (PFAT) were used in this experiment. Blood samples were collected via jugular cannulas every 6 hours from time 0 to 48 hours of feed deprivation for the determination of plasma glucose, NEFA, BHOB and lactate concentration. From 12 hours to 48 hours of feed deprivation plasma glucose concentration decreased ($P<0.05$) by 25% from 4.04 ± 0.032 mmol/L to 3.04 ± 0.032 mmol/L. From 6 hours NEFA concentration increased ($P<0.05$) from 0.15 ± 0.021 mmol/L by almost 10-fold to 1.34 ± 0.021 mmol/L at 48 hours of feed deprivation. Feed deprivation also influenced BHOB concentrations and from 12 to 48 hours it increased ($P<0.05$) from 0.15 ± 0.010 mmol/L to 0.52 ± 0.010 mmol/L. Merino sired lambs had a 8% greater reduction in glucose and 29% and 10% higher NEFA and BHOB response respectively compared to Terminal sired lambs ($P<0.05$). In Merino sired lambs, increasing PWT was also associated with an increase in glucose and decline in NEFA and BHOB concentration ($P<0.05$). In Terminal sired lambs, increasing PFAT was associated with an increase in glucose and decline in NEFA concentration ($P<0.05$). Contrary to the hypothesis, Merino sired lambs showed the greatest metabolic response to fasting especially in regards to fat metabolism.

7.2   Introduction

Under Australian production systems, lambs may undergo periods of nutritional restriction due to various environmental and management factors. Seasonal fluctuations in feed availability and quality mean that animals must mobilise body reserves in order to meet energy demands. Alternatively, during procedures such as shearing and drenching sheep may undergo a period of complete feed deprivation of several hours. Under routine pre-slaughter protocols, lambs may undergo a period of acute feed deprivation usually less than 24 hours, but which may extend up to 48 hours (Jacob et al., 2005a; Ferguson and Warner, 2008).

During periods of acute feed deprivation metabolic energy from the utilisation of glucose or oxidation of volatile fatty acids (VFAs) produced in the rumen ceases and is replaced by increasing levels of adipose tissue mobilisation (Heitmann et al., 1987). This results in normal homeostatic response, characterised by an increase in circulating non-esterified fatty acids (NEFA) and a shift towards hepatic ketogenesis through β-oxidation of long chain fatty acids, producing β-hydroxybutyrate (BHOB), in order to maintain euglycaemia and tissue energy balance (Bergman, 1971; Chilliard et al., 2000; Pethick et al., 2005). In contrast, muscle glycogen concentrations in sheep appear to be unaffected by feed deprivation, even up to several days and in the absence of adrenergic stress, does not affect plasma lactate concentrations (Daly et al., 2006; Jacob et al., 2009).

However, recent work has shown that prime lambs have very high levels of plasma NEFA, although similar levels of plasma glucose and lactate when compared to yearling cattle at slaughter following routine pre-slaughter management (Stewart et al., 2014) (Chapter 4). This could be linked to greater metabolic requirements of lambs (Graham et al., 1974) which has been demonstrated to increase rates of lipolysis in response to periods of nutritional restriction (Reid and Hinks, 1962; Foot and Russel, 1979). Growth potential and body composition influence basal metabolic rate (Graham et al., 1974), which in-turn may influence the metabolic response to feed deprivation. In human studies, skeletal muscle has been shown to have a higher resting metabolic rate than adipose tissue (Usui et al., 2009)), thus animals with proportionately greater amounts of lean tissue and higher growth rates are likely to have greater maintenance energy requirements (Graham et al., 1974; Webster, 1981; Kolstad and Vangen, 1996; Wang et al., 2010). As such, more muscular and higher growth lambs, as commonly seen among terminal breed types, would be expected to have a greater glucose requirement. During acute feed deprivation this may also lead to increased mobilisation of adipose tissue to provide NEFA and BHOB as an alternative source of fuel for muscle to meet their energy requirements (Hocquette et al., 1998).

Currently, it is not well understood how the metabolic response to feed deprivation is influenced by genetic selection for increased growth, muscling and leanness. However, there is evidence that genetically divergent animals differ metabolically. For example, more muscular genotypes have an increased adipose tissue response to adrenaline, leading to increased NEFA mobilisation in sheep (Martin et al., 2011) and cattle (McGilchrist et al., 2011). During fasting, a similar effect is observed with leaner and more muscular lines of sheep which have a greater increase in plasma NEFA concentration in response to feed deprivation (Cameron, 1992). Likewise, rams selected for low back fat thickness had lower glucose concentrations during fasting than those selected for high back fat (Carter et al., 1989; Van Maanen et al., 1989). This indicates that leaner and more muscular animals may utilise more fat as an energy source during feed deprivation.

This study tested the hypothesis that Terminal sired lambs will have decreased plasma glucose and increased plasma NEFA and BHOB concentrations in response to feed deprivation up to 48 hours compared to Merino sired lambs. Selection for growth, leanness and muscling in sheep is achieved through the use of Australian Sheep Breeding Values (ASBV) for post-weaning weight (PWT), post-weaning fat depth (PFAT) and post weaning eye muscle depth (PEMD). Therefore, it was hypothesised that the metabolic changes due to feed deprivation would also be greater in progeny of sires with greater growth (PWT), muscling (PEMD) and leanness (PFAT) breeding values.

7.3 Materials and Methods

7.3.1 Animals and Procedures

This experiment assessed the impact of feed deprivation in lambs over a 48 hour period on plasma metabolite responses, and was repeated for 3 replicate groups. Data were collected from 89 lambs (330 – 350 days old) produced by the Meat and Livestock Australia Genetic Resource Flock, Katanning, Western Australia. The design of the Meat and Livestock Australia Genetic Resource flock (formally known as the Sheep Co-operative Research Centre (Sheep CRC) Information Nucleus Flock) has been described previously (Fogarty et al., 2007; Van der Werf et al., 2010). Merino sired (22 female, 23 wethers) and Terminal sired (23 ewes, 21 wethers) lambs were the progeny of Merino dams artificially inseminated using 15 Terminal (Poll Dorset, Suffolk, White Suffolk, Texel) and 17 Merino (Merino, Poll Merino) sires. Sires used had a range in Australian Sheep Breeding Values for post-weaning eye-muscle depth (PEMD), post weaning fat depth (PFAT) and post-weaning weight (PWT) (Table 7-1).

Lambs were transported to Murdoch University and managed together in a small paddock with minimal dry feed and *ad-libitum* access to a commercial pelleted ration (EasyOne®, Milne Feeds™, Analysis on dry matter basis: Crude Protein (min) 14.5%, Metabolisable Energy 11.0 MJ/Kg, Crude Fibre (max) 20.0%, Urea (max) 1.8%, Vitamin E 60mg/kg, Selenium 300μg/kg, Lasalocid Acid 37.0ppm), for 7 days prior to being randomly assigned to one of three replicates, balanced for sex, sire type and sire identification.

Table 7-1 Mean, standard deviation (SD) and range for live animal measures, sire Australian Estimated Breeding Values (ASBV) and carcass composition parameters in Merino and Terminal sired lambs

Variable (units)	Merino sired lambs (n = 45)			Terminal sired lambs (n = 44)		
	Mean	SD	Range	Mean	SD	Range
Pre-acclimation live weight	51.1	6.98	34.5 – 62.5	52.9	4.18	45.0 – 63.0
Starting live weight (kg)	49.6	6.47	34.5 - 62.5	51.0	3.96	42.5 – 58.5
Feed deprivation live weight loss (kg)	3.5	1.12	1.5 – 6.0	3.61	1.29	0.5 – 8.5
Feed deprivation live weight loss (%)	7.0	1.79	3.3 – 10.6	7.08	2.52	1.0 – 16.7
Sire Australian Estimated Breeding Values						
Post-weaning eye-muscle depth (mm)	0.9	1.01	-0.8 – 2.5	1.4	1.26	-0.6 – 3.9
Post-weaning fat depth (mm)	0.3	0.69	-0.8 – 1.5	-0.6	0.84	-1.9 – 1.2
Post-weaning weight (kg)	4.5	3.2	-0.8 – 12.1	12.7	2.5	9.8 – 17.7
Carcass parameters						
Hot Carcass Weight (kg)	23.2	3.1	17.0 - 29.0	24.97	2.2	19.1 – 29.9
GR tissue depth (mm)	17	4.9	8 - 28	18	4.0	10 – 29
M. *longissimus lumborum* (loin) weight (g)	381	64.6	269 - 538	410	60.6	276 - 528
M. *longissimus lumborum* (loin) fat weight (g)	263	75.1	117 - 457	293	80.8	163 - 595

On day 0, lambs from replicate 1 were walked to the animal house facility via a laneway adjacent to the paddock. Lambs were weighed and randomly assigned to individual pens. Lambs were acclimatised to individual pens for 5 days and fed the same commercial pelleted ration once a day at 4% live weight per day. Individual feed intakes were measured daily and lambs had ad-libitum access to water. On day 4, the neck area on all lambs was clipped to facilitate jugular cannulisation. On day 5, lambs were weighed and indwelling jugular cannulas were inserted. The site was prepared with 2% chlorhexidine and 70% ethanol and 1mL lignocaine hydrochloride (Lignocaine 20, Ilium®) was injected subcutaneously around cannula site. The jugular vein was then punctured with a needle (14 gauge 1.5 inch, Monoject™) and polythene tubing (Microtube extrusions Pty Ltd, Eastwood, New South Wales; internal diameter = 1.00mm, outer diameter = 1.50mm) was passed through the needle and inserted into 20cm into the vein. The cannula was then flushed using heparinised saline via three-way tap (BD LuerLok™) and secured. The neck area was then bandaged (Tensoplast® Vet, BSN medical) to maintain the cannula patency.

On day 6, remaining feed was measured and then removed from all animals at time 0 (8:00am). Blood samples were collected at time 0, 2, 6, 12, 18, 24, 30, 36, 42 and 48 hours of feed deprivation. Prior to blood sample collection, 2mL of blood was withdrawn and discarded. Blood samples were collected into Lithium heparin S-Monovette Vacutainer® (Sarstedt Australia Pty. Ltd, South Australia). Post sampling, cannulas were flushed with 5mL of heparinised 0.9% saline. Blood tubes were immediately placed on ice. Within 30 minutes of sample collection, blood samples were centrifuged at 4°C for 15 minutes at 3000rpm. Plasma samples were pipetted into 2mL micro tubes and then stored at -20°C. Following final blood collection at 48 hours of feed deprivation, lambs were weighed, cannulas were removed from lambs and feed was provided. Lambs were monitored for 24 hours before being moved back to the paddock with the other lamb replicate groups. The same procedures were repeated in succession for replicate 2 lambs from day 9 and replicate 3 lambs from day 18.

Approximately 3 weeks following completion of the third replicate (day), lambs were slaughtered as part of a commercial feed deprivation trial (Stewart et al., submitted, Chapter 8). Carcass weight and composition traits were measured including; hot carcass weight (HCWT), GR tissue depth, M. *longissimus lumborum* (loin) weight (LLWT) and M. *longissimus lumborum* (loin) fat weight (LLFAT) (Table 7-1).

7.3.2   Plasma sample analysis

Frozen plasma samples were allowed to thaw once to approximately 2°C at room temperature and then were gently inverted several times, after which 100µL was pipetted into sample cups for analysis. Laboratory analyses of plasma were carried out as a batch using the Olympus AU400 automated chemistry analyser (Olympus Optical Co. Ltd, Melville, New York). Plasma NEFA concentration was analysed using a NEFA-C kit (C Kit Wako Pure Chemical Ind., Osaka, Japan; modified for the Olympus AU400 Automated Chemistry Analyser). Plasma β-hydroxybutyrate (BHOB) was analysed using a commercial kit (Randox Laboratories kit, County Antrim, UK, Ranbut, Cat. No. RB1007). Glucose analysis was carried out using the glucose reagent kit (Olympus Diagnostics, Tokyo, Japan, Cat. No. OSR6121) and plasma L-Lactate was analysed with a reagent kit (Olympus Diagnostics, Tokyo, Japan, Cat. No. OSR6193).

7.3.3   Statistical analysis

Glucose, NEFA, BHOB, and lactate concentrations were analysed using linear mixed effect models (SAS Version 9.1, SAS Institute Cary, NC, USA). In each model, fixed effects for sire type, sex, time off feed as well as relevant interactions were tested. Random terms included lamb identification within sire identification and replicate.

Plasma glucose was included as a covariate in the base statistical model for NEFA to determine their association during feed deprivation. Likewise, NEFA was included as a covariate in the base statistical model for BHOB to determine their association during feed deprivation. Residuals from the response surface were tested for normality. Non-significant terms (P>0.05) were removed in a stepwise manner.

Hot carcass weight, live weight and percentage live weight change were included separately into each base model as covariates to determine whether animal size or acclimation period had an impact on metabolite response to feed deprivation.

In addition, GR tissue depth, LLWT and LLFAT were included separately into each base model only with HCWT as covariates to represent the impact of relative composition (muscling or fatness) on the metabolic responses to feed deprivation. Non-significant (P>0.05) terms were removed in a stepwise manner.

In a separate analysis, NEFA, BHOB, glucose and lactate responses to feed deprivation were tested for associations with sire ASBVs for PWT, PEMD and PFAT. Initially all three ASBV's were included as covariates in the model, as well as their first order interactions with other terms including sire type and non-significant (P>0.05) terms were removed in a stepwise manner. Due to the difference in PWT ranges for Merino and Terminal sired lambs (Table 1), the PWT term was analysed within sire type. Due to the correlations that exist between ASBVs in this data set (Merino sired lambs: PWT vs. PEMD=0.75; PWT vs. PFAT= 0.62; PEMD vs. PFAT=0.85; Terminal sired lambs: PWT vs. PEMD= 0.33, PWT vs. PFAT = 0.17, PEMD vs. PFAT= 0.71), this process was repeated with the ASBVs one at a time to test the independence of their effects.

7.4   Results

7.4.1   Effect of sire type on the plasma glucose concentration response to feed deprivation

There was a significant association between duration of feed deprivation and plasma glucose concentration (P<0.01, Table 7-2). From 0 hours to 12 hours there was minimal change, however as time off feed increased from 12 hours to 48 hours, plasma glucose concentration decreased by 25% from 4.04mmol/L to 3.04mmol/L, across all lambs. Glucose concentration also differed between sire types (P<0.01, Table 7-2) and the effect of feed deprivation on glucose concentration was greater for Merino sired lambs compared to Terminal sired lambs from 30 hours of feed deprivation (Figure 7-1). In Merino sired lambs, glucose concentration decreased by 27%, from 4.03mmol/L to 2.92mmol/L between 12 to 48 hours of feed deprivation. In Terminal sired lambs, this decrease was smaller, with glucose concentration only decreasing by 22% from 4.06mmol/L to 3.17mmol/L between 12 to 48 hours of feed deprivation. Merino and Terminal sired ewe and wether lambs differed in glucose concentration (P<0.05, Table 7-2). This difference was observed in Merino ewe lambs (3.49 ± 0.06mmol/L), which had lower (P< 0.05) glucose concentrations than Merino wether lambs (3.66 ± 0.06mmol/L) and Terminal sired ewe (3.69 ± 0.06mmol/L) and wether lambs (3.67± 0.06mmol/L).

The sire type differences in glucose response to feed deprivation remained unchanged when the model was corrected for phenotypic covariates such as live weight, HCWT, LLFAT, LLWT or GR tissue depth.

There was a curvilinear association between live weight and glucose concentration (P<0.05) which was evident from 18 hours of feed deprivation (P<0.05). The greatest magnitude of effect was

observed at 42 hours of feed deprivation. Between a live weight of 40 and 50kg, plasma glucose decreased from 3.25 ± 0.092mmol/L to 3.03 ± 0.051mmol/L and increasing live weight from 50 to 62 kg was associated with an increase in plasma glucose from 3.03± 0.051mmol/L to 3.50 ± 0.135.

7.4.2 Effect of feed deprivation on plasma non-esterified fatty acid (NEFA) and β-hydroxybutyrate (BHOB) concentrations

Feed deprivation had a significant effect on NEFA concentration ($P<0.01$, Table 7-2). From 0 to 6 hours of feed deprivation there was no change in NEFA concentration, however as time off feed increased from 6 to 48 hours, NEFA concentration increased by 1.18mmol/L from 0.15mmol/L to 1.33mmol/L. NEFA concentration also differed between sire types ($P<0.01$, Table 7-2) and the effect of feed deprivation on NEFA was greater in Merino sired lambs than Terminal sired lambs ($P<0.01$, Table 7-2). Between 6 and 48 hours of feed deprivation plasma NEFA concentration in Merino sired lambs increased by 1.35mmol/L, whereas in Terminal sired lambs NEFA concentration only increased by 1.02mmol/L (Figure 7-1).

Table 7-2 F-values and numerator and denominator degrees of freedom (NDF, DDF) for the base models for non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHOB), glucose and lactate concentration in lambs during feed deprivation.

Effect	NEFA NDF, DDF	NEFA F-value	BHOB NDF, DDF	BHOB F-value	Glucose NDF, DDF	Glucose F-value	Lactate NDF, DDF	Lactate F-value
Sire type	1,781	14.7**	1,783	2.2na	1,782	5.3*	-	
Sex	-		-		1,782	2.68ns	1,782	0.45ns
Time	9,781	618.9**	9,783	192.3**	9,782	312.03**	9,782	21.9**
Sire type*Time	9,781	12.31**	9,783	3.48**	9,782	8.45**	-	
Sex*sire type	-		-		1,782	4.56*	-	
Sex*Time	-		-		-		9,782	2.0*

* P<0.05; ** P<0.01
ns: non-significant

Feed deprivation had a significant effect on BHOB concentrations (P<0.01, Table 7-2). Initially from 0 to 12 hours, BHOB decreased by 0.18mmol/L from 0.33mmol/L to 0.15mmol/L and from 12 to 48h it increased by 0.37mmol/L from 0.15mmol/L to 0.52mmol/L. The effect of feed deprivation on BHOB concentration differed between sire types (P<0.01, Table 7-2) and was greatest in Merino sired lambs. From 12 hours of feed deprivation BHOB increased by 0.39mmol/L in Merino sired lambs compared to an increase of 0.34mmol/L in Terminal sired lambs. (Figure 7-1). The effects of sire type and feed deprivation on NEFA and BHOB remained unchanged when the model was corrected for phenotypic covariates such as live weight, HCWT, LLFAT, LLWT or GR tissue depth, yet these covariates themselves demonstrated associations with NEFA and BHOB.

Increasing carcass fatness was associated with a decrease in NEFA response during feed deprivation (P<0.05), however this was only significant at 24, 30 and 36 hours of feed deprivation. The magnitude of effect was greatest at 36 hours with an increase in LLFAT from 100 to 500 grams, associated with a decrease in NEFA of 0.29mmol/L from 1.02 ± 0.05 mmol/L to 0.73 ± 0.07 mmol/L. There was a weak negative association between GR tissue depth and average NEFA concentration (P<0.05), which decreased by 0.15mmol/L (from 0.73mmol/L to 0.59mmol/L) across the 8mm to 29mm GR tissue depth range. There was no association (P>0.05) between NEFA and live weight, live weight change or LLWT.

Live weight had a small although inconsistent effect on the BHOB response (P<0.05), an effect that was significant at 18, 24 and 48 hours of feed deprivation. The association was greatest at 48 hours, with an increase in live weight from 40 to 62kg associated with a 16% decrease in BHOB concentration from 0.56 ± 0.022mmol/L to 0.47 ± 0.024mmol/l. Similarly, increasing HCWT was associated with decreasing BHOB concentration at 24 and 48 hours of feed deprivation (P<0.05). The greatest change was observed at 48 hours of feed deprivation where increasing HCWT from 17 to 30kg, was associated with a decrease in BHOB of 0.10 mmol/L from 0.56 ± 0.028mmol/L to 0.47 ± 0.024mmol/L. There was no association (P>0.05) between BHOB and live weight change, LLFAT, LLWT or GRFAT
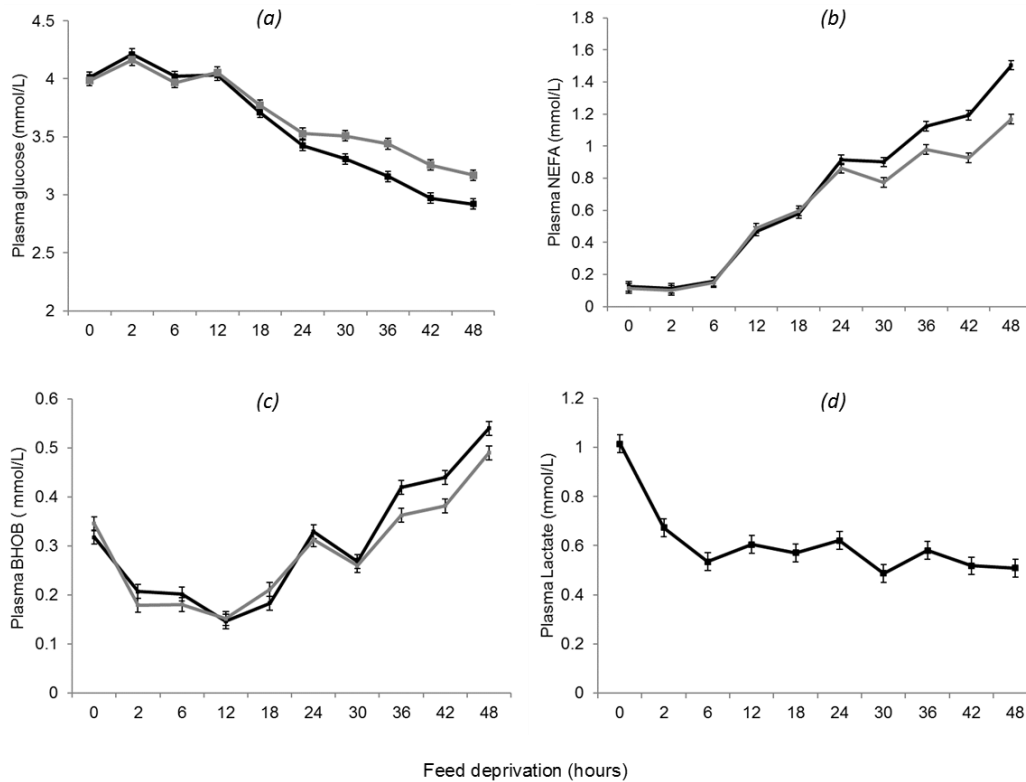
Figure 7-1 Table of figures showing effect of feed deprivation on (a) plasma glucose concentrations ± standard error (SE); (b) non-esterified fatty acid (NEFA) concentrations ± SE; (c) β-hydroxybutyrate (BHOB) concentrations ± SE in Merino sired lambs (black lines) and Terminal sired lambs (grey lines); (d) plasma lactate concentrations ± standard error in all lambs (black line).

7.4.3 Association between plasma non-esterified fatty acids (NEFA) and glucose concentration

From 24 hours of feed deprivation onwards there was a negative association between plasma NEFA and plasma glucose concentration ($P<0.05$). This association was strongest at 48 hours of feed deprivation. At this time point in Merino sired lambs decreasing plasma glucose from 3.5mmol/L to 2.3mmol/L, was associated with a 0.62mmol/L increase in plasma NEFA concentration (Figure 7-2). This effect was less in Terminal sired lambs where a decrease in glucose from 3.7mmol/L to 2.5mmol/L was associated with an increase in NEFA of only 0.41mmol/L (Figure 7-2). This pattern was consistent across all time points from 24 to 48 hours. Furthermore, when glucose was included as a covariate in the NEFA base model, there was no effect ($P>0.05$) on the sire type NEFA response to feed deprivation.
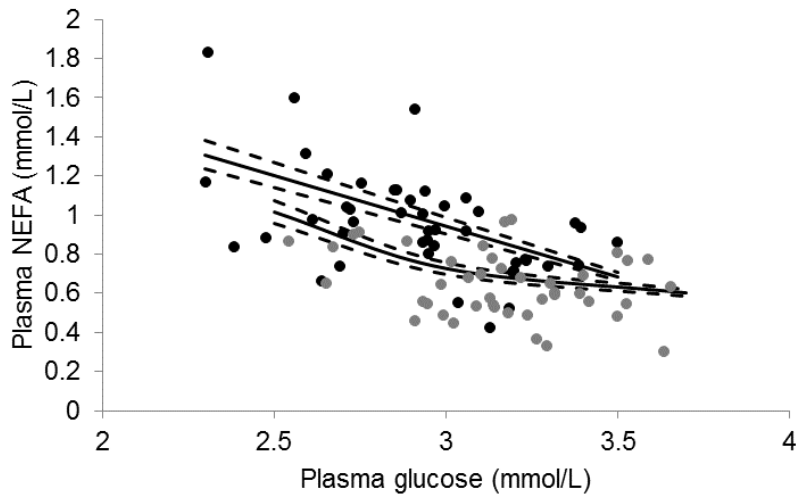
Figure 7-2 Association between non-esterified fatty acid (NEFA) and glucose concentration at 48 hours of feed deprivation ± standard error (dashed lines) in Merino sired lambs (black lines) and Terminal sired lambs (grey lines). Icons denote Merino ● and Terminal ● sire residuals from the response surface.

7.4.4 Association between plasma β-hydroxybutyrate (BHOB) and non-esterified fatty acids (NEFA) concentration

There was an association between plasma BHOB concentration and NEFA concentration which gradually inverted over time ($P<0.05$). At 0 hours, the association was negative with an increase in NEFA from 0.03mmol/L to 0.2mmol/L associated with a decrease in BHOB from 0.36 ±0.014mmol/L to 0.30 ±0.013mmol/L. From 12 hours onwards the association was positive, increasing in magnitude until 42 hours of feed deprivation where an increase in plasma NEFA from 0.6mmol/L up to 1.6mmol/L was associated with an increase in plasma BHOB of 0.19mmol/L (Figure 7-3). This effect was the same for both sire types across all time points.
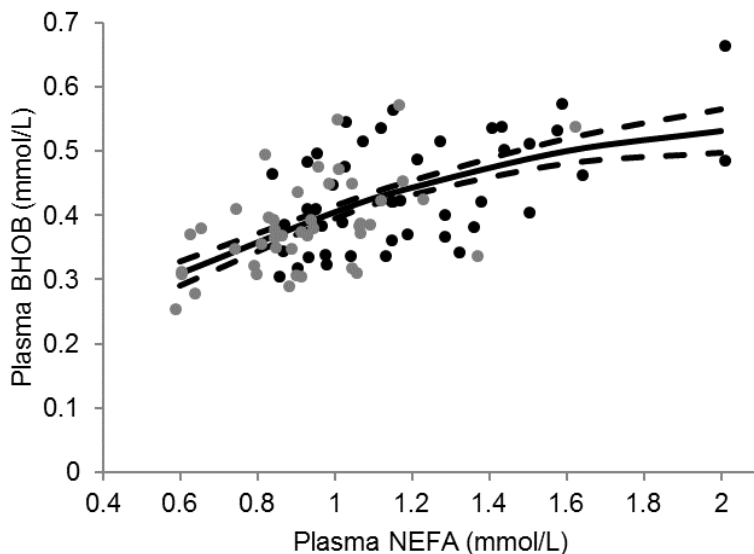
Figure 7-3 Association between β-hydroxybutyrate (BHOB) and non-esterified fatty acid (NEFA) concentration (mmol/L) at 42 hours of feed deprivation ± standard error (dashed lines). Icons denote Merino ● and Terminal ● sire residuals from the response surface.

7.4.5 Effect of feed deprivation on plasma lactate concentration

Plasma lactate concentrations were affected by time ($P<0.05$, Table 7-2). From 0 to 6 hours of feed deprivation plasma lactate concentration decreased by 0.48 mmol/L ($P<0.01$, Figure 7-1) after which levels stabilised, with only small and inconsistent differences observed between time points ($P<0.05$, Figure 7-1). Lactate concentrations were affected by sex ($P<0.05$, Table 7-2) and were 20% higher for female lambs (1.12 ± 0.063mmol/L) compared to male lambs (0.91 ± 0.063mmol/L) but only at 0 hours of feed deprivation. Plasma lactate response was not affected ($P>0.05$) by phenotypic covariates such as live weight, HCWT, LLFAT, LLWT or GR tissue depth.

7.4.6 Effect of sire and sire breeding values on the metabolic response to feed deprivation

When sire ASBVs for PWT, PEMD and PFAT were tested, glucose, NEFA and BHOB levels demonstrated associations with PWT and PFAT. There was no association ($P>0.05$) between PEMD and glucose, NEFA or BHOB concentration.

There was a positive association ($P<0.05$) between glucose and PFAT in Terminal sired lambs that was consistent across all time points from 0 hours to 48 hours of feed deprivation. Increasing PFAT from -1.8mm to 1.2mm was associated with a 0.21mmol/L increase in plasma glucose concentration (Figure 7-4). The effect of PFAT on glucose was no longer significant ($P>0.05$) when it was included alone as a covariate in the base glucose model. The association between glucose levels and PFAT was not evident in Merino sired lambs ($P>0.05$). There was a trend ($P <0.1$) for an association between glucose and PWT in Merino sired lambs. This pattern was consistent across all time points from 0 hours to 48 hours of feed deprivation. Increasing PWT from -2.0mm to 12mm was associated with a 0.33mmol/L increase in plasma glucose concentration (Figure 7-4). The magnitude of this effect was reduced by approximately 35% when PWT was included separately in the base glucose model. There was no association between glucose and PWT in Terminal sired lambs ($P>0.05$).

In Terminal sired lambs, PFAT demonstrated a negative association with plasma NEFA from 36 hours of feed deprivation ($P<0.05$). As PFAT increased from -1.8mm to 1.2mm, plasma NEFA concentration decreased by approximately 0.36mmol/L at 36, 42 and 48 hours of feed deprivation (Figure 7-4). The magnitude of this effect was reduced by approximately 40% when PFAT was included separately in the base NEFA model. There was no association ($P>0.05$) between PFAT and the NEFA response to feed deprivation in Merino sired lambs (Figure 7-4). In Merino sired lambs, PWT was negatively associated with NEFA concentration from 30 hours of feed deprivation ($P<0.05$). The strongest association was at 48 hours of feed deprivation, with an increase in PWT from -1kg to 12 kg associated with 34% decrease in NEFA concentration from 1.75mmol/L to 1.15mmol/L (Figure 7-4). When PWT was included alone in the base NEFA model, the magnitude of this effect was increased by 20% and 10% at 30 hours and 36 hours of feed deprivation and diminished by 23% and 24% at 42 hours and 48 hours. There was no association ($P>0.05$) in Terminal sired lambs (Figure 7-4).

In Merino lambs, PFAT demonstrated a positive association with BHOB ($P<0.05$) which was consistent across all time points. As PFAT increased from -0.75mm to 1.5mm, plasma BHOB concentration increased by approximately 0.06mmol/L (Figure 7-4). PFAT was no longer significant ($P>0.05$) when included as a single covariate in the base BHOB model. There was no association ($P>0.05$) between PFAT and BHOB response to feed deprivation in Terminal sired lambs (Figure 7-4).

The association between PWT and BHOB differed for Merino and Terminal sired lambs and was consistent across all time points ($P<0.01$). In Merino sired lambs, increasing PWT from -2kg to 12.5kg was associated with a 0.1mmol/L decrease in plasma BHOB concentration (Figure 7-4). The opposite association was observed in Terminal sired lambs and as PWT increased from 10kg to 18kg, plasma BHOB increased by 0.06mmol/L (Figure 7-4). This effect remained significant when PWT was included as a single covariate in the base BHOB model, however the magnitude of effect was halved in Merino sired lambs.
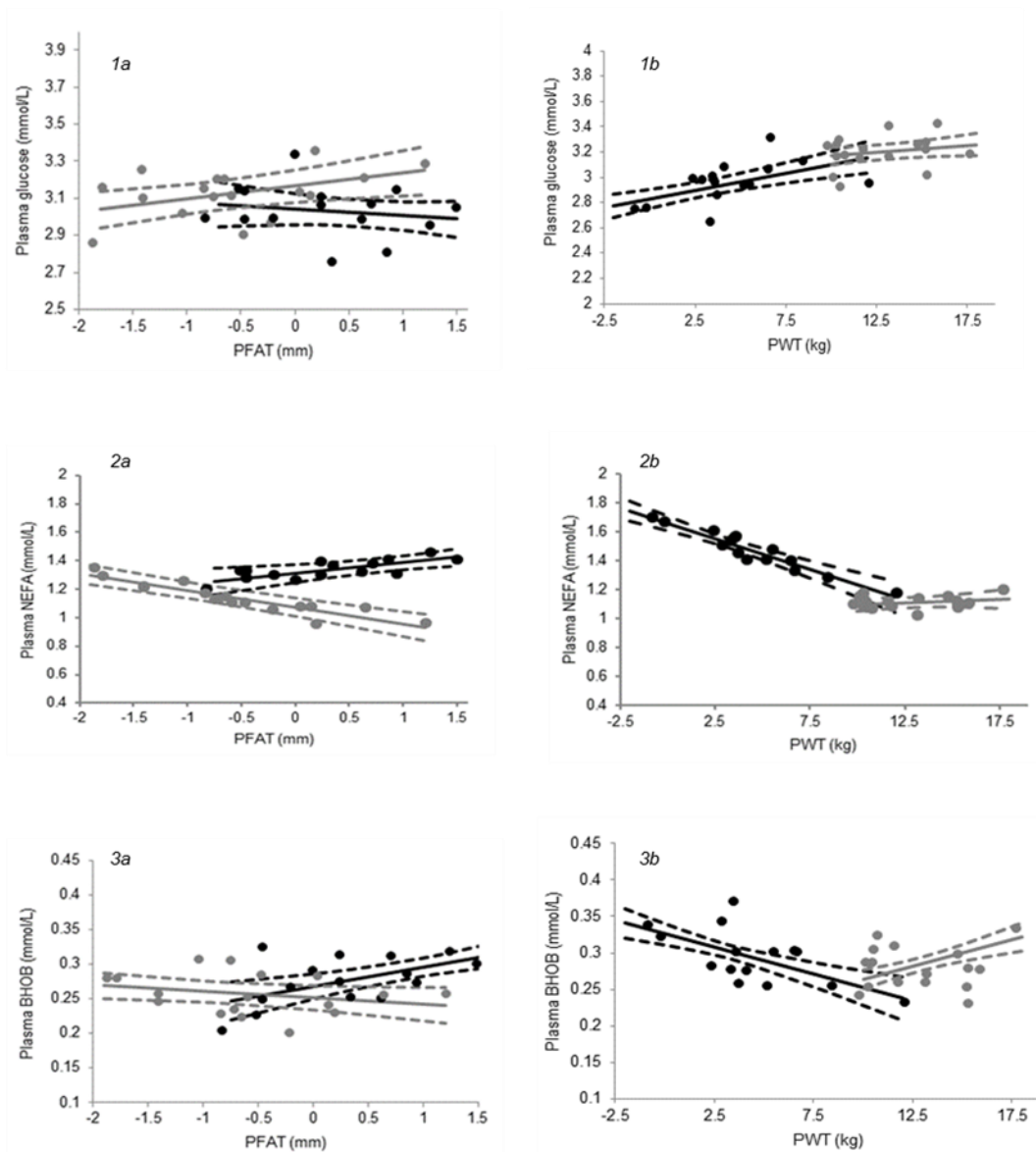
Figure 7-4 Table of figures showing association between plasma glucose (mmol/L), plasma non-esterified fatty acid (NEFA) (mmol/L) and β-hydroxybutyrate (BHOB) and Post-weaning fat depth (PFAT) (mm) and Post weaning weight (PWT) (Kg) Australian Sheep Breeding Values (ASBV) in Terminal (grey line and ± SE) and Merino (black line and ± SE) sired lambs. Icons denote Merino ● and Terminal ● sire residuals from the response surface

7.5   Discussion

7.5.1   Effect of sire type on the metabolic response to feed deprivation

Contrary to the hypothesis, Terminal sired lambs had higher plasma glucose and reduced plasma NEFA and BHOB concentrations in response to feed deprivation compared to Merino sired lambs. For both Merino and Terminal sired lambs there was little change in plasma glucose up to 12 hours of feed

deprivation. This may reflect supply of rumen derived volatile fatty acids from residual feed in the early stages of feed deprivation (Bass and Duganzich, 1980; Bergman, 1990a). The decline in plasma glucose concentrations became evident after this initial period and is likely to have coincided with a decrease in insulin:glucagon concentration ratio (Holness and Sugden, 1989) eliciting an increase in hepatic glycogenolysis and gluconeogenesis and increased rates of lipolysis.

Coinciding with the decline in plasma glucose, plasma NEFA and BHOB concentration increased from 6 and 12 hours of feed deprivation respectively. During feed deprivation, circulating levels of rumen derived butyrate gradually decline. This is offset by the hepatic production of BHOB via ketogenesis in response to elevated NEFA concentration from starvation induced lipolysis (Pethick et al., 2005). Merino sired lambs had the greatest increase in NEFA and BHOB concentration, a response that appears to be driven by the greater decline in plasma glucose (Pethick et al., 2005) in these lambs. The other key metabolite derived from lipolysis is glycerol, which can contribute up to 50% of glucose via gluconeogenesis (Bergman et al., 1968). However, as the lipolytic response (indicated by NEFA response) was lower in Terminal sired lambs, it indicates that glycerol is unlikely to be the substrate driving the higher levels of glucose observed in Terminal sired lambs.

Alternatively, amino acid carbon derived from muscle may also contribute to the synthesis of glucose during fasting (Heitmann and Bergman, 1980; Oddy et al., 1987) and may have contributed to the higher glucose levels in Terminal sired lambs. Increased protein degradation has been associated with reduced feed intake in breeds selected for faster growth (Oddy et al., 1995; Oddy et al., 1998). Therefore a similar effect may be seen in Terminal sired lambs during feed deprivation where higher protein turnover is contributing greater quantities of gluconeogenic substrates, resulting in the higher and more stable glucose concentrations. It is expected that an increased availability of plasma alanine would be utilised as previous work has shown that in comparison to fed sheep, the liver of sheep deprived of feed for up to 3 days removed greater amounts of alanine from portal circulation (Heitmann and Bergman, 1980). This in-turn would then explain the differences in lipolytic response to feed deprivation in Merino and Terminal sired lambs. Further work is required to understand whether a similar metabolic response to feed deprivation occurs in Merino and Terminal sired lambs under commercial conditions (Stewart et al. submitted, Chapter 8).

Unlike the effect of feed deprivation on NEFA, BHOB and glucose, time off feed had no impact on plasma lactate, supporting the initial hypothesis. This is in line with previous work that demonstrated that feed deprivation per se up to 48 hours under resting conditions is unlikely to be a cause of muscle glycogen turnover and thus elevated plasma lactate concentrations (Jacob et al., 2005a; Daly et al., 2006). The higher plasma lactate observed in female lambs at time 0 may reflect greater excitement, muscle contraction and stress (Pethick et al., 1991; Gardner et al., 1999b) in female lambs when feed was removed combined with the first blood samples being collected.

7.5.2 Effect of sire breeding values on the metabolic response to feed deprivation

In support of the hypotheses, the decline in glucose and subsequent increases in plasma NEFA were greatest in the progeny of low PFAT Terminal sires. This indicates that the progeny of lean sires maintain lower plasma glucose concentrations during feed deprivation. This would result in a higher glucagon to insulin ratio, stimulating lipolysis and contribute to the higher NEFA and BHOB observed in these animals (Carter et al., 1989; Van Maanen et al., 1989). Genetically leaner lambs may also be more sensitive to the effects of glucagon, as Carter et al. (1989) showed that progeny from the low back fat line were also more sensitive to the lipolytic effects of glucagon, demonstrated by a greater elevation of plasma NEFA concentrations in response to intravenous administration of this hormone. Alternatively, the progeny of low PFAT sires may have a more glycolytic fibre type (Greenwood et al.,

2006), which is likely to lead to increased glucose consumption by muscle (Hocquette et al., 1998) and in turn may lead to the depressed levels of glucose in plasma evident in this study. This would also have resulted in a higher glucagon to insulin ratio, explaining the elevated NEFA concentrations. This parallels previous work in sheep and cattle that were selected to be more muscular and leaner and were found to have increased response to adrenaline (Martin et al., 2011; McGilchrist et al., 2011). Although feed deprivation is not acting through the adrenergic axis, the associated glucagon release makes use of the same post-receptor reaction cascade leading to the mobilisation of triglyceride and the release of NEFA (Pethick et al., 2005). Therefore, this may implicate a post-receptor difference between these genetically divergent animals, with the genetically leaner lambs having up-regulated lipolytic pathways and rates of fat turnover in times of feed deprivation (Carter et al., 1989). The negative association between NEFA and LLFAT and GR tissue depth found in the current study tends to support this assertion. While it would have been preferable to utilise composition traits measured prior to feed deprivation rather than carcass data (Stewart et al., submitted, Chapter 8). Furthermore, it does align well with the phenotypic leanness of these animals (Anderson et al., 2015) and with previous work which demonstrated that increased selection for fatness reduced live weight losses during periods of sub-optimal nutrition (Blumer et al., 2013). As live weight change is correlated to fat weight change (Adams et al., 2002), high PFAT may be beneficial under periods of extended feed deprivation and have less impact on live weight, fat depths and other labile fat stores such as intramuscular fat. Moreover, under commercial conditions, leaner lambs may lose more weight during pre-slaughter feed deprivation, which may impact on carcass weights (Thompson et al., 1987).

Contrary to the hypothesis, plasma BHOB was higher in high PFAT Merino sired lambs, an effect that was consistent across all time points. It is unclear why this effect was only observed in Merinos, given that a similar association between NEFA and PFAT was not found in the same animals. Furthermore, the association between PFAT and glucose and NEFA in terminal sired lambs suggests that fat mobilisation is lower in higher PFAT animals. However, the association may differ in Merinos. Genetically fatter lambs may have increased rates of ketogenesis and may be better able to maintain euglycaemia, thus reducing rates of lipolysis. A practical outcome of this may be that the progeny of high PFAT Merinos sires may be more adapted to periods of underfeeding (Blumer et al., 2016).

Contrary to the hypothesis, in Merino sired lambs, increased PWT was associated with higher levels of plasma glucose and a reduction in NEFA and BHOB response to extended feed deprivation (>30 hours), likely due to the greater decline in glucagon to insulin ratio (Holness and Sugden, 1989). Furthermore, correcting the model for PWT accounted for a portion of the difference in NEFA response between sire types, highlighting that this difference was partly driven by a difference in genetic growth potential. It is unclear what physiological mechanism underpins this difference between low and high growth lambs, however increased rates of protein degradation have been observed in high growth animals during underfeeding (Bremmers et al., 1988; Oddy et al., 1995). During starvation, this may result in an elevated supply of gluconeogenic amino acids being available to maintain euglycaemia (Heitmann and Bergman, 1980), thus reducing the reliance on fat mobilisation and ketogenesis in high growth animals.

In Terminal sired lambs, there was no association between plasma glucose or NEFA and PWT, but increasing PWT was associated with an increase in plasma BHOB concentrations. It is unclear why this effect was observed, given that there was not a similar association observed between NEFA and PWT. However this effect was small and driven by a high PWT sire, which when its progeny were removed from the dataset it resulted in the association being no longer significant. Therefore greater numbers of progeny of high growth sires with a larger range in ASBVs for PWT would be required to more thoroughly explain this association.

Contrary to the hypothesis, there was no association between greater muscling (high PEMD) and the metabolic response to feed deprivation. This may indicate that there is no impact of muscling on

the metabolic response to feed deprivation. Alternatively, in the current study, correlations between PFAT, PWT and PEMD may have prevented associations to be observed, by co-explaining the associated metabolic effects, particularly in a small dataset. The results from this study indicate the PFAT is a driver of the metabolic response to feed deprivation in Terminal sired lambs. Alternatively, in Merino sired lambs, PWT appears to be a stronger driver of glucose and fat metabolism.

7.6 Conclusion

Significant differences in fat and energy metabolism exist between Merino and Terminal sired lambs during 48 hours of feed deprivation at rest. These differences appear to be partly underpinned by physiological differences due to sire type and genetic selection for growth and leanness. Merino sired lambs had a significantly greater glucose, NEFA and BHOB response to feed deprivation and further work is required to understand the mechanisms that underpin this sire type response. Moreover, work under commercial pre-slaughter conditions is required to determine the impact of extended time off feed on carcass yield and meat quality attributes, particularly in Merino genotypes.

Chapter 8 Feed deprivation in Merino and Terminal sired lambs: The metabolic response under pre-slaughter conditions and impact on meat quality and carcass yield

8.1   Abstract

Under current Australian industry pre-slaughter guidelines, lambs may be off feed for up to 48 hours prior to slaughter. The purpose of this study was to examine what proportion of circulating metabolites at slaughter are due to stress and feed deprivation and if this response differs between Merino and Terminal genotypes. In addition the effect of feed deprivation on carcass weight and meat quality was examined. Jugular blood samples were collected from 88 Merino and Terminal sired lambs at rest and at slaughter following 24, 36 and 48 hours of feed deprivation and plasma analysed for glucose, lactate, non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHOB). From the same carcasses hot carcass weight (HCWT) were measured as well as a suite of meat quality traits measured such as M. *longissimus lumborum* (loin) and M. *semitendinosus* pH at 24 hours post mortem. Loin samples were also analysed for intramuscular fat content and Warner-Bratzler Shear Force. Merino sired lambs had a higher NEFA response compared to Terminal sired lambs at slaughter after 24, 36 and 48 hours of feed deprivation, with NEFA levels up to 35% higher than previously reported in the same animals at rest in animal house conditions, whereas BHOB response to feed deprivation was not affected by sire type (P>0.05) and similar to previously reported at rest. In addition to the metabolic effects, increasing feed deprivation from 36 hours was associated with a 3% reduction in HCWT and dressing percentage as well as causing increased ultimate pH in the M. *semitendinosus* in Merino sired lambs. There was no effect of feed deprivation on intramuscular fat or Warner-Bratzler shear force. Findings from this study demonstrate that Merino and Terminal sired lambs differ in their metabolic response to feed deprivation under commercial slaughter conditions. In addition, commercial feed deprivation appears to have a negative effect on ultimate pH and carcass weight and warrants further investigation.

8.2   Introduction

Under Australian pre-slaughter management, lambs will normally undergo a period of feed deprivation prior to slaughter up to 48 hours (Jacob et al., 2005b). Pre-slaughter feed deprivation reduces visible contamination of sheep during transportation and facilitates hygienic carcass processing and accurate prediction of dressing percentage (Pointon et al., 2012). Feed deprivation

results in a progressive decline in circulating glucose causing the up-regulation of lipolysis, resulting in increases in plasma non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHOB) (Pethick et al., 2005). Liver glycogen phosphorylase is activated by fasting, with depletion of liver glycogen (Jacob et al., 2009), yet the role of glycogen in skeletal muscle is thought to be less influenced by pre-slaughter feed deprivation and more by the effects of stress or contraction-linked energy demands of muscle (Harris, 2006).

Although changes in plasma metabolites are expected, recent work by Stewart et al. (2014) (Chapter 4) has shown that prime lambs have higher levels of plasma NEFA at slaughter than previously reported (Warriss et al., 1989; Pethick et al., 2005), following routine pre-slaughter management, which included feed deprivation for up to 48 hours. Evaluating the metabolic effect of time off feed in a commercial pre-slaughter environment is challenging as levels of circulating metabolites at slaughter are also likely to be impacted by acute stress (Bassett, 1970; Chilliard et al., 2000; Martin et al., 2011). Therefore, to understand the role of feed deprivation on plasma metabolite response and the factors that modulate this impact, experimental designs are required that control for acute stress.

The confounding of the effects of feed deprivation and acute stress on pre-slaughter metabolism are also likely to extend to breed types. Based on the work of Martin et al. (2011), Terminal breed types, which are typically leaner and more muscular, may be expected to have a greater adipose response during higher stress conditions such as those experienced during commercial slaughter (Ferguson and Warner, 2008). Alternatively, Merino sired lambs which are not selected for rapid lean growth have been shown under resting conditions to demonstrate a greater NEFA and BHOB response and lower glucose concentration during 48 hours of feed deprivation compared to Terminal sired lambs (Stewart et al. accepted, Chapter 7). Therefore, further understanding of the impact of breed type (Merino vs Terminal) on the response to feed deprivation under commercial conditions is required, to dis-entangle the metabolic response from that of acute stress.

Genotype has also been shown to alter the metabolic response to both feed deprivation and acute stress. During feed deprivation, lambs with high genetic potential for rapid growth had a reduced NEFA response (Stewart et al., accepted, Chapter 7), where an increase in Australian Sheep Breeding Values (ASBVs) for post-weaning weight (PWT) from 1 to 12 kg was associated with a 34% reduction in the NEFA response to feed deprivation in Merino lambs. Likewise, genetically leaner lambs had a larger adipose tissue response under feed deprivation, with a decrease in ASBVs for Post-Weaning Fat Depth (PFAT) from 1.2 to -1.8 mm associated with a 42% increase in NEFA response in Terminal sired lambs. The acute stress response may also be affected by genetic selection, but in this case via animals selected for muscularity to increase lean meat yield. Martin et al. (2011) and McGilchrist et al. (2011) demonstrated in sheep and cattle that animals with higher breeding values for muscling had a higher adipose tissue response following exogenous adrenaline, resulting in elevated plasma NEFA concentrations.

Therefore, under commercial feed deprivation and the imposed stress associated with pre-slaughter conditions, it was hypothesised that Terminal sired lambs would initially demonstrate higher NEFA and BHOB concentrations at slaughter than Merino sired lambs. However, these effects will diminish under conditions of extended feed deprivation (>36 hours) due to the greater metabolic response of the Merino genotype to time off feed. In addition, it was hypothesised that the progeny of sires with increased muscling (Post-weaning eye-muscle depth (PEMD)) breeding values will have greater NEFA and BHOB at slaughter due to their greater adipose response to adrenaline. Alternatively, the progeny of sires with lower PWT and PFAT breeding values will have higher NEFA and BHOB due to their greater response to feed deprivation and these differences will increase with the duration of feed deprivation.

8.3   Materials and Methods

This study was approved by the Animal Ethics Committee, Murdoch University, Perth, Australia (Approval number: R2724/15).

8.3.1   Experimental design and slaughter details

Data were collected from 88 lambs produced by the Meat and Livestock Australia Genetic Resource Flock in Katanning, Western Australia. Details of the design on this flock (previously known as the Sheep Co-operative Research Centre (Sheep CRC) Information Nucleus Flock) has been previously (Fogarty et al., 2007; Van der Werf et al., 2010). Female (n= 44) and wether (n = 44) lambs were the progeny of Merino dams artificially inseminated using industry Terminal and Merino sires with a range in Australian Sheep Breeding Values for PEMD, PFAT and PWT (Table 8-1). Merino sired (n = 45) and Terminal sired (n = 43) lambs had previously been used in a prior experiment (Stewart et al, submitted, Chapter 7) and were randomly allocated to three treatment groups for 24 hours (n = 29) 36 hours (n =30) and 48 hours (n = 29) of pre-slaughter feed deprivation. Treatment groups were the same as previously allocated groups (Stewart et al, submitted, Chapter 7). Groups were balanced for sex, sire type and sire identification.

Table 8-1 Number of sires, number of lambs and mean (min, max) of live weight and Australian Sheep Breeding Values (ASBVs) for post-weaning eye muscle depth (PEMD), post-weaning fat depth (PFAT) and post-weaning weight (PWT) of each siretype.

Sire type	No. Sires	No. lambs	Live weight (kg)	PEMD (mm)	PFAT (mm)	PWT (kg)
Merino	15	45	57.2 (42.5 – 71.5)	0.9 (-0.8, 2.5)	0.3 (-0.8, 1.5)	4.5 (-0.8, 12.1)
Terminal	17	43	59.3 (45.0 – 69.0)	1.3 (-0.6, 3.9)	-0.6 (-1.9, 1.2)	12.7 (9.8, 17.7)

Lambs were managed in a small paddock with minimal dry feed and ad-libitum access to a commercial pelleted feed (EasyOne®, Milne Feeds™, Western Australia) for 3 weeks. Three days prior to the commencement of feed deprivation, all lambs were weighed in the paddock and then transported (<2 km) to an undercover shed and maintained under these conditions with ad libitum access to the same pelleted feed and water for 3 days.

On day 4 at 0700 hours, jugular blood samples were collected from group 48. Blood samples were collected via venepuncture into 9mL lithium heparin Vacuette® tubes (Greiner bio-one, Austria) and immediately placed on ice for approximately 1 hour.

Following blood collection, all feed was removed and ad libitum water provided. The same protocol was applied to the 36 hour treatment group at 1900 hours on day 4 and the 24 hour treatment group at 0700 hours on day 5. For all groups, care was taken to avoid excitement or exercise during blood collection. On day 5 at 0900 hours, all lambs were transported as a mixed group via truck to a commercial abattoir (3.5 hours, 350km) where they were held in lairage yards, without feed but with free access to water. On day 6, all lambs were slaughtered at 0700 following electrical stunning and exsanguination. Blood samples were collected from each lamb at exsanguination into 9mL lithium heparin Vacuette® tubes (Greiner bio-one, Austria). Tubes were immediately placed in ice for approximately 2 hours until centrifugation at 3000rpm for 15 minutes. Following centrifugation, plasma samples were pipetted in two separate 2mL aliquots which were stored at -20°C until processing.

8.3.2   Carcass measurements and sample collection

Following slaughter, lambs were dressed according to AUS-MEAT standards and hot carcass weight (HCWT) recorded. Dressing percentage was calculated as HCWT divided by live weight and expressed as a percentage. Carcasses underwent medium voltage electrical stimulation and then chilled overnight at 3°C. At 24 hours post mortem, pH was measured on the left portion of the M. *longissimus lumborum* (pH24LL) as described by Pearce et al. (2010). At 24 hours post mortem, pH was also measured in the M. *Semitendinosus* (pH24ST). At 24 hours post slaughter, the loin (M. *longissimus lumborum*) was removed from the carcass saddle region caudal to the middle of 12th/13th rib (Anonymous, 2005). From this, the subcutaneous shortloin fat was removed and weighed (LLFAT) and the entire trimmed shortloin weighed (LLWT). GR tissue depth (mm) was measured using a GR knife on the external fat of the carcass 110mm from the midline and over the 12th rib. C-site fat depth (mm) was measured adjacent to the exposed loin eye at the 12th/13th rib.

Approximately 40g of diced loin muscle was then collected and stored at -20°C until subsequent freeze drying using a Cuddon FD 1015 freeze dryer (Cuddon Freeze Dry, New Zealand). Intramuscular fat (IMF) was measured on the loin samples using a near infrared procedure (Perry et al., 2001) in a Technicon Infralyser 450 (Unity Scientific, Australia) that were validated with chemical fat determinations using solvent extraction. Intramuscular fat (IMF) was expressed as percentage fat in wet tissue. For the measurement of loin shear force (SHEAR5), approximately 65g of loin muscle was vacuum packed and aged for 5 days at 1ºC before being frozen at −20 °C. Frozen samples were defrosted and then cooked in a water bath to an internal temperature of 71°C and then cooled in running water. Shear force was measured on 6 replicate samples using a Lloyd texture analyser (Model LRX, Lloyd Instruments, Hampshire, UK) with a Warner–Bratzler shear blade fitted (Hopkins et al., 2010).

8.3.3   Plasma sample analysis

Frozen plasma samples were allowed to thaw to approximately 2°C at room temperature and then were gently inverted several times, after which 100μL was pipetted into sample cups for analysis. Laboratory analyses of plasma were carried out as a batch using the Olympus AU400 automated chemistry analyser (Olympus Optical Co. Ltd, Melville, NY) and commercially available reagent kits for glucose, NEFA, BHOB and lactate as previously described (Stewart et al., submitted, Chapter 8).

8.3.4   Statistical analysis

Basal and slaughter concentrations of plasma NEFA, BHOB, glucose and lactate as well as live weight, HCWT, dressing percentage and composition measures (LLWT, LLFAT, c-site fat depth and GR tissue depth) were analysed using linear mixed effect models (SAS Version 9.1, SAS Institute Cary, NC, USA). In each model, fixed effects for sire type, sex and feed deprivation group were included and relevant interactions tested.

For basal concentration models, live weight was included as a covariate. For slaughter concentration of metabolites, live weight and basal metabolite concentration were included as covariates. Slaughter levels of glucose were included as a covariate in NEFA models and NEFA was included as a covariate in BHOB models. HCWT and carcase composition indicators (HCWT tested with one of LLFAT, LLWT, c-site fat depth, or GR tissue depth) were also included as covariates to determine if weight or composition had an effect on the metabolic response to feed deprivation.

In a separate analysis, slaughter levels of NEFA, BHOB, glucose and lactate response to feed deprivation were also tested for associations with sire ASBVs for PWT, PEMD and PFAT. Initially all 3

ASBVs were included as covariates in the model, as well as their first order interactions with other terms, and non-significant (P>0.05) terms were removed in a stepwise manner. Due to the correlations that exist between ASBVs in this data set (Merino sired lambs: PWT vs. PEMD=0.75; PWT vs. PFAT= 0.62; PEMD vs. PFAT=0.85; Terminal sired lambs: PWT vs. PEMD= 0.33, PWT vs. PFAT = 0.17, PEMD vs. PFAT= 0.71), this process was repeated with the ASBVs one at a time to test the independence of their effects.

Live weight and carcass parameters (HCWT, dressing percentage), composition indicators (LLFAT, LLWT, GR tissue depth, c-site fat depth) and meat quality variables (SHEARF5, IMF, pH24LL and pH24ST) were analysed using linear mixed models. Fixed effects for feed deprivation group (group 24, group 36, group 48), sire type (Terminal, Merino) and sex (wether, female) were included and relevant interactions tested. For the HCWT models, pre-slaughter live weight was included as a covariate. For composition variables, HCWT was included as covariate. In all models, sire identification was included as a random term. Non-significant (P>0.05) terms were removed in a stepwise manner.

8.4   Results

8.4.1   Animal live weight

Pre-slaughter live weight differed (P=0.0513, Table 8-2) between treatment groups. Lambs in the 24 hour group (60.1 ± 1.01 kg) were heavier compared to the 36 hour group (57.6 ± 0.99 kg) and 48 hour groups (57.2 ± 1.01 kg). Pre-slaughter live weight also differed (P<0.05, Table 8-2) between sire type and groups. Merino lambs sired from the 24 hours group (60.9 ± 1.4 kg) were heavier (P<0.05) than in the 36 hour (56.2 ± 1.4 kg) and 48 hour (54.7 ± 1.4 kg) group. There was no difference in live weight (P>0.05) between the 36 or 48 hour group in Merino sired lambs or the 24, 36 or 48 hour groups for Terminal sired lambs. Merino lambs in the 48 hour group were lighter (P<0.05) than Terminal sired lambs in the 24 hour (59.3 ± 1.4 kg), 36 hour (59.0 ± 1.4 kg) and 48 hour (59.8 ± 1.4 kg) treatment groups. There was no other differences in live weight (P>0.05) between sire types for any other treatment groups.

8.4.2   Basal concentrations of plasma non-esterified fatty acid (NEFA), β-hydroxybutyrate (BHOB), glucose and lactate

Basal concentrations of NEFA and glucose differed between treatment groups (P<0.05, Table 8-2). The 48 hour treatment group had the highest NEFA and lowest glucose (Table 8-3), however in both cases these differences were small. Treatment group, sire type, sex or live weight did not affect (P>0.05) basal BHOB or lactate concentrations.

Table 8-2 F values, P values and numerator and denominator degrees of freedom (NDF, DDF) for the effects of the base model on non-esterified fatty acid (NEFA), β-hydroxybutyrate (BHOB) and glucose concentrations at slaughter, basal concentrations of NEFA and glucose, pre-slaughter live weight and carcass variables in Merino and Terminal sired lambs.

Effect	NDF, DDF	NEFA	NDF,DDF	BHOB	NDF, DDF	Glucose	NDF, DDF	Basal NEFA	Basal Glucose
Sire type	1, 52	28.67**		ns		ns		ns	ns
Feed deprivation group	2, 52	36.92**	2,53	30.56**	2, 54	6.09*	2,54	6.12**	5.67**
Sire type*Feed deprivation group	2, 52	4.85*		ns		ns		ns	ns

Effect	NDF, DDF	Pre-slaughter live weight	HCWT†	NDF, DDF	pHST24
Sire type	1, 52	2.23^{ns}	9.35**	1,49	14.45**
Feed deprivation group	2, 52	3.15*	3.29*	2,49	8.48**
Sire type*Feed deprivation group	2, 52	3.73*	ns	2,49	2.85β

β $P<0.1$; * $P<0.05$; ** $P<0.01$, † Hot carcass weight (HCWT) model corrected for live weight; pHST24: M. *Semitendinosus* pH at 24 hours post-mortem; ns: non-significant

Table 8-3 Predicted Least squared means ± standard error (SE) and range for basal and slaughter levels of plasma non-esterified fatty acid (NEFA), β-hydroxybutyrate (BHOB), glucose and lactate, and live weight and carcass variables measured for lambs in Group 24, Group 36 and Group 48 of feed deprivation

Variable (units)	24 hours	36 hours	48 hours
Basal plasma metabolic indicators (mmol/L)			
Non-esterified fatty acid	0.10 ± 0.010b	0.07 ± 0.010a	0.12 ± 0.010b
β -hydroxybutyrate	0.36 ± 0.024	0.36 ± 0.024	0.31 ± 0.024
Glucose	4.64 ± 0.067a	4.58 ± 0.065a	4.37 ± 0.067b
Lactate	1.93 ± 0.270	2.66 ± 0.265	2.36 ± 0.270
Slaughter plasma metabolic indicators (mmol/L)			
Non-esterified fatty acid	1.08 ± 0.056a	1.25 ± 0.056b	1.70 ± 0.057c
β-hydroxybutyrate	0.43 ± 0.014a	0.42 ± 0.014a	0.55 ± 0.015b
Glucose	4.79 ± 0.099a	4.74 ± 0.098a	4.46 ± 0.100b
Lactate	1.92 ± 0.172	2.04 ± 0.169	2.07 ± 0.172
Carcass weight and composition parameters			
Hot carcass weight (kg)	25.2 ± 0.46a	24.0 ± 0.46b	23.2 ± 0.47b
Dressing percentage (%)	41.8 ± 0.36a	41.3± 0.36b	40.6 ± 0.36b

Variable (units)	24 hours	36 hours	48 hours
Loin weight [f] (g)	401 ± 9.0	397 ± 8.7	388 ± 8.9
Loin fat weight [f] (g)	278 ± 12.5	264 ± 12.2	289 ± 12.4
GR tissue depth (mm) [f]	18 ± 0.7	19 ± 0.7	17 ± 0.7
C-site fat depth (mm)[f]	6 ± 0.5	7 ± 0.4	7± 0.5
Meat quality parameters			
M. *Longissimus lumborum* pH at 24 hours post-mortem	5.60 ± 0.011	5.58 ± 0.011	5.61 ± 0.011
M *Semitendinosus pH* at 24 hours post-mortem	5.63 ± 0.021a	5.66 ± 0.020a	5.74 ± 0.020b
M. *Longissimus lumborum* Warner Bratzler Shear force[x] (N)	30.49 ± 1.131	30.65 ± 1.115	31.89 ± 1.137
M. *Longissimus lumborum* intramuscular fat (%)	6.16 ± 0.288	6.58 ± 0.283	6.29 ± 0.284

[a,b,c] means within rows with different letters are significantly different ($P<0.05$); GR tissue depth: measured tissue depth (mm) 110mm from midline over the 12th rib; c-site fat depth: measured fat depth (mm) adjacent to the exposed loin eye at the 12th/13th rib.; [f]corrected for hot carcass weight; [x]measured on samples aged for 5 days

8.4.3　Metabolic response to feed deprivation

Duration of feed deprivation had an impact (P<0.05; Table 8-2) on plasma NEFA, BHOB and glucose concentrations at slaughter, although no effect (P>0.05) on plasma lactate concentrations.

8.4.3.1　Effect of feed deprivation on plasma non-esterified fatty acid (NEFA) concentrations

The base model described 62% of the variance for NEFA concentrations at slaughter. Feed deprivation had the largest effect on plasma NEFA concentration (P<0.01; Table 8-2), with lambs undergoing 48 hours of feed deprivation having NEFA levels 57% and 36% higher than at 24 hours, and 36 hours of feed deprivation (Table 8-3). The effect of feed deprivation on NEFA concentration also differed between sire types (P<0.01, Figure 8-1). NEFA concentrations in Merino sired lambs were 29%, 23% and 48% higher compared to Terminals sired lambs at 24, 36 and 48 hours of feed deprivation (P<0.05, Figure 8-1). Between 24 and 48 hours of feed deprivation, this represented an increase of 66% in Merinos, compared to only 44% in Terminal sired lambs.

There was no effect (P>0.05) of sex, live weight, carcase composition or basal NEFA concentration on the NEFA response to feed deprivation. A positive association was observed between plasma NEFA and plasma glucose concentrations, however this effect was only significant in male lambs (P<0.05). Increasing glucose concentrations from 3.8mmol/L to 5.8mmol/L were associated with a 47% increase in plasma NEFA concentration from 1.07 ± 0.096mmol/L to 1.58 ± 0.111 mmol/L.
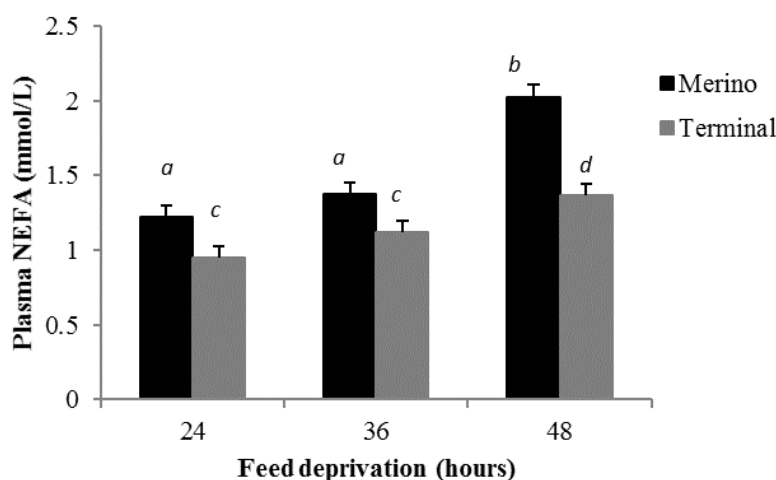
Figure 8-1 Effect of feed deprivation (hours) on plasma non-esterified fatty acid (NEFA) (mmol/L) levels in Merino (black bars) and Terminal (grey bars) sired lambs. Letters[a,b,c,d] with different superscripts are significantly (P<0.05) different.

8.4.3.2　Effect of feed deprivation on plasma β-hydroxybutyrate (BHOB) concentrations

The base model described 51% of the variance for BHOB concentrations at slaughter. Plasma BHOB concentrations were affected by feed deprivation (P<0.05, Table 8-2), with no change between 24 hours and 36 hours, but then increasing by 29% from 36 hours to 48 hours (P<0.05, Table 8-3). These changes were not influenced by either sex or sire type (P>0.05). There was an association between basal BHOB concentration and levels measured at 36hours of feed deprivation (P<0.1). As

basal BHOB increased from 0.2mmol/L to 0.8mmol/L there was a 46% increase in BHOB at 36 hours of feed deprivation BHOB from 0.37 ± 0.020 mmol/L to 0.54 ± 0.043 mmol/L. This association had no impact on the magnitude of the feed deprivation treatment response described above. There was no association (P>0.05) of live weight, HCWT, LLFAT, LLWT or c-site fat depth on the BHOB response to feed deprivation. However, increasing GR tissue depth from 8mm to 30mm was associated with a 0.13mmol/L increase in plasma BHOB from 0.41 ± 0.024 mmol/L to 0.54 ± 0.039mmol/L. Inclusion of GR tissue depth in the model had no impact on the magnitude of the BHOB response to feed deprivation.

There was a positive association observed between average BHOB concentrations and NEFA concentrations following feed deprivation (P<0.01), which also differed between sire types (P<0.05, Figure 8-2). In Merino sired lambs, increasing plasma NEFA from 0.85mmol/L to 2.7mmol/L was associated with a 25% increase in plasma BHOB from 0.44 ± 0.022 mmol/L to 0.54 ± 0.032 mmol/L. There was a stronger association observed in Terminal sired lambs, despite having an overall lower NEFA response to feed deprivation (Figure 8-1). As plasma NEFA concentrations increased from 0.7mmol/L to 1.8mmol/L, plasma BHOB concentrations increased by 47% from 0.38 ± 0.022 mmol/L to 0.56 ± 0.030mmol/L.
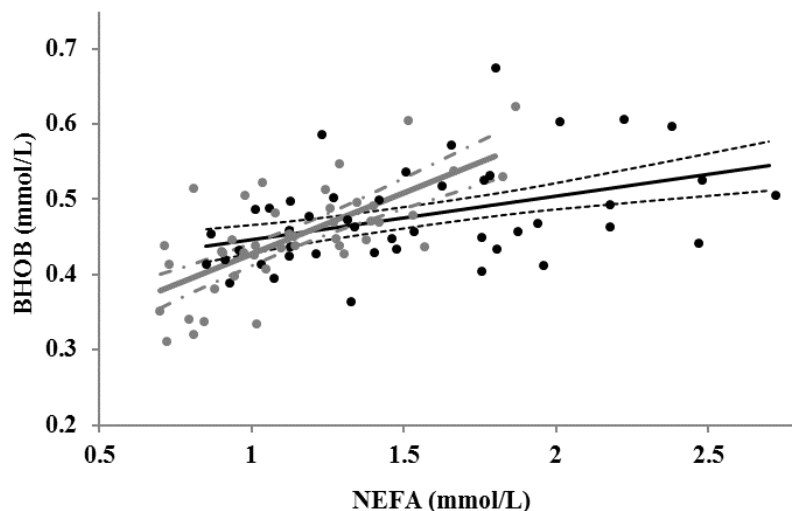
Figure 8-2 Association between post feed deprivation β-hydroxybutyrate (BHOB) and non-esterified fatty acid (NEFA) concentrations in Merino (black lines) and Terminal (grey lines) sired lambs. Lines represent least squared means ± standard error. Icons denote Merino ● and Terminal ● residual sire estimates from the response surface.

8.4.3.3   Effect of feed deprivation on plasma glucose concentration

The base model described 54% of the variance for glucose concentrations at slaughter. Increasing duration of feed deprivation from 24 to 48 hours was associated with a 7% decrease in plasma glucose concentration at slaughter (P<0.05, Table 8-3), with the bulk of this change occurring between 36 hours and 48 hours. There was no effect (P>0.05) of sex or sire type on the glucose response to feed deprivation.

There was a strong association between glucose concentrations at slaughter and basal glucose (P<0.05). As basal glucose increased from 3.75mmol/L to 5.5mmol/L, glucose concentration at slaughter increased by 15% from 4.37 ± 0.133 mmol/L to 5.03 ± 0.156 mmol/L. This association was

consistent in magnitude across each of the feed deprivation treatment groups. In addition, correcting the model for basal glucose levels partially accounted for the effect of feed deprivation on plasma glucose concentration at slaughter, but this effect was small with the 48 hour treatment group having concentrations that were only 1% lower at slaughter. There was an association between plasma glucose concentration at slaughter and live weight ($P<0.1$). Increasing live weight from 45kg to 70kg was associated with an 8.5% increase in plasma glucose from 4.46 ± 0.141 mmol/L to 4.84 ± 0.128 mmol/L. However, this association did not influence the response to increasing feed deprivation, as it was consistent across all feed deprivation treatment groups.

8.4.4 Effect of sire breeding values on metabolic response to feed deprivation

PFAT did not influence the BHOB response to feed deprivation. However, PFAT did show a consistent association with BHOB, with an increase in PFAT from -1.5mm up to 1.5mm, associated with an increase in plasma BHOB of 0.068 mmol/L from 0.43 ± 0.018 mmol/L up to 0.50 ± 0.021 mmol/L ($P<0.05$,Figure 8-3). Correcting for HCWT and phenotypic indicators of carcass composition had no effect on the magnitude of this association. There was no effect ($P>0.05$) of PEMD or PWT on BHOB concentration. There was also no effect ($P>0.05$) of post weaning weight (PWT), post-weaning eye-muscle depth (PEMD) or post-weaning fat depth (PFAT) on the plasma NEFA, glucose or lactate response to feed deprivation.
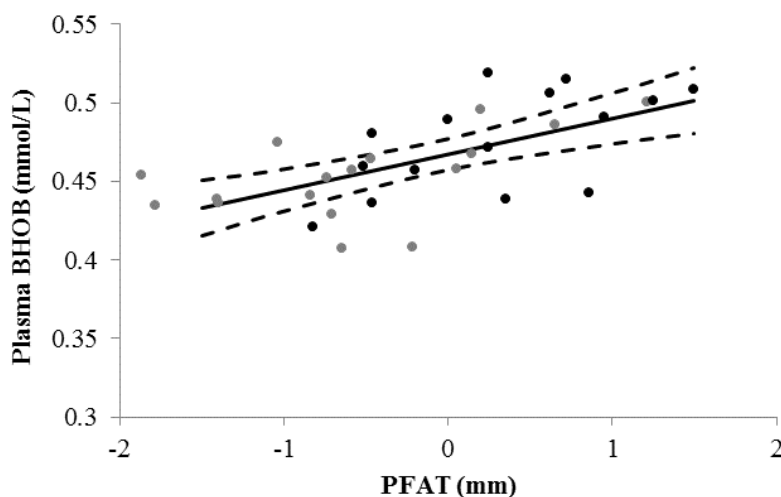
Figure 8-3 Association between β-hydroxybutyrate (BHOB) concentration (mmol/L) in lambs at slaughter and Post-Weaning Fat Depth (PFAT) (mm), averaged across the three feed deprivation treatment groups. Line represents predicted mean ± standard error. Icons denote Merino ● and Terminal ● residual sire estimates from the response surface.

8.4.5 Effect of feed deprivation on carcass parameters and meat quality

Hot standard carcass weight (HCWT) and dressing percentage were affected by duration of feed deprivation and sire type ($P<0.05$). Increasing feed deprivation from 24 to 48 hours was associated with a 3% decrease ($P<0.05$, Table 8-3) in HCWT, with the bulk of this change occurring between the 24 and 36 hour treatment groups. Likewise, increasing duration of feed deprivation from 24 to 48

hours caused a decrease in dressing percentage by 1.2 dressing percentage units, an effect that was the same for both sire types. There was also no difference (P>0.05, Table 8-3) in LLFAT, LLWT, c-site fat depth or GR tissue depth between feed deprivation groups when adjusted for HCWT. There was a significant effect of feed deprivation on pH24ST (P<0.01, Table 8-2) which differed between sire types (P< 0.1). The effect of feed deprivation on pH24ST was greatest in Merino sired lambs, where feed deprivation from 24 to 48 hours was associated with an increase in pH24ST from 5.65 ± 0.030 to 5.83 ± 0.028. There was no effect of feed deprivation on pH24ST in Terminal sired lambs (P>0.05). Feed deprivation had no impact on pH24LL, IMF or SHEAR5 (P>0.05).

8.5   Discussion

8.5.1   Metabolic response to feed deprivation

Up to 48 hours of feed deprivation under commercial pre-slaughter conditions caused significant changes in intermediary metabolism in lambs assessed by the concentration of blood metabolites at exsanguination. Increasing feed deprivation up to 48 hours caused an increase in plasma NEFA and BHOB concentration, reduced glucose concentration and had no effect on plasma lactate concentrations. Contrary to the initial hypothesis, the NEFA concentration in Merino sired lambs had a greater NEFA response compared to Terminal sired lambs at 24 hours of feed deprivation. However, in support of the hypothesis, the increase in NEFA concentration between 36 and 48 hours was markedly greater in Merinos. This difference in NEFA response is the observed effect of feed deprivation, as the level of stress imposed during curfew, transport and lairage was the same across each treatment group, indicating that the Merino sired lambs had a greater metabolic response to feed deprivation than Terminal sired lambs.

While NEFA concentrations at slaughter at each time point reflect the combined effect of the stressors associated with pre-slaughter transport and lairage as well as feed deprivation, NEFA concentrations at 24 hours may be more strongly reflective of stress responsiveness. Supporting this assertion, previous work has shown that there are no sire type difference in NEFA response to feed deprivation up to 30 hours under resting or animal house conditions (Stewart et al, accepted, Chapter 7, Table 8-4). This indicates that Merinos have a greater NEFA response to acute stress under commercial pre-slaughter conditions. This result in Merinos was unexpected as previous work has shown that more muscular, higher yielding genotypes have greater adipose tissue responsiveness to adrenaline in both sheep and cattle (Martin et al., 2011; McGilchrist et al., 2011). On this basis, it was expected that Terminal sired lambs would have had a greater adipose response than Merino lambs under the stress of commercial slaughter conditions in the early stages of feed deprivation.

However, an alternative interpretation is that feed deprivation was the stronger driver of NEFA concentration at slaughter. At the level of the adipose tissue, although the more muscular Terminal lambs may have responded more to adrenergic stimulation, the differential in NEFA response between sire types may have been due to greater adipose tissue mobilisation as a result of feed deprivation in the Merinos.

The levels of NEFA at 24 hours in Merino sired lambs in the current study were up to 33% higher than previously reported in the same lambs at rest (Stewart et al. accepted, Chapter 7, Table 8-4). By contrast, the NEFA concentration at slaughter in Terminal sired lambs were only 10% higher than at rest (Table 8-4). In addition, the levels of NEFA at 48 hours in Merino sired lambs observed in this study were up to 35% higher than previously reported in the lambs at rest (Stewart et al. accepted, Chapter 7, Table 8-4). These findings indicate that not only are Merinos more stress responsive than Terminal sired lambs under commercial slaughter conditions but they also respond more to feed deprivation (Gardner et al., 1999b).

Contrary to the hypothesis, the BHOB response to feed deprivation was not influenced by sire type. This was unexpected as, the lower NEFA response in Terminal sired lambs is also likely to have also produced differences in ketone production. However, an association between BHOB and NEFA was found which also differed between sire types. In Terminal sired lambs, there was a more rapid increase in BHOB response as NEFA concentration increased compared to Merino sired lambs, despite having a lower NEFA response. This indicates that higher yielding, Terminal sired lambs may have altered intrahepatic mechanisms to allow greater uptake of NEFA and overall ketogenic response (Cameron, 1992). This may reflect an increased use of NEFA as an energy source by lean tissue (Cameron, 1992; Hocquette et al., 1998), which is proportionately greater in Terminal compared to Merino breed type (Anderson et al., 2015).

The stronger association between BHOB and NEFA in Terminal lambs may also reflect a slower utilisation of BHOB, thus resulting in higher circulating levels observed for this genotype. Selection for increased muscling and leanness is linked to a shift from oxidative to glycolytic muscle fibre type (Greenwood et al., 2006). This shift may have altered substrate (BHOB) utilisation by Terminal lambs under extended periods feed deprivation, as they may be less reliant on fat catabolism due to a reduction in oxidative capacity (Gardner et al., 2014).

Indicators of fat metabolism during feed deprivation may also be linked to phenotypic fatness. Increasing GR tissue depth, a crude indicator of phenotypic fatness was found to be associated with an increase in BHOB concentration across all-time points. This suggests that the difference in fat mobilisation during feed deprivation may be partly driven by differences in whole body fatness (Chilliard et al., 2000). Although this association did not differ between sire types previous work has shown that Terminal breed types have lower percentages of body fat (Anderson et al., 2015). Therefore, animals with a greater GR tissue depth may have had increased fat turnover contributing to the higher BHOB concentrations.

The positive association between average NEFA concentrations and glucose concentration in male lambs was unexpected and opposite to the association found at rest (Stewart et al. accepted, Chapter 7). During feed deprivation NEFA concentrations increase as a result of a declining plasma glucose and decreasing insulin:glucagon ratio (Pethick et al., 2005). However, in this study the association between NEFA and glucose is likely to reflect both hepatic glycogenolysis and lipolysis due to the combined interactions of fasting (Pethick et al., 2005) and adrenergic stimulation due to stress (Martin et al., 2011). Hence, the stress driven mobilisation of all substrates is likely to have driven the positive association observed in this study.

Contrary to the hypothesis, there was no sire type difference in the glucose response to feed deprivation. It was expected that Merinos would have the greater decline in plasma glucose due to increasing duration of feed deprivation (Pethick et al., 2005), contributing to the higher NEFA and BHOB response observed (Stewart et al. accepted, Chapter 7). However under commercial slaughter conditions, plasma glucose may be elevated due to adrenergic stimulation and increased rates of hepatic glycogenolysis (Brockman and Laarveld, 1986). The effect of this may have been more pronounced in Merinos, which are more stress susceptible (Gardner et al., 1999b). Thus a stress induced increase in plasma glucose at slaughter in Merinos may have masked the hypoglycaemic effect of feed deprivation (Pethick et al., 2005) and prevented a sire type difference in glucose response at slaughter. The association between slaughter levels of blood glucose and live weight was not unexpected. As live weight increases, proportionately the liver represents a larger portion of the animals mass (Butterfield, 1988) and may have contributed to higher glucose levels due to the size related glycogen depot. This result suggests that larger lambs could be more capable of maintaining glucose levels under feed deprivation.

At slaughter, the plasma lactate concentration did not differ between feed deprivation groups, supporting previous work showing that extended feed deprivation has no impact on plasma lactate concentrations (Daly et al., 2006). In addition, basal and slaughter levels of lactate in this study (Table 8-3) were above normal for ruminants. This is likely to reflect contraction-linked energy demands of muscle associated with exercise and adrenergic stimulation (Pethick, 1993).

Table 8-4 Levels of non-esterified fatty acid (NEFA) concentration (mmol/L) in Merino and Terminal sired lambs at rest and under commercial slaughter conditions

	Merino		Terminal	
	Resting[f]	Slaughter[b]	Resting[f]	Slaughter[b]
Basal	0.13 ± 0.034	0.10 ± 0.010	0.11 ± 0.034	0.09 ± 0.082
24 hours	0.92 ± 0.029	1.22 ± 0.079	0.86 ± 0.030	0.95 ± 0.081
36 hours	1.12 ± 0.029	1.38 ± 0.079	0.98 ± 0.030	1.12 ± 0.078
48 hours	1.50 ± 0.029	2.03 ± 0.079	1.17 ± 0.030	1.37 ± 0.081

[f] Resting levels of NEFA (Chapter 7); [b]Basal samples collected prior to feed removal.

8.5.2 Effect of sire breeding values on the non-esterified fatty acid (NEFA) and β-hydroxybutyrate (BHOB) response to feed deprivation

Contrary to the hypothesis, there was no association between PEMD breeding values and NEFA and BHOB concentrations at slaughter. Therefore, it would be expected that under higher stress commercial slaughter conditions, progeny of sires with high PEMD breeding values would demonstrate a higher NEFA and BHOB response, independent of the metabolic effects of feed deprivation. Moreover in the current study, lower muscled Merinos, demonstrated a greater response to stress than the more muscular terminal sired lambs, as shown by a greater NEFA response at 24 hours of feed deprivation. This contradicts the earlier work by Martin et al. (2011) and McGilchrist et al. (2011) that showed the adipose response to adrenaline is greater in animals genetically selected for increased muscularity. Alternatively, the sustained metabolic impact of feed deprivation may have explained more variation in the adipose response compared to the rapid, short acting effects of adrenaline at slaughter (Boisclair et al., 1997; Pethick et al., 2005) thereby preventing any stress linked association between PEMD and NEFA and BHOB to be observed. Additionally, as there is no evidence suggesting that these breeding values produce differences in stress response, we therefore conclude that the differences across all groups are driven by their differential response to feed deprivation.

Contrary to the hypothesis, progeny with lower PFAT demonstrated a greater BHOB response, which was consistent across feed deprivation groups. However, this effect was small and not mirrored by a similar NEFA response in the same animals. The positive association observed between PFAT and BHOB in the current study indicates that genetically fatter animals may have a greater capacity for ketogenesis, which would enable them to more efficiently utilise adipose tissue during periods of nutritional restriction (Pethick et al., 2005). Supporting this assertion, Merino lambs from higher PFAT sires have been shown to have higher BHOB concentrations during feed deprivation (Stewart et al. accepted, Chapter 7). Alternatively, this result may indicate that genetically leaner lambs have a reduced capacity for ketogenesis during extended periods of feed deprivation under acute stress and

which could result in greater protein turnover to meet energy demands (Hocquette et al., 1998) and may result in greater carcass weight losses (Thompson et al., 1987).

Contrary to the hypothesis, there was no association between NEFA and PFAT or PWT. It is unclear why this association was not found, given the association observed in Terminal and Merino sired lambs during feed deprivation under resting conditions (Stewart et al. accepted, Chapter 7). In the current study, lower growth Merino lambs had higher stress responsiveness evident at 24 hours and greater responsiveness to feed deprivation at 48 hours demonstrated by higher NEFA concentrations. Therefore, a possible explanation is that the higher stress responsiveness of lower growth impetus Merino lambs at slaughter masked the association between PWT and the NEFA response to feed deprivation.

8.5.3 Effect of feed deprivation on carcass yield and meat quality

Results from this study demonstrate that feed deprivation greater than 36 hours is having a detrimental effect on carcase weight, aligning with previous work in sheep (Thompson et al., 1987; Warriss et al., 1987). From 36 to 48 hours of fasting, lambs lost approximately 3% of carcass weight. Similarly, there was a loss of approximately 3% in dressing percentage between 24 and 48 hours of feed deprivation. Carcass weight has been shown to be reduced by feed deprivation (Kirton et al., 1967; Carr et al., 1971; Thompson et al., 1987; Warriss et al., 1987). Thompson et al. (1987) showed that carcass weight is reduced by 0.1% per hour after 12 hours of fasting, although (Daly et al., 2006) reported opposing results. In 18 month old merino wethers deprived of feed for 0, 2 and 4 days under low stress pre-slaughter conditions, it was found that time off feed had no significant effect on carcass weights or GR fat depth. Similarly, Jacob et al. (2005b) found inconsistent effects of increasing lairage times on carcass weight, with only 50% of consignments showing a significant effect of time from 24 to 48 hours off feed. Likewise, variation in duration of on-farm curfew is likely to have influenced the inconsistent findings by (Jacob et al., 2005b) and indicates that total time off feed rather than lairage time is likely to affect carcase yields.

The association between feed deprivation and an increase in the ultimate pH of the M. *semitendinosus* (particularly in Merinos) was unexpected. Previous work has indicated that feed deprivation does not have an effect on glycogen concentrations or ultimate pH (Kirton et al., 1968; Tarrant, 1989; Jacob et al., 2005a; Daly et al., 2006). However, extended feed deprivation combined with pre-slaughter factors could be imposing additional stress (Bray et al., 1989) which may be causing increased glycogen turnover in the pre-slaughter period. Moreover, this result supports the earlier findings of (Gardner et al., 1999b) who demonstrated the susceptibility of Merinos to dark cutting under commercial slaughter protocols that involved feed deprivation. In the current study, 33% of Merino sired lambs had a pH24ST value above 5.8 compared to only 7% in Terminal sired lambs, with the majority of these lambs in the 48 hour group. Therefore, the susceptibility of Merinos to dark cutting may be reduced by ensuring that they are slaughtered within 24 hours of feed removal. There was no impact of feed deprivation on pH24LL, SHEAR5 or IMF percentage found in this study. In addition, all carcasses had a pH24LL < 5.8 and indicates that loin muscle glycogen levels were sufficient prior to slaughter. This is in agreement with previous work (Jacob et al., 2005a; Jacob et al., 2005b; Daly et al., 2006; Toohey and Hopkins, 2006) showing that feed deprivation (< 48hours) is unlikely to affect eating quality in lamb.

8.6 Conclusion

Merino and Terminal sired lambs differ significantly in in the metabolic response to feed deprivation and stress under commercial slaughter conditions, particularly in regards to adipose tissue turnover. In addition, commercial feed deprivation up to 48 hours appears to have a negative effect

on ultimate pH in Merinos and requires further research at an industry level. Importantly, increased feed deprivation beyond 36hrs is detrimental to the carcass weight and dressing percentage in lambs, which could affect the profitability of lamb producers and warrants further investigation.

Chapter 9 General Discussion

9.1   Influence of pre-slaughter stress and feed deprivation on ultimate pH, tenderness and intermediary metabolism – benefits to industry

Within the Australian lamb industry there has been little research benchmarking physiological or metabolic indicators of stress, nor their association with meat quality and carcass weight. This thesis set out to determine whether acute and chronic stress can be quantified using indicators in plasma at slaughter and if they relate to ultimate pH and tenderness in lamb. The impact of selection for carcass yield on the metabolic response to feed deprivation was also examined under resting and commercial conditions. In addition to these genotype differences, it also afforded a greater understanding of the relative contributions of acute stress and feed deprivation to the metabolic response at slaughter.

Chapter 4 quantified the impact of a range of environmental and production factors that influence these physiological indicators in plasma. An important early finding was the very high levels of NEFA compared to lactate and glucose at slaughter, indicating that significant fat mobilisation occurs in lambs in the pre-slaughter period, higher than previously described in the literature. This suggests that adipose tissue may be more important than glycogen as an energy source during commercial feed deprivation and acute stress. Given that the lambs used in this experiment were processed under best practice pre-slaughter management, the effect of feed deprivation on intermediary metabolism in lambs selected for carcase yield was further examined in Chapter 7 and Chapter 8.

The association between plasma indicators of acute and chronic pre-slaughter stress and lamb loin ultimate pH and tenderness was investigated in Chapter 5 and Chapter 6. An important finding was the positive association between plasma glucose and lactate and ultimate pH in lamb and represents the first large scale study in lamb to show that plasma indicators of stress relate to ultimate pH in lamb. The association be of stress on WBSF was investigated and demonstrated no relationship with the plasma indicators measured. However, there was an association with kill order which itself may be an indicator of duration of acute stress, hence we cannot discount that acute stress may be having an influence on lamb tenderness. From an industry perspective, the suggestion that tenderness is impacted by the immediate pre-slaughter practices is important and warrants further investigation.

The influence of feed deprivation in lambs genetically selected for carcase yield under resting and commercial conditions was examined (Chapter 7 and Chapter 8). Under resting conditions, differences in intermediary metabolism exist between Merino and Terminal sired genotypes. This difference also appeared to be partly linked to genetic selection for increased growth and leanness through the use of ASBVs for PWWT and PFAT. The sire type difference in fat mobilisation was also mirrored under commercial conditions but showed that Merino sired lambs mobilise significantly more adipose tissue under commercial feed deprivation, highlighting the sensitivity of adipose tissue to stress.

9.2   Quantifying acute and chronic stress in lambs at slaughter

This study was one of the first large scale studies to measure physiological indicators of acute and chronic stress in plasma at slaughter (Chapter 4). Overall, plasma indicators reflecting acute stress and feed deprivation were elevated at slaughter, with indicators of muscle damage and dehydration generally within the normal range. Differences between sites and kill groups existed, however these

effects were small in magnitude and likely reflected differences in pre-slaughter management, such as handling, time off feed and water, and transport times.

As expected, plasma indicators reflecting acute stress and feed deprivation were elevated at slaughter, however levels found in this experiment were significantly higher than previously reported in the literature (Pethick et al., 2005). One of the most interesting findings of this experiment was that plasma NEFA concentrations were markedly elevated (Heitmann et al., 1987) while more moderate increases in lactate, glucose and BHOB were observed. The metabolic response at slaughter also demonstrated associations with carcass weight and fatness and leanness, suggesting that lambs selected for increased lean meat yield may respond differently to feed deprivation. These findings underpinned the design for experiments outlined in Chapter 7 and Chapter 8, where it was examined how selection for increased carcass yield affects the metabolic response under resting conditions and at slaughter. In addition, these experiments also helped tease apart the combined effects of acute stress and feed deprivation on adipose tissue turnover at slaughter.

Levels of AST were mostly within normal range (Radostits et al., 2007) at slaughter, indicating that minimal long term tissue damage occurred from farm to slaughter. However, CK levels were above normal levels indicating that some degree of tissue damage had occurred in the pre-slaughter period (Boyd, 1988). Elevations in CK are widely considered specific and sensitive measures of acute muscle damage and bruising in ruminants (Tarrant, 1990; Russell and Roussel, 2007) due to their high concentration and activity in skeletal muscle. Elevations in CK can be transient (Grigor et al., 1997; Pettiford et al., 2008) and are also observed as a result of adrenergic stimulation (McVeigh and Tarrant, 1981) and handling (Hemsworth et al., 2011), which may have contributed to the higher levels seen at slaughter.

The levels of total protein and sodium found in this study indicate that overall, lambs were not dehydrated at slaughter. This contradicts the earlier study by Jacob et al. (2006c) which showed that approximately 50% of lambs were sub-clinically dehydrated after lairage in two Australian commercial abattoirs. One possibility is that the current study used weaned lambs which are familiar with drinking from troughs, unlike sucker lambs (Jacob et al., 2006c) and were consigned directly from farm to abattoir, rather than through saleyards. Sheep are resilient to dehydration, being able to maintain plasma volume and osmolarity during periods of water restriction. In part this is achieved by producing smaller and more concentrated volumes of urine (Jacob et al., 2006c), therefore plasma sodium and total protein may not accurately quantify dehydration in this study. Ideally, packed cell volume would have been also measured on blood samples. However, given the number of samples collected this was too cost-prohibitive.

Not all lambs will drink in lairage (Jacob et al., 2005b) due to unfamiliar environments (Knowles et al., 1993; Ferguson and Warner, 2008) with hydration status also affected by ambient temperature (Jacob et al., 2006b), and stress (Parker et al., 2003). Furthermore, the pre-slaughter period was long enough to cause dehydration if lambs failed to drink in lairage (Jacob et al., 2006c). Given the potential carcass yield losses from dehydration, further work is required to investigate new methods or technologies to quantify hydration status on a commercial level to manage dehydration and its potential impact on meat quality and yield.

For this experiment it would have been ideal to have more control over the pre-slaughter management of lambs, while retaining their commercial focus. Different stress treatments (acute stress, feed deprivation, water restriction) could have been applied to lambs within the same consignments, which may have provided a greater variation in stress response and in the plasma indicators of stress measured.

In addition, it would have been interesting to understand how blood indicators changed within the immediate pre-slaughter period. Cannulating lambs and repeat blood sampling throughout lairage and pre- and post- slaughter would provide information on the variation in plasma stress indicators, particularly those influenced most by adrenergic stimulation. This would be challenging and cost prohibitive on a commercial scale, however it would afford a more comprehensive understanding of the impact of time on fluctuations on metabolic indicators and stress hormone responses at slaughter.

9.3   Opportunities for carcass based grading systems in lamb

The grading systems for Australian lamb currently exist as a voluntary pathways-based grading system. This system is relatively simple by comparison to beef grading operating at a supply chain level whereby producers, processors and retailers follow a series of regulations and recommendations of critical control points that are known to impact lamb eating quality encompassing the pre-slaughter period through to retail. This process aims to ensure a minimum eating quality standard for all Australian lamb graded under Meat Standards Australia.

Under Meat Standards Australia grading systems for beef, individual carcasses are assessed for loin ultimate pH and must be below a pH of 5.71 at the time of grading. Carcasses that are non-compliant receive a penalty of approximately $AUD 0.50 per kg carcass weight (McGilchrist et al., 2012). While this penalty is passed onto the producer, the processor also has to carry the cost of processing factors and carcass wastage.

A key issue with high ultimate pH carcasses is the accelerated spoilage that occurs due to the less acidic pH, a particular concern in chilled export markets where aging can extend beyond 90 days. One option would be that lamb carcasses that have high ultimate pH are not eligible to enter into export markets. This would help maximise food safety and shelf life, particularly for chilled product entering into the USA.

However, applying penalties on the basis of cut-off type rules for ultimate pH means that a greater understanding of glycogen metabolism from farm gate to slaughter is required. What is not clear from the current research is whether glycogen levels were adequate when lambs left the farm, or whether they were depleted during the pre-slaughter transport and lairage period as a result of stress. So further understanding of the effects of supply chains is important to understand who bears the cost of dark cutting carcasses.

Alternatively, for indicators that predict shear force, segregating carcasses into tenderness categories would be potentially valuable for the industry. This system could underpin a grading system to segregate carcasses into eating quality categories, which would attract relative increases in carcass and cut price per kg (Lyford et al., 2010).

However, as this system has been broadly adopted across industry it no longer differentiates between individual lamb carcases. Hence, in order to maximise the value extracted from carcasses and deliver consistency in eating quality to consumers, industry is pushing for an individual carcass grading system for lamb. The challenge of implementing such a system in the lamb industry is that the cost of a human grader grading each carcass is not economically viable. Therefore, individual carcass grading in lamb lends itself to automation and to technologies that align with pre-existing operations within the plant.

The lamb processing sector has an interest in understanding whether acute and chronic stress is linked to reduced eating quality. If this were the case, a plasma metabolite measure may enable the identification of stressed animals at slaughter, enabling the segregation of carcasses into carcass

grades. Utilisation of a plasma measure of stress to predict ultimate pH or tenderness would need to operate at line speed and be able rapidly identify carcasses of inferior meat quality. Importantly, predictions would need to be highly accurate, precise and repeatable. Collection of a blood sample and interpretation of the result would also need to take place at the point of exsanguination. This would require installation of new carcass tracking systems at the point of slaughter, which would need to be traceable following skin removal and carcass dressing. For indicators predicting ultimate pH and tenderness, identification of carcasses of inferior quality would be useful to segregate carcasses as they enter chillers, which would have flow on effects in boning room and meat packing efficiencies.

9.4   Association between plasma indicators of stress and loin ultimate pH

An important finding of Chapter 5 was the positive association between plasma glucose and lactate and ultimate pH in lamb. This represents the first large scale study in lamb which has shown that plasma indicators of acute stress at slaughter relate to ultimate pH. This result highlights that acute stress prior to slaughter is a significant driver of glycogen turnover which is having a negative influence on ultimate pH in lamb.

It is well established that to maximise the concentration of muscle glycogen at slaughter, thereby reducing the risk of dark cutting, pre-slaughter stress and associated adrenaline release must be minimised (Tarrant, 1981). A number of studies have shown that stress events such as handling (Sutherland et al., 2016), exercise (Gardner et al., 2001a; Warner et al., 2005), transport (Jacob et al., 2005a), and time in lairage (Toohey and Hopkins, 2006) may cause stress and increased glycogen turnover.

However, previous work has shown that the immediate short term effects of stress before slaughter are unlikely to reduce muscle glycogen below the threshold for dark cutting if muscle glycogen levels are adequate (Immonen and Puolanne, 2000). Therefore, it is plausible in the current study that muscle glycogen levels were borderline, and combined with an acute stress response at slaughter, continuing to mobilise glycogen has resulted in an association being observed between ultimate pH and stress indicators. If glycogen concentrations had been adequate, it is possible that this experiment would not have exposed this association. A number of studies have shown that stress immediately before slaughter does not impact on ultimate pH (Warner et al., 2007).

An individual's stress susceptibility may also explain the association found between plasma lactate and glucose and ultimate pH. Lambs which are more susceptible to stress (e.g. poorer temperaments) may have greater rates of glycogenolysis in response to routine management practices such as mustering, handling and novel environments and therefore have lower resting muscle glycogen levels and be at risk of dark cutting (Coombes et al., 2014). The influence of temperament on the incidence of high ultimate pH has been well documented in beef (Tarrant, 1989; Coombes et al., 2014) but further work is required in lamb. A possibility in this study is that lambs with high glucose and lactate at slaughter due to stress, also had a greater adrenergic response throughout the pre-slaughter pathway due to handling, exposure to novel environments and loading/unloading. Thus, these peaks in adrenergic responses would have had the cumulative effect of gradually reducing muscle glycogen over the pre-slaughter period, potentially leading to glycogen levels below the threshold required to reach a low ultimate pH. Future experiments may be required to determine whether the plasma lactate and glucose response throughout the pre-slaughter period is linked to the change in muscle glycogen from farm to slaughter and its impact on ultimate pH.

However, despite there being a significant association between plasma lactate and glucose at slaughter with ultimate pH, the effect of plasma indicators was very small in comparison to the

production factors. Demonstrating this further, these indicators only explained 10% of the variation in loin ultimate pH. Moreover, the effect of stress on loin ultimate pH appears to be relatively small in comparison to the production and environmental factors accounted for in this study, which described 26% of the variance.

In the current study, factors such as site, year and kill group had the largest impacts on ultimate pH, with these factors likely to reflect environmental and other circumstantial stressors. A key difference between sites, years and kill groups would have been nutrition, which has previously been shown to play a crucial role in determining muscle glycogen concentration (Pethick and Rowe, 1996). These differences could also have been explained by management practices within extensive grazing enterprises, with factors such as mustering, frequency of handling, location of watering points and environmental factors such as weather likely to have affected muscle glycogen levels (Gardner et al., 2014). Therefore, the ability to predict ultimate pH will be improved by better understanding the role of glycogen turnover throughout the pre-slaughter period.

In addition to the production factors, lamb genotype also had an effect on loin ultimate pH. In line with previous studies, Merino genotypes had a higher ultimate pH in this experiment than Terminal and Maternal genotypes. Merino genotypes have been shown to have a higher stress susceptibility and greater risk for high ultimate pH (Gardner et al., 1999a). Genetic selection for muscling has been shown to reduce the propensity to mobilise muscle glycogen during acute stress and thus may have a protective effect against dark cutting. In this experiment it was shown that Terminal sired lambs, which are bred for increased lean meat yield (Anderson et al., 2015) had a lower ultimate pH than Merino sired lambs. Work by McGilchrist et al. (2011) and Martin et al. (2011) showed that high muscling genotypes have a reduced muscle response to adrenaline and therefore are less likely to mobilise muscle glycogen in response to acute stress. Further demonstrating this, McGilchrist et al. (2012) showed that cattle with larger eye-muscle areas had a reduced incidence of dark cutting.

Therefore, further work is required on a large industry scale to identify factors from farm to slaughter that cause acute stress within the pre-slaughter period, and quantify their impact on muscle glycogen levels on farm and at slaughter and how this affects ultimate pH in Australian lamb. A limitation of the current study was that muscle glycogen levels were not collected pre- and post-slaughter, which would have provided a greater understanding of the impact of pre-slaughter management and stress on loin ultimate pH in this study. Future work investigating the role of nutrition, genotype and pre-slaughter management requires a more tightly controlled experimental design.

9.5 Association between plasma indicators of stress and loin shear force

The aim of experiment 3 (Chapter 6) was to assess the association between plasma indicators of acute and chronic stress and lamb loin tenderness, as measured objectively by Warner Bratzler Shear Force (WBSF). This was an important study as tenderness is a key driver of consumer eating quality of lamb (Pannier et al., 2014), yet even the higher quality cuts such as the loin (M. *longissimus lumborum*) are known to vary markedly in tenderness. This was well demonstrated in a study by Pannier et al. (2014) who showed that approximately 40% of lamb loins rated as good every day (3 star) or lower by consumers under the Meat Standards Australia grading system with a significant proportion (7%) rated as unsatisfactory.

Contrary to the hypothesis, there was no association between WBSF and plasma indicators of stress, contradicting earlier studies in beef (Warner et al., 2007; Gruber et al., 2010; Pighin et al., 2015)

which showed a link between acute stress and tenderness. This was unexpected, as a clear association was demonstrated between glucose and lactate and loin ultimate pH (Chapter 5). In addition concentrations for plasma stress indicators were mostly above basal levels with a large range in concentration (Radostits et al., 2007) indicating that there was a degree of stress and variability in responsiveness in the lambs during the pre-slaughter period (Pighin et al., 2014).

Whilst this may indicate that there is no association between stress and tenderness, more likely it suggests that plasma indicators themselves are poor indicators of stress. The homeostatic regulation of glucose, fat and muscle metabolism, hydration status represents a normal physiological response to pre-slaughter stressors. Multiple factors such as physical activity, variable nutrition, and previous handling experiences would influence the slaughter responses (Ferguson and Warner, 2008), reducing the accuracy of plasma indicators as indicators of immediate pre-slaughter stress. Furthermore, the challenge with measuring indicators at slaughter and then relating them to meat quality traits is that the stress event, blood collection and physiological impact on shear force does not happen simultaneously. This would have also uncoupled the link between acute stress, plasma indicators and shear force. Future work should focus on applying stress treatments under more controlled experimental conditions and examining whether acute stress affects eating quality as assessed by untrained consumers, rather than only objective measurements of tenderness.

One unexpected finding was the positive association between WBSF and kill-order, which may reflect the duration of exposure to immediate pre-slaughter processes during the lead up to slaughter. Previous behavioural studies have shown that cattle observed to be more nervous or more stressed tend to move through the race at the end of the group (Grandin, 1980; Orihuela and Solano, 1994; Stockman et al., 2012). This may apply in this experiment, where lambs that are killed later within a kill group were more stressed and/or were exposed to that stress for longer (due to the later kill-time), underpinning the link between kill-order, acute stress and the resulting impact on tenderness.

A better understanding of the factors driving the kill-order effect are required. From a practical industry perspective animals must be killed one at a time. However, future work could be directed at understanding how different handling methodologies cause stress (Hargreaves and Hutson, 1997; Deiss et al., 2009; Sutherland et al., 2016) and influence both the kill-order effect on plasma metabolites (Chapter 4) and shear force. An example of where this theory has been applied is the use of automated yards and gates in the lairage yards installed in some pig abattoirs. Opportunities could exist for the lamb industry to install similar handling equipment.

Similar to the findings of Chapter 5, production factors had the greatest impact on loin WBSF in lamb. However there was still considerable variation not accounted for, with the base model only explaining 50% of the variation in WBSF. Considerable variation existed between years and kill groups, which varied by up to 20 N after correcting for other environmental factors such as site and genotype. This may be related to differences in growth rate or nutrition that have been shown to influence tenderness (Hopkins et al., 2005) as well as processing effects (Johnston et al., 2001). However, it may also reflect differences in stress experienced in these cohorts, which was not captured by plasma stress indicators.

Importantly, the average WBSF values found in this study were higher than 27 N, which is associated with a 10% failure rate for lamb eating quality (Hopkins et al., 2006a). In addition, there was a clear positive association found between WBSF and ultimate pH. This highlights that significant improvement in WBSF can be achieved by implementing strategies to reduce the incidence of high ultimate pH in lamb (Chapter 5).

The results from Chapter 5 and Chapter 6 clearly demonstrates that the use of plasma indicators of stress as a predictive tool to identify high pH carcasses or high WSBF carcases on a commercial basis is not practical or realistic and should be avoided in future research.

9.6   Metabolic response to feed deprivation in low and high yielding lambs

The aim of Chapter 7 was to examine the metabolic response to feed deprivation in lambs genetically selected for high and low carcass yield characteristics under resting conditions. The purpose of this was to further understand the mechanisms that underpinned the markedly elevated NEFA levels at slaughter demonstrated in Chapter 4. The key findings from this experiment were that differences in the metabolic response to feed deprivation existed between Merino and Terminal sired lambs. In addition, selection for increased genetic growth, leanness and muscling also influenced the NEFA response to feed deprivation.

Rejecting the hypothesis, Merino sired lambs had a greater decrease in plasma glucose concentration and a greater NEFA and BHOB response compared to Terminal sired lambs during 48 hours of feed deprivation. The decline in plasma glucose concentrations became evident after this initial period and is likely to have coincided with a decrease in insulin:glucagon concentration ratio (Holness and Sugden, 1989) eliciting an increase in hepatic glycogenolysis and gluconeogenesis and increased rates of lipolysis in Merino lambs.

Alternatively, higher glucose levels observed in Terminal sired lambs during feed deprivation may be related to up-regulated gluconeogenic pathways in Terminal sired lambs. However, as the lipolytic response (indicated by NEFA response) was lower in Terminal sired lambs, it indicates that glycerol is unlikely to be the substrate driving the higher levels of glucose observed in Terminal sired lambs. During decreased glucose availability, there is an increase in the release of glucose precursors, including amino acids from muscle (Khoo et al., 1973) and greater removal of amino acids from portal circulation (Heitmann and Bergman, 1980). Protein turnover in breeds selected for faster growth is higher (Oddy et al., 1995; Oddy et al., 1998), therefore a similar effect may be seen in Terminal lambs where higher protein turnover is contributing greater quantities of gluconeogenic substrates, resulting in the higher and more stable glucose concentrations observed during feed deprivation.

Previous work has shown that greater amounts of alanine are removed from the portal circulation in lambs undergoing feed deprivation (Heitmann and Bergman, 1980). Supporting this there was a trend for plasma glucose concentrations to increase with selection for increased PWT. In addition, NEFA response to feed deprivation was found to decrease in response to selection for increased PWT. Although these associations were only significant in Merino sired lambs, it demonstrates that increased genetic growth reduced the lipolytic response. Moreover, higher PWT Terminal sired lambs had the lowest NEFA response during feed deprivation. Future studies examining the role of feed deprivation on the metabolic response in sheep divergent for genetic growth should examine protein metabolism, to understand what their role is in maintaining glucose homeostasis.

The differences in glucose and NEFA response may be driven by differences in breed insulin regulation. Ponnampalam et al. (2012) showed that Merino lambs had a greater rebound in plasma NEFA concentration compared with cross-bred lambs on a low plane of nutrition following an insulin challenge. The authors proposed that this was most likely a counter-regulatory response to rebound hypoglycaemia, which was also greater in Merino lambs, rather than a direct response to the hyperinsulinemia *per se*. This observation suggests that Merino lambs have a greater capacity to produce energy via breaking down adipose tissue when energy production from carbohydrate (glycogen) is lower.

In support of the hypothesis, the decline in glucose concentration and increase in plasma NEFA were greatest in the progeny of genetically leaner (low PFAT) sires. This indicates that the progeny of lean sires maintain lower plasma glucose concentrations during feed deprivation. This would result in a higher glucagon to insulin ratio, stimulating lipolysis and contributing to the higher NEFA and BHOB observed in these animals (Carter et al., 1989, Van Maanen et al., 1989). A greater proportion of muscle to adipose tissue will increase the uptake of glucose in response to insulin (Prior and Smith, 1982). Thus in leaner animals that proportionately have more muscle, basal levels of insulin may be higher (Briegel and Adams, 2002) resulting in a more rapid decline in glucose concentrations during acute feed restriction. Low glucose levels would also potentiate greater increases in glucagon. Furthermore, it has been suggested that genetically leaner animals may also be more sensitive to the effects of glucagon (Carter et al., 1989), which would further enhance lipolysis. In addition, measuring the insulin and glucagon responses in sheep divergent for phenotypic and genotypic leanness and fatness will improve our understanding of the hormonal regulation of intermediary metabolism in lambs during feed deprivation.

The glucose and NEFA responsiveness in the progeny of low PFAT sires may be linked to the greater proportion of glycolytic fibre types (Greenwood et al., 2006) in these animals. This is likely to lead to increased glucose consumption by muscle (Hocquette et al., 1998), which in turn would lead to the lower levels of glucose in plasma evident in low PFAT sired lambs in this study. This would also have resulted in a higher glucagon to insulin ratio, explaining the elevated NEFA concentrations. Previous work by Martin et al. (2011) and McGilchrist et al. (2011) has shown a similar response as a result of adrenaline administration in sheep and cattle selected to be more muscular and leaner. Although feed deprivation is not acting directly through the adrenergic axis, the associated glucagon release makes use of the same post-receptor reaction cascade leading to the mobilisation of triglyceride and the release of NEFA (Pethick et al., 2005). However, feed deprivation has been shown to cause psychological stress (Bourguet et al., 2011) and therefore could potentiate an adrenergic response. The overall effect would be for genetically leaner lambs to have an up-regulated lipolytic pathways and rates of fat turnover in times of feed deprivation (Carter et al., 1989). To examine the interaction between genotype fibre type and adrenergic effects on the metabolic response to feed deprivation more thoroughly, it would be useful to determine the number and type of β-adrenergic receptors in both muscle and fat tissue. This would provide a greater understanding of how feed deprivation influences the stress response to feed deprivation.

Clearly, further work is required in this area to understand the how genetic selection for body composition and phenotypic leanness and fatness affects intermediary metabolism. A limitation to the current study was that HCWT in conjunction with loin fat weight or loin muscle weight were used as indicators of body composition and were only measured at slaughter. It would have been ideal to document the composition of lambs at the time before and after feed deprivation trial. Future studies could utilise computer tomography (CT) to measure live animal composition more accurately (Anderson et al., 2015) to more determine the role that composition has on energy and fat metabolism during feed deprivation. In particular, it would facilitate a more comprehensive evaluation of the impact of selection for reduced PEMD, PFAT and PWT on fat distribution, particularly abdominal fat. Furthermore, CT scanning lambs post-feed deprivation would enable a greater understanding on whether feed deprivation causes significant losses in lean and fat tissue. Future studies should also utilise commercial technologies such as dual x-ray absorptiometry (DEXA) which would more accurately quantify the effect that feed deprivation has on lean meat yield.

9.7 Impact of commercial feed deprivation on metabolic response and carcass traits in lamb

The aim of Chapter 8 was to re-examine the metabolic effects of feed deprivation found in Chapter 7, but under commercial slaughter conditions. This is an important study to follow on from Chapter 4, which showed that prime lambs have higher levels of plasma NEFA at slaughter than previously reported (Warriss et al., 1989; Pethick et al., 2005), following routine pre-slaughter management, which included feed deprivation for up to 48 hours. Examining the metabolic effect of feed deprivation under commercial conditions is challenging, as levels of circulating metabolites are likely to be affected by acute stress.

The confounding of the effects of feed deprivation and acute stress on pre-slaughter metabolism are also likely to extend to genotype and selection for greater growth, leanness and muscling. Selection for increased carcass yield appears to drive a higher adipose tissue response in sheep in response to feed deprivation (Chapter 7). Increasing selection for PWT in Merino sired lambs is associated with a significant reduction in the glucose response and elevated NEFA response to feed deprivation, while in Terminals genetic leanness was driver of greater glucose decline and increased fat turnover in response to feed deprivation. While it is known that selection for increased muscling increases the fat response to adrenaline (Martin et al., 2011) , it was not clear how this response would be affected by concurrent feed deprivation, however it was expected that adrenaline would initially be a stronger driver of adipose tissue metabolism leading to a greater NEFA response in Terminals, early in feed deprivation. This difference in NEFA response would be expected to diminish over time due to the Merino sire types greater decline in glucose and NEFA response to extended feed deprivation.

Contrary to the hypothesis, yet supporting the earlier findings of Chapter 7, Merino sired lambs showed a significantly greater adipose tissue response than Terminal sired lambs measured during commercial slaughter. As the level of imposed stress was the same across all treatment groups, this difference in NEFA response is the observed effect of feed deprivation. However, it was found that the NEFA response in Merino sired lambs was 20% higher under commercial conditions, whereas in Terminal sired lambs, there was little difference in NEFA response between the two experiments. This demonstrates that the adipose tissue is highly sensitive to both feed deprivation and stress in Merino genotypes.

Unlike the findings of Chapter 7, sire type differentials in NEFA response in the current study were not driven by differences in glucose decline in response to feed deprivation. One hypothesis is that this is due to the combined, yet opposing effects of adrenergic stimulation and feed deprivation on hepatic glycogenolysis and gluconeogenesis. It would be expected that initially during feed deprivation, plasma glucose levels would be more greatly affected by the hyperglycaemic effects of adrenaline. However as liver glycogen levels decline with extended feed deprivation, greater quantities of glucose from other gluconeogenic substrates would occur. The positive association between NEFA and glucose in this study tends to support this assertion. Furthermore, this highlights the challenge in utilising metabolites as indicators of acute stress at slaughter (Chapter 4).

An experimental design limitation was that lambs were not re-randomised at the commencement of the trial but remained in their groups from the resting feed deprivation trial (Stewart, et al. submitted, Chapter 7). The authors acknowledge this weakness in design and animals should have been re-randomised to groups, which would have allowed the authors to test the effect of replicate. However, in the author's opinion a period of three weeks was more than adequate for there to be no carry-over effects from the previous experimental treatments (Chapter 7).

This assertion is based upon several reasons. Firstly, the live weight of each group was similar to the average live weight of each replicate in Chapter 7, suggesting that animals had re-alimented sufficiently. Secondly, the basal levels of metabolites in all lambs, were within normal range, and the only difference evident between treatment groups was for the 48 hour treatment lambs in their glucose concentrations and the 24 hour and 48 hours group in their NEFA concentration. Fluctuations in NEFA and glucose level are more likely to reflect slight differences in adrenergic stimulation between the groups at the time of sampling. In addition the 48 hour group in the current chapter equates to the first replicate in the resting feed deprivation trial (Chapter 7) and was therefore least likely to show a carry-over effect of the previous experiment. Moreover, all metabolite responses were corrected for basal metabolite concentration, helping to negate any potential carry-over effects from Chapter 7 and in almost all cases these basal metabolites were dropped due to non-significance.

Thirdly, previous work in cattle has demonstrated that following acute feed restriction of 24 to 48 hours, 7 to 14 days of re-alimentation is long enough to regain pre-existing levels of dry matter intake and rumen fermentative capacity (Cole and Hutcheson, 1985). Fisher et al. (2010) also demonstrated that following transport of 12, 30 or 48 hours (with complete food and water deprivation) there was a decline in live weight which after 72 hours of recovery, sheep had recovered 91 to 96% of their pre-transport live weight. Elevation in metabolites recorded had largely returned to basal levels by 72 hours of recovery. In addition, replicates of 12, 30 and 48 hours within this experiment were tested only 1 week apart. Replicate and its interaction with treatment group had no effect on live weight or BHOB response. Furthermore, in sheep undergoing hormone challenges with insulin and adrenaline, which were run twice daily for 5 consecutive days, there was no evidence of a day effect on metabolic responses, or of the challenge administered between 4-14 hours prior. This was tested in metabolic response models for NEFA, glucose and lactate published by (Martin et al., 2011), although the sampling time effect was dropped due to non-significance (K. Martin, pers comm). Therefore, on the basis of the evidence presented the authors believe that the 3 week lag between treatments described in Chapter 7 and Chapter 8 was sufficient to negate any potential carry-over effect on results.

As expected feed deprivation had minimal effects on meat quality and carcass traits. Feed deprivation did not affect loin ultimate pH, in line with previous work that showed time off feed has no impact on muscle glycogen mobilisation (Daly et al., 2006). Likewise feed deprivation did not influence other meat quality attributes such as loin intramuscular fat content or tenderness. Although intramuscular fat is a highly labile source of adipose tissue (Pethick et al., 2004), the duration of feed deprivation may have been too short to cause measurable reductions of adipose tissue in this depot.

However, an unexpected finding of Chapter 8 was the negative effect of feed deprivation on the ultimate pH of the M. *semitendinosus* (ST) in Merino sired lambs. Increasing feed deprivation from 24 to 48 hours was associated with a 2% increase in ultimate pH from 5.63 to 5.74. This effect may be driven by the intrinsic properties of the ST muscle as well as the stress responsiveness of the Merino breed. The ST muscle has a greater proportion of fast-twitch glycolytic fibres (Conlee et al., 1978) and generally has lower glycogen levels with slow rates of glycogen synthesis. Terminal sired lambs selected for increased muscling have decreased proportions of oxidative myofibres (Greenwood et al., 2006) and reduced muscle response to adrenaline (Martin et al., 2011). Feed deprivation is known to increase β-adrenergic receptor affinity (Houseknecht et al., 1995) as well as cause a stress response. These effects may be amplified further in the Merino breed, which have been shown to have greater muscle glycogen turnover in response to stress greater compared to cross bred lambs (Gardner et al., 1999a). From an industry perspective, this result highlights that single point measures of loin pH in the loin (as for beef) on an industry level may not be appropriate for the lamb industry as part of an individual carcass grading system. Moreover, utilising loin pH as a proxy for whole carcass pH may result in high ultimate pH carcasses entering into chilled export markets, which could limit shelf life.

Validation of this result is required by repeating this experiment with larger numbers of animals. In addition, muscle glycogen samples from pre- and post-feed deprivation would add significantly to the understanding of glycogen metabolism during commercial feed deprivation.

The lack of effect of feed deprivation on meat quality traits such as IMF% and WSBF was not unexpected. However, IMF% has been reported as a highly labile source of adipose tissue, therefore if depots are mobilised during feed deprivation, surely this would be a significant contributor to NEFA turnover. In addition, larger studies, with more diverse/extreme sires and a greater range in IMF may help to examine this effect. It is possible that the range in IMF% was simply not large enough in the current dataset to see an effect.

Given the relatively small size of this study, it was an unexpected finding that feed deprivation had a negative impact on carcase weight and dressing percentage. Increasing time off feed from 36 hours to 48 hours was associated with a 0.72kg or 3% loss in HCWT. Previous work has shown that feed deprivation is detrimental to carcass yield. This is in alignment with earlier work by Thompson et al. (1987), whom showed that carcass weight is reduced by 0.1% per hour after 12 hours of fasting. This is an important finding for the lamb industry as it indicates that significant losses in carcass weight gains could be avoided by limiting duration of total time off feed to 36 hours. For example, if 50% of lambs consigned to slaughter underwent 48 hours of feed deprivation, for a 21kg carcass this would equate to carcass weight loss of approximately 0.6kg. At the prices for lamb of 650c/kg (source: MLA), if processing 5000 lambs per day, this equates to a loss of approximately $10,000 per day. This result indicates that the current industry best practice recommendations for time off feed need to be re-evaluated. In addition, the reasons underpinning the requirement for feed deprivation (biosecurity, fleece bacterial loads, food safety) should be re-evaluated.

A limitation of this study was that it was not possible to determine what proportion of carcass weight losses were from fat or lean tissue. While there was no effect of feed deprivation on carcass LLFAT, LLWT and GR tissue depth, it would have been more valuable to determine the effect on whole carcass composition. Future studies could involve repeating the current experiment but utilising newer technologies to predict carcass composition. Opportunities may exist to utilise devices such as DEXA or CT imaging to assess whether significant carcass yield losses occur as a result of feed deprivation. This would eliminate the need to utilise point measure traits such as GR tissue depth or c-site fat depth as predictors of overall carcass fatness. This is particularly important in a genetically diverse population, these traits may be a biased measure of fat distribution within the carcass.

Given the large metabolic response to feed deprivation, combined with the carcass weight losses and increased incidence of high ultimate pH, it is evident that the duration of pre-slaughter feed deprivation should be re-evaluated on a larger industry scale. If consistent with findings reported here, this would warrant updating lamb pathways based grading systems and best practice pre-slaughter management to include a total time off feed limit of 36 hours.

9.8   Summary

This thesis reveals some important impacts of acute stress and feed deprivation on plasma physiological responses at slaughter and the influence on lamb ultimate pH, tenderness and carcass weight. This was the one of the first comprehensive studies to quantify stress at slaughter using plasma indicators of acute stress, feed and water deprivation and muscle damage. However as discovered, the physiological response to acute and chronic stressors is highly variable and not easily or accurately quantified by plasma indicators at slaughter.

However, there are several key findings that are highly relevant and important to the Australian lamb industry. The association between plasma glucose and lactate with loin ultimate pH shows that acute stress is influencing glycogen concentrations in the immediate pre-slaughter period. Future work should be directed at developing new strategies to manage lambs during this period to reduce acute stress and to mitigate pH issues to maximise meat quality and food safety. The negative effects of kill order on tenderness further support this and warrant further investigation.

A significant finding was the adipose tissue response at slaughter, demonstrated by markedly elevated NEFA levels at slaughter in lambs slaughtered under best practice pre-slaughter management. The potential causes underpinning this were further explored in the feed deprivation trials under resting and commercial conditions, to understand how acute stress and feed deprivation influenced fat turnover. While the Merino breed continues to demonstrate greater responsiveness to feed deprivation and stress, the metabolic response to feed deprivation also appears to be driven by genetic potential for growth and leanness, indicating that recommendations for feed deprivation may need to be updated to accommodate more modern lamb genotypes. In addition, the negative impact of feed deprivation on M. *semitendinosus* ultimate pH and carcass weight is an important finding and indicates that best practice pre-slaughter management may need to be updated to include no more than 36 hours off feed before slaughter to maximise meat quality, carcass weights and animal wellbeing. However this would require further investigation at an industry level to validate the findings in the current study.

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