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Supplementation to reduce the impact of mycotoxins and insufficient magnesium

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Abstract

The Southern beef compliance project (B.SBP.0110) identified that low pasture magnesium (**Mg**) concentration was associated with an increased incidence of dark cutting in grazing cattle. Hypomagnesaemia in grazing beef cattle originates because of a) low pasture mineral concentrations; b) insufficient intake of pastures to provide sufficient levels of mineral; or c) reduced mineral absorption. This project aimed to evaluate the benefits of supplementing grass-fed cattle with Mg; the influence of dietary intakes of potassium (**K**) and protein on Mg absorption, muscle glycogen synthesis and breakdown; and the impact of feeding a mycotoxin binder on growth rate, muscle glycogen and incidence of dark cutting.

The dietary K and protein experiments did not elicit the anticipated responses in insulin sensitivity, insulin responsiveness, glucose disappearance rates, adrenaline sensitivity or glycogen metabolism. The null-effect within these experiments was likely associated with the presentation of the diet to the cattle, i.e. oaten chaff and formulated pellets, compared to the lush, rapidly growing pastures on a commercial farm. As such, these rations had a greater dry matter when compared with a typical pasture diet. Due to the high dry matter content of the diets fed in these experiments, it is probable that rumen passage rates were slower than what is typically observed in grazing cattle possibly resulting in an increase, or at least more efficient, Mg absorption in these cattle.

On farm short-term Mg supplementation was not consistently associated with an increase in muscle glycogen or a reduced incidence of dark-cutting across the 14 producers. Magnesium supplementation was associated with an increase in plasma Mg concentration for 4 of the 14 producers. In addition, 2 of the 14 producers observed an increase in muscle glycogen in response to Mg supplementation. Results from this experiment highlight that pasture mineral imbalances were present for some producers during autumn, winter and early spring and as such some grazing stock were at risk of Mg deficiencies during these periods. Overall Mg supplementation during these periods may be beneficial, although further research is required to understand the underlying mechanisms in which hypomagnesaemia influences the incidence of dark cutting in grazing beef cattle.

Finally, supplementing cattle with a mycotoxin binder was not associated with improved growth or carcass characteristics. Nor did mycotoxin binder supplementation influence the incidence of dark cutting, although the incidence of dark cutting in this experiment was minimal (2.9%), regardless of mycotoxin binder supplementation.

Executive summary

Dark cutting has been identified as a major cause of carcass non-compliance within the Meat Standards Australia (**MSA**) program. The 2017 Australian beef quality audit highlighted that between 2015 and 2017 the national incidence of carcass non-compliant grass-fed carcasses was 10.8 % from 3.1 million MSA grass-fed graded carcasses. Dark cutting beef has been estimated to cost the Australian beef industry approximately $55 million per year. Dark cutting is the consequence of low muscle glycogen concentrations at slaughter, which is the result of glycogen concentration on farm minus the amount used during the pre-slaughter period. As a result, interactions between animal management, transport, lairage, climate conditions and processing may have a cumulative impact on the incidence of dark cutting. Additionally, the Southern beef compliance project (B.SBP.0110), determined that low pasture magnesium (**Mg**) concentrations were associated with an increased incidence of dark cutting in grazing cattle. This report also defined that the incidence of dark cutting increased during late summer through to early winter. Furthermore, in these southern regions, perennial pastures may contain endophytes that can produce high concentrations of alkaloids (mycotoxins), some of which are toxic to cattle. There is evidence to suggest that mycotoxins from perennial pastures and low Mg are associated with increased dark cutting during the late summer to early winter period. For grazing cattle low pasture mineral concentrations; insufficient intake of pastures to provide sufficient levels of mineral; and/or reduced mineral absorption are associated with hypomagnesaemia. Magnesium is completely reliant on consumption and absorption by the body. Furthermore, Mg has an inherent role in glucose metabolism as an enzymatic cofactor for major metabolic pathways and acts as a secondary messenger for insulin. Subclinical Mg deficiency results in reduced glycaemic control and insulin resistance by reducing both insulin secretion and peripheral tissue sensitivity. Subclinical Mg deficiency also causes an increased stress response (driven by adrenaline), decreased appetite and increased muscle contraction, which leads to glycogen breakdown. The combination of these mechanisms indicates that glycogenesis (glycogen production) during the post-prandial period is impaired and additionally the rates of glycogenolysis (glycogen breakdown), will be increased by subclinical hypomagnesaemia. Thus, it is anticipated that this impaired glycogenesis as a fuction of hypomagnesaemia has an intrinsic role in the incidence in dark cutting of grass fed beef from Southern Australia. Furthermore, it has been demonstrated that pasture mineral imbalances are widespread during autumn, winter and early spring, suggesting that grazing cattle are predisposed to Mg deficiencies in these regions. During these periods, grass dominant pastures that are young, short and rapidly growing are likely to have high crude protein levels, high K, high Dietary Cation-Anion Difference (**DCAD**) balance and low Mg concentrations. As such three experiments have been conducted to evaluate the influence of diet on the mechanisms associated with dark cutting in grass fed cattle. Therefore this project was undertaken to evaluate benefits on supplementing grass fed cattle with Mg and mycotoxin binders to reduce the incidence of dark cutting beef in Southern Australia. Additionally, experiments were conducted to investigate the influence of dietary K and protein on Mg absorption, glycogen synthesis and breakdown.

Experiment 1, Dietary Protein. The purpose of this experiment was to evaluate the impact of varying levels of dietary protein on the Mg status of cattle and its impact on: i) responses to exogenous insulin and glucose disappearance following an glucose tolerance test; ii) adrenaline sensitivity during an exogenous adrenaline challenge; and iii) the impact on glycogen depletion and repletion during and after an exercise challenge. Eighteen Angus steers were randomly allocated to three dietary protein treatment groups, thus there were six animals per treatment. The diets consisted of: 1) low (≈ 10 %); 2) medium (≈ 20 %); and high (≈ 30 %) crude protein levels. Cattle were subjected to a series of challenges to evaluate the influenced of dietary protein on whole body insulin responsiveness, glucose utilisation and clearance and responsiveness to adrenaline. Muscle glycogenolysis was also evaluated where muscle samples from *Longissimus thoracis* (LT), *semitendinosus* (ST) and *semimembranosus* (SM) prior to, directly after exercise and then 3 and 6 days post exercise. Results from this experiment identified that increasing dietary protein within the

current experiment had no impact on insulin sensitivity, insulin responsiveness, glucose disappearance rates, adrenaline sensitivity or glycogen metabolism during and after an exercise challenge for these cattle, against expectation. This null result is likely associated with the highly controlled conditions within this experiment, predominantly associated with diet presentation with a consistent quality and composition. Specifically, that these cattle were confined to pens thus restricting locomotion and foraging activity required to elicit the anticipated responses. Although the results presented here were against expectation, the data suggests that that dark cutting in cattle consuming high protein diets may not be elicited by changes in insulin sensitivity or altered sensitivity to adrenaline.

Experiment 2, Dietary Potassium. The purpose of this experiment was to evaluate the impact of varying levels of dietary K on the Mg status of cattle and its impact on: i) responses to exogenous insulin and glucose disappearance following an glucose tolerance test; ii) adrenaline sensitivity during an exogenous adrenaline challenge; and iii) the impact on glycogen depletion and repletion during and after an exercise challenge. Eighteen Angus steers were randomly allocated to three dietary K treatment groups, thus there were six animals per treatment. The diets consisted of: 1) low (≈ 1.5 %); 2) medium (≈ 2.7 %); and high (≈ 3.8 %) K levels. Cattle were subjected to a series of challenges to evaluate the influenced of dietary K on whole body insulin responsiveness, glucose utilisation and clearance and responsiveness to adrenaline. Muscle glycogenolysis was also evaluated where muscle samples from *Longissimus thoracis* (LT), *semitendinosus* (ST) and *semimembranosus* (SM) prior to, directly after exercise and then 3 and 6 days post exercise. Similar to the previous experiment, dietary K did not influence insulin sensitivity, insulin responsiveness, glucose disappearance rates, adrenaline sensitivity or glycogen metabolism during and after an exercise challenge. The variations of dietary K in these cattle did not generate subclinical hypomagnesaemia, because neither glycogenesis or glycogenolysis were influenced by K concentrations. High K diets within this experiment were used in an attempt to decrease absorption of Mg in the rumen, thereby inducing hypomagnesaemia. Due to the high dry matter content of the feeds fed in this experiment, the rumen passage rates were likely much slower in comparison with grazing cattle and as such, this could increase Mg absorption from these diets contributing to the null result here. This suggests that further investigations into the effect of pasture composition on hypomagnesaemia and subsequent incidence of dark cutting in grazing cattle is warranted.

Experiment 3, Mg Supplementation. This experiment investigated the influence of Mg supplementation to reduce the incidence of dark cutting in grazing cattle. Commercial grass-fed beef cattle (n = 1075) from 14 producers located on King Island, were randomly separated into groups (n = 44). Cattle were supplemented with a pellet that either contained or did not contain Mg, for 7 to 14 days prior to slaughter. The pellets were isoenergetic and isonitrogenous, the only difference being the Mg content. Supplementing cattle with increased dietary Mg did not influence overall muscle glycogen concentration or incidence of dark cutting. Although, Mg supplementation was associated with increased muscle glycogen for 2 of the 14 producers. Within this experiment, FOO had the greatest influence on muscle glycogen concentration, suggesting that graziers should ensure that there is enough FOO (≥ 1500 kg/ha) to ensure that cattle consumption rates are adequate to avoid hypomagnesaemia. Alternatively, producers could consider supplementing cattle with a high fibre roughage to reduce rumen passage rates, increasing Mg absorption. The results suggest that supplementing cattle with Mg, may be useful when cattle are at risk of hypomagnesaemia and have limited FOO. However, it appears the hypomagnesaemia risk can probably be managed more cost effectively by: i) ensuring high levels of FOO, which will also have a positive impact on muscle glycogen concentration and subsequently reducing the incidence of dark cutting; ii) graze ryegrass pastures at the 3 leaf stage to avoid pasture mineral imbalances; and iii) provide access to roughage if the DM content of grasses is less than 15%, to slow rumen passage rates.

Experiment 4, Mycotoxin Binder Supplementation. This experiment investigated the influence of supplementing cattle with a mycotoxin binder on ii) the growth performance of cattle; ii) glycogen storage and metabolism[1] and iii) the incidence of dark cutting in cattle grazing pastures containing endophytes. One hundred and one Black Angus, Hereford and Hereford × Angus steers (577.1 ± 28.6 kg) were used enrolled into this experiment. Upon enrolment, cattle were allocated to one of two treatments: 1) Control (n = 51) or 2) supplemented with a commercially available mycotoxin binder for 50 days prior to slaughter (binder, n = 50). Cattle in the binder cohort were offered 50 g/head/day mycotoxin binder (Mycofix ® plus, Biomin Holding GmbH, Austria), whereas control cattle were offered the same supplement base but were not offered the mycotoxin binder. The initial findings from this experiment suggest that the mycotoxin binder did not greatly influence the performance of these cattle. However, the control cattle had a larger weight change during the experiment (12.1 ± 2.1 kg*; P* < 0.0001) and had a higher ADG (0.25 ± 0.04; *P* ≤ 0.0001) when compared with cattle in the binder treatment. However, this increased growth did not translate into increased differences in final live weight or carcass performance. The incidence of dark cutting in both the binder and control cattle was also minimal (2.9%). Regardless, the results from this experiment are inconclusive and as such it is difficult to make specific recommendations regarding mycotoxin binder supplementation.

This project has provided additional knowledge towards understanding of the nutritional factors and the mechanisms in which these influence the incidence of dark cutting in grazing beef enterprises in Southern Australia. Developing a greater understanding of the 'on farm' factors that are associated with an increased risk of dark cutting is required. Enhancing this knowledge will allow for the development of effective management strategies to reduce the incidence of dark cutting within the Australian grass fed industry.

[1] Due to the impact of COVID-19 on travel, an on farm muscle sample was unable to be collected from these cattle. A muscle sample from slaughter has been collected, however samples remain in storage in Tasmania due to logistical constraints. Pasture samples also remain on King Island due to logistic challenges of frozen transport.

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1 Background

The Meat Standards Australia (**MSA**) program was developed for the Australian beef industry to ensure a consistent eating quality experience for consumers. Dark cutting is a major cause of non-compliance within the MSA program. Carcasses are classified as dark cutting when ultimate pH (**pHu**) of the striploin (*longissimus thoracis*) is > 5.7. Dark cutting reduces a consumers eating experience (Tarrant 1989; Ferguson *et al.* 2001) and is also associated with variable tenderness (Purchas and Aungsupakorn 1993; Watanabe *et al.* 1996); difficulty cooking (Cox *et al.* 1994); and increased risk of microbial spoilage (Faustman and Cassens 1990). As such dark cutting carcasses are devalued at the retail level as an inferior product, with both beef processors and producers experiencing economic loss (Jose *et al.* 2015). Dark cutting beef is estimated to cost the Australian beef industry approximately $55 million per year (Jose *et al.* 2015). McGilchrist *et al.* (2012) estimated that the cost of dark cutting to producers equated to approximately $0.50 (AUD) per kg carcass weight or $7.09 (AUD) for every carcass graded within the MSA program during 2009. According to the Australian Bureau of Statistics inflation increases this value to $8.71 (AUD) in 2019 (Australian Bureau of Statistics 2019), however this does not account for the change in number of carcasses graded within the MSA program. The 2017 Australian beef quality audit highlighted that between 2015 and 2017 the national incidence of carcass non-compliant grass-fed carcasses was 10.8 % from 3.1 million MSA grass-fed graded carcasses (Meat Standards Australia 2017). September 2019 grid values indicate that the producer penalties range between 20 c/kg and 45 c/kg for grass-fed cattle, suggesting that the current economic impact of non-compliant carcasses to grass-fed beef produces is between 18 and 41 million (AUD), based on an average carcass weight of 281 kg. However, it is important to consider that this value describes the impact of dark cutting to producers and does not encompass the economic impact of dark cutting beef to the processing sector, specifically via restricted market access and subsequent processing of an inferior product.

Ultimate pH is largely determined by the muscle glycogen concentration at slaughter, where anaerobic respiration post slaughter increases muscle acidification (Tarrant 1989). The importance of glycogen metabolism in the conversion of muscle to meat is well understood by the beef industry and has been extensively studied with comprehensive reviews on the subject and its relationship to meat quality (Pethick *et al.* 1999; Gardner *et al.* 2014). Dark cutting and the balance between glycogenesis and glycogenolysis reflects numerous complex interactions that exists between the animal, their nutritional status, environmental and pre-slaughter factors. A key determinant of glycogenesis potential is daily metabolisable energy (**ME**) intake (Pethick and Rowe 1996; Pethick *et al.* 1999). It has been established that the incidence of dark cutting fluctuates in pasture-fed cattle with higher incidences being observed in Mediterranean climates in late summer and early winter when feed quality and/or availability declines (Pethick *et al.* 1999). Pre-slaughter glycogenolysis can result from strenuous physical activity or acute catecholamine-induced psychological stress and has a major influence on the incidence of dark cutting meat (Tarrant 1989; Ferguson *et al.* 2001; Ferguson and Warner 2008). A degree of stress during the pre-slaughter period is an unavoidable part of animal transport and pre-slaughter guidelines and approved pathways within the MSA program provide guidelines to alleviate excessive exposure to these stressors. However, there is variation in dark cutting incidence at certain times of the year in pasture fed cattle that is unable to be unexplained by ME content or available food on offer (**FOO**) (Loudon *et al.* 2018).

In Southern Australia, the incidence of dark cutting has been shown to increase during late summer to early winter (B.SBP.0110). During these periods, pasture-finished cattle are most likely consuming pastures in the vegetative-phase (McGilchrist *et al.* 2014). Grass species this vegetative phase are typically rapidly growing, with a high crude protein concentration, but also often have high potassium (**K**) and phosphorus (**P**) concentrations in conjunction with variable calcium (**Ca**) levels and moderate Mg concentrations (Metson *et al.* 1966; Metson and Saunders 1978). These high

concentrations of protein and high DCAD, especially due to high K, are thought to reduce Mg absorption, which may lead to clinical and subclinical hypomagnesaemia in cattle (Schonewille 2013). Specifically, the absorption of Mg is impacted by pasture K content (Suttle 2010). Dietary K concentrations have a curvi-linear effect on Mg absorption, where inhibition is most pronounced between 1 to 3 % K DM (Rahnema and Fontenot 1986; Suttle 2010). Additionally, the grass tetany index (K / (Ca + Mg) is used to determine if pasture K content is sufficient to elicit hypomagnesaemia or hypocalcaemia (Kemp and t Hart 1957). Magnesium is an essential macronutrient predominantly associated with the microsomes, functioning as catalyst for a wide range of enzymatic reactions (Mayland 1988; Suttle 2010) and as a co-factor for the metabolism of carbohydrates, lipids and proteins (Mayland 1988). However, there is no substantial storage or active control over excretion, thus daily consumption of sufficient Mg is required. Periods of subclinical Mg deficiency are associated with reduced glycaemic control and insulin resistance by reducing both insulin secretion and peripheral tissue sensitivity (Lentz *et al.* 1976; Miller *et al.* 1980; Matsunobu *et al.* 1990; Suárez *et al.* 1995). Subclinical Mg deficiency also causes an increased stress response, decreased appetite and increased muscle contraction (Ebel and Günther 1980; Mayland 1988; Schonewille 2013). Furthermore, in these regions perennial pastures may contain endophytes that can produce high concentrations of alkaloids, some of which are toxic to cattle. In addition, adrenaline is released as a component of the fight and flight response, during handling, loading, transportation and unloading. Combined these may result in an excessive depletion of muscle glycogen reservoirs prior to slaughter, increasing the risk of dark cutting. Thus, a series of experiments were conduction within this project to evaluate: i) the benefits on supplementing grass-fed cattle with Mg and mycotoxin binders on the incidence of dark cutting; ii) the influence of dietary protein and K on Mg absorption, glycogen synthesis and breakdown. The outcomes from this project will support cost-effective solutions to minimise the impact of mycotoxins and low Mg grass-fed beef production systems throughout Southern Australia.

2 Project Information

2.1 Objectives

The objectives of this project were:

1. Determine the impact of varying levels of dietary protein on the Mg status of cattle and its impact on glycogen synthesis and glycogen depletion;
2. Determine the impact of varying levels of minerals and DCAD on the Mg status of cattle and its impact on glycogen synthesis and glycogen depletion;
3. Determine the effectiveness of commercially available Mg supplements to reduce the impact of low Mg levels on dark cutting rates and whole farm profitability in southern grass fed production systems;
4. Determine the ability for commercial mycotoxin binder products to reduce the impact of mycotoxins originating from pastures on animal growth rates and dark cutting rates, in addition to whole farm profitability; and
5. Maintain the employment of early career, connected, scientist with understanding of the non-compliance issues that face southern Australian grass fed production systems

2.2 Outcomes

The project has been successful in achieving the objective identified above, with the exception of objective 4, which is only partially completed due to complications associated with methodology and subsequent restrictions due to COVID-19. Briefly, the outcomes of the experiments conducted within this project have identified and achieved are described below:

1. *The impact of varying levels of dietary protein on glycogen synthesis and glycogen depletion (Section 3)*

Increasing dietary protein had no impact on insulin sensitivity, insulin responsiveness, glucose disappearance rates, adrenaline sensitivity or glycogen metabolism within this experiment. It was anticipated that increased dietary protein intake would reduce Mg absorption and increase adrenaline sensitivity in these cattle. The controlled conditions within this experiment, predominantly associated with diet composition based on chaff and a pelletised ration, were probably insufficient to elicit the anticipated responses due to restricted movement and consistent quality of available feed. Future studies resembling commercial conditions more closely are required to develop an understanding of the influence of protein content in grazing pastures on the mechanisms involved in glycogen deposition and utilisation, which may lead to dark cutting.

2. *The impact of varying levels of dietary potassium on energy synthesis and glycogen depletion (Section 4)*

This experiment showed that under highly controlled conditions that increasing dietary K did not influence insulin sensitivity, insulin responsiveness, glucose disappearance rates or adrenaline sensitivity. In addition, glycogen metabolism was not altered before or after exercising. Therefore results reject the hypotheses that increasing the dietary K would reduce insulin sensitivity, insulin responsiveness and increase adrenaline sensitivity in these cattle. However, as the diets were consistent in quality the cattle may not have entered a state of hypomagnesaemia.

3. *Impact of short term Magnesium (Mg) supplementation prior to slaughter in pasture finished beef (Section 5)*

Supplementing cattle with increased dietary Mg did not influence overall muscle glycogen concentration or incidence of dark cutting. However, short-term Mg supplementation prior to slaughter was effective at improving muscle glycogen concentration in groups of cattle from 2 of 14 producers investigated. Additionally, Mg supplemented cattle from four producers was associated with an increase in plasma Mg concentration. The experiment highlighted that some grazing stock were at risk of Mg deficiencies during autumn, winter and early spring. Further research

investigating an objective measurement of detecting risk of hypomagnesaemia to finishing stock would be beneficial for commercial industries.

4. Commercial Mycotoxin Binder Supplementation (Section 6)

The initial findings from this experiment suggest that the mycotoxin binder did not positively influence the performance of these cattle, in fact mycotoxin binder supplementation was associated with reduced growth rates. Specifically, the control cattle had a larger weight change during the experiment (12.1 ± 2.1 kg*; P* < 0.0001) and had a higher ADG (0.25 ± 0.04; *P* ≤ 0.0001) when compared with cattle in the binder treatment. However, this increased growth did not translate into increased differences in final live weight or carcass performance. In addition, the incidence of dark cutting in these cattle was also minimal (2.9%) and was not associated with treatment. As such, it is difficult to make any assumptions about the ability of the mycotoxin binder to reduce toxicity effects and the subsequent relationship with dark cutting. Further analysis of muscle glycogen at slaughter and pasture samples will need to be evaluated prior to providing a definitive conclusion from the data presented here.

As dark cutting is typically associated with low muscle glycogen stores at slaughter, a component of this experiment was developed to investigate the change in muscle glycogen between farm departure and slaughter. Determining muscle glycogen prior to slaughter involves the collection of a muscle biopsy, via a purposely designed biopsy drill (Gardner *et al.* 2001c). As this is a unique request for a commercial producer there was an initial delay in sourcing cattle for this experiment. A cohort of cattle from King Island were identified to provide the research team with access to the 160 cattle required to conduct this experiment. Initially, these cattle were scheduled for slaughter between December 2019 and February 2020. However, delays were then experienced due to drought conditions and reduced growth rates in the sourced cattle. Further delays were experienced due to the COVID-19 pandemic. Subsequently, a second cohort of 101 cattle were sourced for this experiment and these cattle slaughtered in July 2020. An on-farm muscle biopsy from these cattle was not able to be collected due to COVID-19 travel restrictions. However, data collation and analysis for cattle growth (live weight) and carcass characteristics have been completed and presented in the appropriate section below. Pasture samples were also collected for this experiment and muscle samples at slaughter were collected from these cattle, however due to logistical constraints around COVID-19 these samples remain in storage on Tasmania. Once these samples are shipped from Tasmania and subsequent muscle glycogen analysis and statistical interrogation of this data can be conducted, an update will be provided to MLA in an agreeable format.

5. Engagement with an early career scientist

Under the mentorship of Dr Peter McGilchrist this project has engaged with two post-doctoral research scientists and a PhD Candidate;

a. Dr Kate Loudon (PhD Candidate, PhD conferred during 2019), whom achieved objective 3 (section 5)
b. Dr Flavio Alvarenga (post-doctoral scientist), whom achieved objective 1 (section 3) and 2 (section 4)
c. Dr Angela Lees (post-doctoral scientist), whom contributed to objectives 1 and 2 and is progressing towards achieving outcome 4 (section 6)

Here we have identified the overall objectives of this project and provided a brief summary of the outcomes achieved. The following sections of this report have been dedicated to objectives 1 to 3, as described above. Objective 4 remains incomplete, although the preliminary findings for live weight and carcass characteristics have been reported here in section 6. The achievement of these outcomes is described in full detail throughout the remainder of this report. This report has been constructed to present the outcomes of objectives 1 to 3 in detail within their own sections. Within

these sections comprehensive details pertaining to the materials and methods, statistical analysis, results, discussion and conclusions for each objective are outlined.

3 Impact of varying dietary protein concentrations on whole body insulin responsiveness, adrenaline sensitivity and muscle glycogen

Project Objective 1. Determine the impact of varying levels of dietary protein on the Mg status of cattle and its impact on glycogen synthesis and glycogen depletion.

3.1 Introduction

Dark cutting is particularly prevalent in southern Australian cattle coming off pasture during autumn and early winter (McGilchrist *et al.* 2014). During these periods, grass dominant pastures that are young, short and rapidly growing are likely to have high crude protein levels, high K (particularly if fertilised with potash), high Dietary Cation-Anion Difference (**DCAD**) imbalance and low Mg concentrations which could result in low Mg absorption and clinical grass tetany (Schonewille 2013). The Na:K ratio is especially important in the rumen where the 'Mg pump' operates to absorb Mg from the gut. Subsequently, cattle grazing pastures with high levels of protein potentially have an increased ammonia absorption from the rumen to the blood stream. This ammonia absorption can negatively influence the central nervous system eliciting muscle and skin tremors (Helmer and Bartley 1971), potentially promoting glycogen breakdown and adrenaline responsiveness to stress. Low Mg also increases glycogen breakdown due to neuromuscular hyperexcitability and increased adrenaline responsiveness to stress. In addition, adrenaline released due to fight and flight response, which can be elicited during handling, loading, transportation and unloading, has been associated with the activation of the hypothalamic-pituitary-adrenal axis, promoting the liberation of glucose and NEFA from muscle and adipose tissues. Combined these may result in an excessive depletion of muscle glycogen reservoirs prior to slaughter. It is well accepted that animals with low muscle glycogen concentrations on farm have an increased risk of dark cutting (McGilchrist *et al.* 2014). Muscle glycogen available at slaughter is a reflection of muscle glycogen deposition on farm, minus the quantity of glycogenolysis that occurs during the pre-slaughter period (McGilchrist *et al.* 2014). Muscle glycogenolysis has been associated with nutritional status (Knee *et al.* 2004), particularly in grazing systems (McGilchrist *et al.* 2012; McGilchrist *et al.* 2014). However, the mechanisms through which high protein diets, balanced for mineral and energy concentrations, influence muscle glycogen deposition and subsequent glycogenolysis in response to systemic challenges has not been elucidated. Therefore, the aims of this experiment were to evaluate the variations of dietary protein concentration on i) responses to exogenous insulin and glucose disappearance following an glucose tolerance test; ii) adrenaline sensitivity during an exogenous adrenaline challenge; and iii) Increasing the protein content of a total mixed ration and determining the impact on glycogen deposition and depletion. It was hypothesised that increased dietary protein intake by ruminants would reduce Mg absorption and increase adrenaline sensitivity of beef cattle.

3.2 Materials and Methods

This experiment was conducted with the approval of the University of New England's animal ethics committee (AEC17-108; Appendix 1).

3.2.1 Animals and Housing

The experiment was conducted using 18 Angus steers (284 ± 23 kg), that were approximately 15 months of age. Steers in this experiment were sired by three bulls, where each bull sired two steers within each dietary treatment (described below). Steers were housed in individual animal pens for a total of 60 days. There was a 30 day acclimation period for steers to adjust to the dietary treatments, followed by a 30 day data collection period.

3.2.2 Nutritional Management

Cattle were randomly allocated to three dietary protein treatment groups consisting of: 1) low (≈ 10 %); 2) medium (≈ 20 %); and high (≈ 30 %) protein levels (Table 3.1). Diets were balanced for minerals and metabolizable energy (**ME**) in order to compare the three protein concentrations. In addition, diets were formulated in consultation with the guidelines described by the NRC (2016) to achieve an average daily gain of 1 kg/day. Cattle were fed once daily at 1600 h. Prior to the next feed offering any refusals were removed and weighed, these data were then used to calculate refusals per animal per day.

Table 3.1. Percentage and chemical composition of the diets

Ingredient, % of DM	Protein level , %		
	10	**20**	**30**
Oat chaff	56.43	24.64	3.94
Lucerne chaff	-	17.36	36.69
Lucerne pellet	-	-	6.88
Canola meal	-	8.61	29.84
Wheat millrun	10.67	16.30	9.31
Wheat ground	31.88	31.08	11.92
Limestone	0.81	0.89	0.71
Magnesium oxide	0.1	0.11	0.09
Mineral premix	0.1	0.11	0.09
Urea	-	0.89	0.89
Chemical composition			
Dry matter (%)	88.0	90.81	90.25
Crude protein (% DM)	10.4	20.12	29.67
Neutral detergent fiber (% DM)	41.99	35.25	34.09
Acid detergent fiber (% DM)	23.3	21.69	24.39
Metabolizable energy (ME MJ/kg DM)	9.57	10.45	9.90

3.2.3 Phenotypic measures and acclimation

Cattle were divided in three groups of six animals consisting of two animals from each dietary treatment. On the day prior to the first challenge, cattle were weighed (non-fasted) and real-time ultrasound measurements (3.5 MHz/180-mm linear array animal science probe, Esoate Pie Medical, Maastricht, Netherlands) of subcutaneous fat depth between the 12/13th rib (**rib fat**) and P8 rump (rump fat) site were determined. Cattle were then fitted with two catheters, one in each external jugular vein. The cannulation site was clipped to remove all hair and sterilized using betadine surgical scrub and ethanol swabs. The site was infiltrated with 2 ml of 2% Lignocaine into the skin (dermis). After 5 minutes an 18-gauge needle was introduced into the jugular vein through the site and a sterile teflon coated surgical wire guide passed through the needle. The needle was then removed and a treated sterile polyethylene catheter with a 2 mm outer diameter and 1 mm inner diameter (Portex, Hythe, Kent, England) was passed over the wire guide and fed into the jugular vein. The wire guide was then withdrawn through the catheter, with the catheter sutured in place at the site of protrusion on the animals' neck. Catheters were then fixed to the neck, providing access for sample collection during the challenges. Catheter were flushed and filled with a heparin/saline to remain patent.

3.2.4 Hyperinsulinaemic, euglycaemic clamp (HIEG) procedure

The day after the catheters were fitted the hyperinsulinaemic, euglycaemic clamp (HIEG) technique, as described by DeFronzo *et al.* (1979) for use in humans and subsequently adapted Bergman *et al.* (1989) for use in ruminants by, was implemented to determine the whole body glucose use in response to insulin. Prior to commencing insulin infusions, basal blood glucose level was determined by successive blood samples collected at 15 minutes over a 60 minute period. An 8 mL sample was collected using S-Monovette Vacutainer® tubes (Sarstedt Australia Pty. Ltd. SA Australia,

Cat. No. 02.1066.001) containing ethylenediaminetetraacetic acid (**EDTA**) to prevent clot formation. From these samples, blood glucose concentration was determined immediately using portable blood glucose meters (AscensiaTM Esprit® 2, Bayer, USA). The remaining samples were centrifuged and the harvested plasma frozen at -20°C, for later laboratory determination of glucose. The five glucose meter readings were used to estimate blood glucose concentration, prior to insulin infusion. Glucose concentration was later confirmed by laboratory analysis of plasma samples.

The insulin infusion commenced with a single dose of 6mU/kg live weight of insulin (Actrapid®, Novo Nordisk Pharmaceuticals Pty. Ltd., Baulkham Hills, NSW) administered via a singular infusion catheter. From then a continuous insulin infusion occurred, commencing at a rate of 0.6mU/kg live weight per minute using a dual channel infusion pump (LIFECARE® 5000 Plum™ Infusion system, Abbott Laboratories, USA). Concurrently, glucose (50% w/v; Baxter Healthcare, Old Toongabbie, NSW) was infused, utilizing the same infusion pump as preciously described. Initially the glucose infusion occurred at a rate of ≈ 125 ml/h. Every 5 minutes, a 2 mL blood sample was collected and blood glucose concentration was rapidly determined using the portable blood glucose meter. Depending on the blood glucose concentration, the glucose infusion rate was adjusted to establishing a constant level of blood glucose at the same level as the pre-infusion level (basal blood glucose) or euglycemia. Once euglycemia was observed, the glucose infusion rate was held constant for 60 minutes and defined as the steady state glucose infusion rate (**SSGIR**). An 8 ml blood samples was collected at 15 minutes intervals S-Monovette Vacutainer® tubes (Sarstedt Australia Pty. Ltd. SA Australia, Cat. No. 02.1066.001), with one drop used to ensure that euglycaemic glucose concentrations were being maintained. The remaining blood samples were then centrifuged and plasma harvested. Plasma samples were then frozen at -20°C until laboratory determination of glucose occurred. Once complete, the rate of insulin infusion was then increased to 6 mU/kg live weight per minute and the above process repeated to establish the SSGIR of the higher insulin infusion rate. Insulin infusion was then ceased and glucose infusion continued until blood glucose concentrations were above the determined euglycaemic concentration and stable or rising. The SSGIR required was then used to determine whole body insulin responsiveness.

3.2.5 Insulin challenge

Throughout the 2 days following the HIEG, steers were subjected to two insulin challenges and a dextrose challenge. Therefore, a total of three different challenges given in the morning (1000h) or afternoon (1400h). Steers were subjected to the challenges in the morning and afternoon were randomized and in the morning of the day 2, all six animals have had received all three challenges. The insulin challenges consisted of 0.5 ug/kg live weight and 2.0 ug/kg live weight, whilst the dextrose challenge was applied at a rate of 0.35 g/kg live weight. A 5 ml blood sample was taken from the catheter at -30, -15, -10, -5, 0, 2.5, 5, 10, 15, 20, 30, 45, 60, 120, 125, and 130 minutes relative to the administration of the respective challenge. Whole blood was collected into S-Monovette Vacutainer® tubes (Sarstedt Australia Pty. Ltd. SA Australia, Cat. No. 02.1066.001) containing EDTA. Immediately after collection, blood samples were stored on ice to minimize further metabolic activity of red blood cells converting glucose to lactate. Within 20 minutes of collection, blood samples were centrifuged at 3500 rpm for 15 minutes at 5 °C. Plasma decanted into two separate sample tubes, where one samples was stored at -20 °C for glucose and lactate analysis and second sample was stored at -80 °C for non-esterified fatty acid (**NEFA**) analysis.

3.2.6 Adrenaline Challenge

After Biopsy 4 on Day 9, steers were scanned at the P8 rump and in between 12-13th rib with real-time ultrasound measurements (3.5 MHz/180-mm linear array animal science probe, Esoate Pie Medical, Maastricht, Netherlands) for subcutaneous fat depth. The steers were then weighed and fitted with one catheter into the external jugular vein. The site for cannulation was clipped to remove all hair and sterilised using betadine surgical scrub and ethanol swabs. A 2% Lignocaine (2ml/steer)

was infiltrated into the skin (dermis) and after 5 minutes an 18 gauge needle was introduced into the jugular vein through the site and a sterile teflon coated surgical wire guide passed through the needle. The needle was then removed and a treated sterile polyethylene catheter (2mm o.d. and 1mm i.d., Portex, Hythe, Kent, England) was passed over the wire guide (at the end protruding from the animal) and fed into the jugular vein. The wire guide was then withdrawn through the catheter, with the catheter sutured in place at the site of protrusion on the animals' neck. The exposed length of the catheter was tied behind the animals' neck for easy access during sampling. The catheter was filled with heparin/saline mixture to prevent thrombis formation.

In the following three days after the catheters were fitted the steers were subjected to three different adrenaline challenges (1.0 ug/kg LW, 3.0 ug/kg LW and 5.0 ug/kg LW) given in the morning (1000h) or afternoon (1400h). The challenges given to the steers in the morning and afternoon were randomized and in the end of the 2nd day, all six animals have had received all three challenges. A 5 mL blood sample was collected, using S-Monovette Vacutainer® tubes containing EDTA to prevent clotting, from the catheter at -30, -15, -10, -5, 0, 2.5, 5, 10, 15, 20, 30, 45, 60, 120, 125, and 130 minutes relative to the administration of the adrenaline challenge. Immediately after the blood collection the tubes were stored on ice to minimize further metabolic activity of red blood cells converting glucose to lactate. Within 20 minutes of collection, the samples were centrifuged at 3500 rpm for 15 minutes at 5^{o}C and the plasma was separated into two eppendorf of 2mL where one was stored at -20^{o}C for glucose and lactate analysis and the other tube was stored at -80^{o}C for NEFA analysis.

3.2.7 Muscle biopsies

Muscle samples from *Longissimus thoracis* (LT), *semitendinosus* (ST) and *semimembranosus* (SM) were collected at day 0 (Biopsy 1) in order to obtain a base line muscle glycogen concentration, in the specified muscles. On Day 3 the steers were weighed and then exercised at 65% of VO_2 max for one hour to deplete muscle glycogen concentration and a biopsy was taken immediately after the exercise (Biopsy 2). An additional two biopsies were collected on Day 6 (Biopsy 3) and Day 9 (Biopsy 4). Muscle samples were collected via the protocols described by McGilchrist *et al.* (2011b). Briefly, a purpose built 12 volt powered motorized biopsy drill was used to sample the muscle while the steers were in a restrained crush. For sampling the SM and ST, the area between 15 and 25 cm below the anus, and over the poverty groove with both muscles accessed through the same incision; the area was prepared by clipping the hair from a 10 x 10 cm area around the point of incision. The site prepared for the LD was at the top of the 12th-13th rib, approximately 5 cm from the backbone.

Muscle samples were analyzed for lactate and glycogen concentration at Murdoch University. The muscle samples were homogenized in 30 mM HCl in a ratio of 1 part muscle to 10 parts acid. An enzymatic method kit (Trinder 1969; Badham and Trinder 1972) was used to analyze lactate on the homogenate. A method outlined by method (Passonneau and Lauderdale 1974) was used to hydrolyze the glycogen in the homogenate into glucose and then it was measured by the Olympus AU 400 using a glucose kit (Cat.No OSR6121). Total glycogen (g/100 g) was then determined by halving the lactate concentration and adding it to the glucose concentration.

3.2.8 Plasma analysis

Laboratory determination of plasma samples were carried out in duplicate using enzymatic methods for glucose (Kunst *et al.* 1984) and lactate (Marbach and Weil 1967) using an integrated chemistry system auto-analyser (Siemens Dimension® XPand Plus). Similarly, NEFA concentrations were analyzed in duplicate using a NEFA Kit from Siemens Dimension ®. The Flex® reagent cartridge Kit was used to measure glucose and lactate concentration.

3.2.9 Modelling of response curves

Basal concentrations of glucose, lactate and NEFA were calculated as the mean of samples collected at -30, -15, -10, -5 and 0 minutes relative to the insulin challenges. The concentrations of each substrate were then plotted against time for each challenge administered to each animal and a derived function with multiple exponential components was fitted to the raw data as described by McGilchrist *et al.* (2011a) and McGilchrist *et al.* (2011b). This showed a classic increase and then tapering off in response which was modeled using the following function:

$$\gamma(t)= A + ([-\gamma/(\beta - \alpha) + \gamma/(\beta-\alpha-\Delta) - \theta]\, e^{-\beta t} + \gamma/(\beta - \alpha)e^{-\alpha t} - \gamma/(\beta-\alpha-\Delta)\, e^{-(\alpha+\Delta)t + \theta}) \; ; \varepsilon(0,\sigma)$$

where γ(t) is the plasma metabolite concentration at time *t*; *t* is time (min); *A* is basal metabolite concentration (average of 5 samples taken in the 30 minute period pre-challenge); β,γ,α,Δ = exponential constants; θ = the adjustment from the basal metabolite concentration; and ε = error

This function was used to determine the time to maximum substrate concentration, the maximum substrate concentration and the area under the response curve (**AUC**) between 0 and 10 minutes (**AUC_{10}**) for glucose relative to administration of the dextrose challenge. For insulin challenge the function was used to determine the time to minimal substrate concentration, the minimal substrate concentration and the area under the response curve between 0 and 10 minutes (**AUC_{10}**). The function was also used to model plasma glucose concentration following the intravenous glucose challenge, with glucose area under curve between 0 and 130 minutes (**AUC_{130}**) reflecting glucose disappearance rate.

3.2.10 Statistical analysis

Data exploration and statistical analyses were conducted in R (R Core Team 2019). Data merging and manipulation, data visualizations and summary data were prepared using the 'dplyr' (Wickham *et al.* 2019b) and 'ggplot2' (Wickham *et al.* 2019a) packages. Data from the SSGIR, insulin challenges and dextrose were analysed using a linear mixed effects model from the 'lme4' package (Bates *et al.* 2019) and estimated marginal means were generated using the 'emmeans' package (Length 2019). Models pertaining to the insulin challenges investigated glucose disappearance, plasma lactate and plasma NEFA.

The SSGIR was not influenced by rib fat ($P > 0.05$), thus rib fat was excluded from the final model. The final SSGIR model incorporated diet, insulin infusion rate, live weight, rump fat and diet × insulin infusion rate as fixed effects. Similarly, the models for glucose disappearance during the insulin challenges incorporated diet, insulin challenge (0.6 mU/kg/min and 6 mU/kg/min), live weight, a measure of fat (rib or rump), and diet × challenge as fixed effects. For these models, neither rib fat or rump fat contributed to significance (P = 0.24), thus these parameters were excluded from the model. Basal glucose concentration was not influenced by diet, insulin infusion rate or diet × insulin infusion rate, thus was not incorporated as a covariate in any of the glucose disappearance models. For the dextrose challenge, the glucose clearance models incorporated diet and live weight. Animal id nested within sire was incorporated into all models described above as a random effect.

For the adrenaline challenges models the investigated plasma glucose, lactate and NEFA concentrations. These models incorporated diet, adrenaline challenge (1.0 ug/kg LW, 3.0 ug/kg LW and 5.0 ug/kg LW), live weight, and diet × challenge as fixed effects. Animal id nested within sire was incorporated into all models described above as a random effect. Models were refined to remove relevant insignificant interactions in a step-wise manner. Fat measures (rib fat or rump fat depth) were investigated within all models, but did not contribute significance (P > 0.05), thus, were excluded from the final models. Additionally, basal glucose concentration was investigated as co-

variate, however but did not contribute significance (P > 0.05), thus were excluded from the final models.

Finally, muscle glycogen and lactate concentrations from the muscle biopsies were analysed using a repeated measures linear mixed effects model, models incorporated diet, muscle biopsy and diet × biopsy as fixed effects and animal id nested within sire as a random effect, for each muscle.

3.3 Results

3.3.1 Influence of dietary protein on SSGIR during HIEG clamp

The responsiveness to insulin during the HEIG clamp was influenced by dietary protein concentration (P = 0.03). There were no differences in the SSGIR between diets containing 10 % protein or 20 % protein (P = 0.3034), nor were there differences between diets containing 20 % protein and 30 % protein (P = 0.3034). However, the SSGIR for diets contacting 30 % protein had a glucose infusion rate 67.2 ± 24.9 ml/hr greater than diets containing 10 % protein (P = 0.05). During the insulin infusion, the SSGIR the 6 mU/kg/min insulin infusion rate required 38.6 ± 4.67 ml/hr more glucose to maintain euglycaemia (P < 0.0001; Figure 1). At the 0.6 mU/kg/min insulin infusion rate there were no differences in the volume of glucose required to maintain euglycaemia across the the three diets (P ≥ 0.23). Similarly, during the 6 mU/kg/min insulin infusion rate there were no differences in the glucose requirement between the 10 % protein and 20 % protein diets (P = 0.9876). Whereas glucose requirement to maintain euglycaemia was 88.56 ± 25.52 ml/hr and 73.37 ± 20.83 ml/hr greater for the 30 % protein when compared with the 10 % and 20 % protein diets, respectively (P ≤ 0.04; Figure 3.1). Rump fat was also identified to influence SSGIR (P = 0.003), however the variability in rump fat was small (1.5 mm to 3 mm).

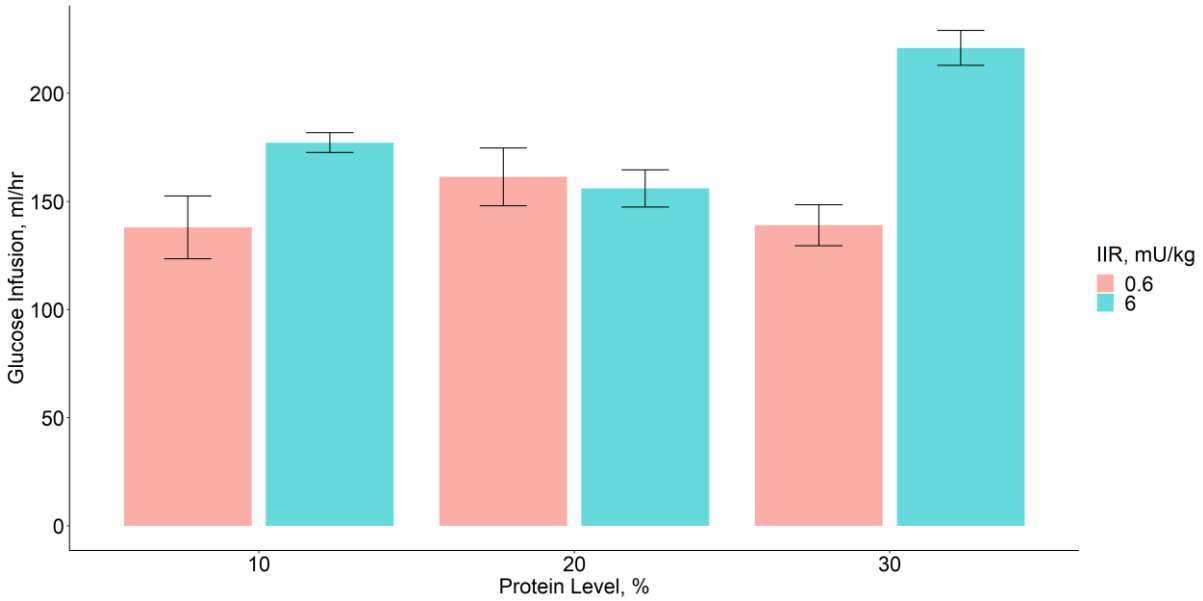

Figure 3.1. The influence of dietary protein level (%) on steady state glucose infusion rate (SSGIR; glucose infusion, ml/hr) at insulin infusion rates (IIR) of 0.6 mU/kg/min and 6 mU/kg/min. Values are means ± SEM.

3.3.2 Glucose disappearance, plasma lactate and plasma NEFA during insulin challenges

Dietary protein concentration did not influence basal glucose concentration, time to maximum concentration, or glucose disappearance between AUC_{10} and AUC_{130} (P ≥ 0.2909; Table 3.2). Furthermore, basal glucose concentration, time to maximum concentration, or glucose disappearance between AUC_{10} and AUC_{130} were not influenced by live weight (P ≥ 0.5506) or diet × insulin challenge (P ≥ 0.4006). The insulin infusion rate during the challenges did not influence basal glucose concentration, time to maximum concentration, or glucose disappearance between AUC_{10} and AUC_{30}

($P \geq 0.06$). Glucose disappearance at AUC_{30} was 13.2 ± 6.51 mM greater during the 6 mU/kg/min insulin infusion rate when compared with the 0.6 mU/kg/min insulin infusion rate ($P = 0.06$). Similarly, the glucose disappearance at AUC_{45}, AUC_{60} and AUC_{130} during the 6 mU/kg/min insulin infusion rate were 28.0 ± 9.94 mM, 42.7 ± 13.0 mM and 78.4 ± 21.0 mM greater than the 0.6 mU/kg/min insulin infusion rate ($P \leq 0.01$).

Table 3.2 Estimated marginal means (± SE) for glucose disappearance measures in response to insulin challenges for steers fed three different levels of dietary protein

Glucose Parameter	Dietary Protein, %		
	10	20	30
Basal Concentration	5.03 ± 0.21	5.08 ± 0.20	5.03 ± 0.20
AUC_{10}	34.2 ± 9.46	34.3 ± 9.34	29.9 ± 9.17
AUC_{20}	-3.68 ± 1.48	-0.62 ± 1.46	-3.23 ± 1.43
AUC_{30}	-13.18 ± 3.70	-6.32 ± 3.66	-13.13 ± 3.59
AUC_{45}	-25.3 ± 5.95	-15.7 ± 5.87	-26.6 ± 5.76
AUC_{60}	-43.4 ± 9.16	-31.6 ± 9.05	-47.5 ± 8.88
AUC_{130}	-58.8 ± 12.1	-46.2 ± 11.9	-65.8 ± 11.7

Basal plasma lactate was influenced by dietary protein concentration, where the basal plasma lactate concentration was 0.07 ± 0.03 mM greater in the 30 % protein diet when compared with the 20 % protein diet (Table 3.3). Time to maximum plasma lactate concentration was not influenced by the insulin challenges ($P = 0.92179$), live weight ($P = 0.14267$) and diet × insulin challenge ($P = 0.63343$). Although dietary protein level tended to influence the time to maximum concentration, where maximum plasma lactate occurred 28.6 ± 12.5 min earlier in the 30 % protein diet when compared with the 20 % protein diet ($P = 0.0895$; Table 3.3). Plasma lactate concentrations between AUC_{10} and AUC_{45} were not influenced by diet ($P \geq 0.1268$), insulin challenge ($P \geq 0.4335$), live weight ($P \geq 0.4585$) or diet × insulin challenge ($P \geq 0.584$). Dietary protein levels tended to influence plasma lactate concentration at AUC_{60} ($P = 0.08$; Table 3.3). However, the AUC_{130} plasma lactate concentration was 8.85 ± 3.28 mM lower for the 30 % protein diet when compared with the 20 % protein diet ($P = 0.0448$; Table 3.3).

Table 3.3. Estimated marginal means (± SE) for lactate during insulin challenges for steers fed three different levels of dietary protein

Lactate Parameter	Dietary Protein Level, %		
	10	20	30
Basal Concentration	0.24 ± 0.02	0.22 ± 0.02[a]	0.29 ± 0.02[a]
Time to max concentration (min)	14.53 ± 9.06	23.31 ± 8.95	-5.33 ± 8.79
AUC_{10}	0.20 ± 0.27	0.64 ± 0.27	0.02 ± 0.26
AUC_{20}	0.53 ± 0.73	1.69 ± 0.72	-0.04 ± 0.70
AUC_{30}	0.81 ± 1.14	2.62 ± 1.12	-0.27 ± 1.10
AUC_{45}	1.06 ± 1.58	3.54 ± 1.56	-0.88 ± 1.53
AUC_{60}	1.15 ± 1.83	4.00 ± 1.81	-1.63 ± 1.77
AUC_{130}	0.24 ± 2.56	3.74 ± 2.39[a]	-5.11 ± 2.37[a]

[a] within row, means with a common superscript differ ($P \leq 0.04$)

Basal NEFA concentrations were 0.01 ± 0.01 mM greater in the 10 % protein diet, when compared with the 20 % ($P = 0.04$) and 30 % ($P = 0.02$) protein diets (Table 3.4). Time to maximum NEFA concentration did not differ over the three dietary protein levels ($P = 0.7146$; Table 4), nor was it influenced by insulin challenge ($P = 0.5766$), live weight ($P = 0.2187$) or diet × insulin challenge ($P = 0.2498$). Plasma NEFA concentration between AUC_{10} and AUC_{130} were not influenced by live weight ($P = 0.29628$) or diet × insulin challenge ($P = 0.57738$) or insulin challenge ($P = 0.5766$). However at AUC_{45} and AUC_{60} plasma NEFA concentration tended to be 0.20 ± 0.11 mM ($P = 0.0889$) and 0.23 ± 0.12 mM ($P = 0.0789$) lower during the 6 mU/kg/min insulin infusion rate. Dietary protein levels had a tendency to influence plasma NEFA concentration between AUC_{10} and AUC_{130} ($P \geq 0.05 \leq 0.10$), generally this

was associated with numerically greater plasma NEFA concentration within cattle offered the 10 % protein diet (Table 3.4).

Table 3.4. Estimated marginal means (± SE) for non-esterified fatty acid (NEFA) during insulin challenges for steers fed three levels of dietary protein

NEFA Parameter	Dietary Protein Level, %		
	10	20	30
Basal Concentration	0.02 ± 0.01[ab]	0.01 ± 0.01[a]	0.009 ± 0.01[b]
Time to max concentration (min)	-19.3 ± 10.3	-21.7 ± 10.2	-10.7 ± 9.99
AUC_{10}	-0.07 ± 0.02	-0.02 ± 0.02	-0.01 ± 0.02
AUC_{20}	-0.23 ± 0.06	-0.05 ± 0.06	-0.06 ± 0.05
AUC_{30}	-0.39 ± 0.09	-0.10 ± 0.09	-0.10 ± 0.88
AUC_{45}	-0.56 ± 0.12	-0.15 ± 0.12	-0.16 ± 0.12
AUC_{60}	-0.66 ± 0.14	-0.19 ± 0.14	-0.19 ± 0.14
AUC_{130}	-0.75 ± 0.17	-0.26 ± 0.16	-0.19 ± 0.16

[a b] within row, means with a common superscript differ ($P \leq 0.04$)

3.3.3 Dextrose Challenge

At the commencement of the dextrose challenge there were no differences in basal glucose concentrations across the three dietary protein levels ($P = 0.5593$; Table 3.5). Additionally, live weight ($P = 0.8140$) and dietary protein levels ($P = 0.7477$) did not influence the time to maximum glucose concentration during the dextrose challenge. However, beyond these parameters dietary protein levels influenced glucose clearance throughout the dextrose challenge. Plasma glucose concentrations were largely greater in the 10 % protein diet between AUC_{10} and $AUC_{130,}$ particularly when compared with the 30 % protein diet (Table 3.5).

Table 3.5. Estimated marginal means (± SE) for glucose measures during a dextrose challenge for steers fed three levels of dietary protein

Glucose Parameter	Dietary Protein Level, %		
	10	20	30
Basal Concentration	5.11 ± 0.17	5.00 ± 0.17	4.85 ± 0.17
Time to max concentration (min)	1.62 ± 0.31	1.80 ± 0.30	1.48 ± 0.30
AUC_{10}	134.0 ± 5.74	126.0 ± 5.67	114.0 ± 5.57
AUC_{20}	242.0 ± 9.75	223.0 ± 9.63	196.0 ± 9.45
AUC_{30}	324.0 ± 12.9[a]	294.0 ± 12.8	250.0 ± 12.6[a]
AUC_{45}	411.0 ± 17.7[a]	367.0 ± 17.5	298.0 ± 17.1[a]
AUC_{60}	470.0 ± 22.6[a]	413.0 ± 22.4[b]	324.0 ± 21.9[ab]
AUC_{130}	577.0 ± 43.1[a]	484.0 ± 42.6[b]	337.0 ± 41.8[ab]

[a b] within row, means with a common superscript differ ($P \leq 0.03$)

3.3.4 Plasma glucose, plasma lactate and plasma NEFA during adrenaline challenges

Basal glucose concentration was not influenced by diet ($P = 0.4135$; Table 3.6), challenge ($P = 0.3040$; Table 3.7) or diet × challenge ($P = 0.93$). Nor was the time to maximum glucose concentration, challenge ($P = 0.15525$) or diet × challenge ($P = 0.40825$). However time to maximum glucose concentration was influenced by dietary protein ($P = 0.4135$; Table 3.6) where the time to reach maximum glucose concentration was 4.59 ± 1.68 min longer in cattle within the 10 % CP diet when compared with animals in the 30 % CP treatment ($P = 0.0408$; Table 3.6). Dietary protein influenced glucose availability between AUC_{30} and AUC_{130}, where glucose concentrations were lower for cattle in the 30 % CP diet when compared with the 10 % CP diet (Table 3.6). Glucose availability was also influenced by the adrenaline challenges between AUC_{10} and AUC_{130} as exogenous adrenaline increased glucose availability regardless of AUC time point (Table 3.7).

Table 3.6. Estimated marginal means (± SE) for glucose availability measures in response to adrenaline challenges for steers fed three different levels of dietary protein

Glucose Parameter	Dietary CP Level, %		
	10	20	30
Basal Concentration	5.24 ± 0.18	5.19 ± 0.18	4.93 ± 0.18
Time to max concentration (min)	11.89 ± 1.20[a]	9.91 ± 1.19[ab]	7.30 ± 1.16[b]
AUC_{10}	16.7 ± 1.59	17.3 ± 1.58	14.7 ± 1.55
AUC_{20}	41.1 ± 3.22	39.8 ± 3.18	30.7 ± 3.12
AUC_{30}	62.2 ± 4.64[a]	58.9 ± 4.58[ab]	43.1 ± 4.50[b]
AUC_{45}	87.2 ± 6.35[a]	81.4 ± 6.24[a]	56.6 ± 6.12[b]
AUC_{60}	105.9 ± 7.57[a]	98.2 ± 7.48[a]	66.0 ± 7.34[b]
AUC_{130}	145.3 ± 10.4[a]	129.5 ± 10.3[a]	83.2 ± 10.1[b]

[ab] within row means without a common superscript differ (P ≤ 0.0324)

Table 3.7. Estimated marginal means (± SE) for glucose availability measures for the adrenaline challenges (ug/kg LW) in steers fed three different levels of dietary protein

Glucose Parameter	Adrenaline Challenge (ug/kg LW)		
	1.0	3.0	5.0
Basal Concentration	5.22 ± 0.13	5.12 ± 0.13	5.02 ± 0.13
AUC_{10}	9.04 ± 1.21[a]	16.40 ±1.21[b]	23.22 ± 0.21[c]
AUC_{20}	21.4 ± 2.35[a]	37.3 ± 2.35[b]	53.0 ± 2.35[c]
AUC_{30}	31.0 ± 3.36[a]	55.0 ± 3.36[b]	78.2 ± 3.36[c]
AUC_{45}	40.9 ± 4.65[a]	76.0 ± 4.65[b]	108.4 ± 4.65[c]
AUC_{60}	47.0 ± 5.74[a]	91.7 ± 5.74[b]	131.4 ± 5.74[c]
AUC_{130}	54.7 ± 8.59[a]	120.9 ± 8.59[b]	182.4 ± 8.59[c]

[a-c] within row means without a common superscript differ (P ≤ 0.0001)

Basal lactate concentration was not influenced by the different dietary protein levels (*P* = 0.3558; Table 3.8), adrenaline challenge (*P* = 0.1039), or diet × adrenaline challenge (*P* = 0.8863). There were no influences of or dietary protein levels or diet × adrenaline challenge on the time to maximum lactate concentration. Time to maximum lactate concentration was influenced by adrenaline challenge (P < 0.0001), where increasing exogenous adrenaline was associated increase time to maximum lactate concentration. Specifically there was a 5.61 ± 1.22 min increase in time to maximum concentration between 1.0 ug/kg LW and 5.0 ug/kg LW adrenaline challenges (*P* = 0.0002). The adrenaline challenge influenced plasma lactate, where plasma lactate for the 1.0 ug/kg LW challenge had lower lactate concentrations when compared with the 5.0 ug/kg LW challenge and at times 3.0 ug/kg LW (*P* ≤ 0.028; Table 3.9). Although, plasma lactate concentrations between AUC_{10} and AUC_{130} were not influenced by diet (*P* ≥ 0. 4055) or diet × insulin challenge (*P* ≥ 0.365; Table 3.8).

Table 3.8. Estimated marginal means (± SE) for lactate concentration (µmole/g) in response to adrenaline challenges for steers fed three different levels of dietary protein

Lactate Parameter	Dietary CP, %		
	10	20	30
Basal Concentration	0.25 ± 0.02	0.26 ± 0.02	0.28 ± 0.02
Time to max concentration (min)	11.7 ± 0.98	15.3 ± 0.97	12.3 ± 0.98
AUC_{10}	4.18 ± 0.54	3.28 ± 0.49	4.44 ± 0.50
AUC_{20}	10.97 ± 1.15	9.32 ± 1.14	11.00 ± 1.13
AUC_{30}	16.7 ± 1.79	14.9 ± 1.77	16.3 ± 1.76
AUC_{45}	23.1 ± 2.53	20.9 ± 2.50	21.7 ± 2.49
AUC_{60}	27.5 ± 3.03	24.5 ± 3.00	25.1 ± 2.99
AUC_{130}	37.0 ± 4.00	30.0 ± 3.96	30.6 ± 3.96

Table 3.9. Estimated marginal means (± SE) for plasma lactate measures for the adrenaline challenges (ug/kg LW) in steers fed three different levels of dietary protein

Lactate Parameter	Adrenaline Challenge (ug/kg LW)		
	1.0	3.0	5.0
Basal Concentration	0.27 ± 0.02	0.28 ± 0.01	0.23 ± 0.01
AUC_{10}	3.10 ± 0.37[a]	3.76 ± 0.36[ab]	5.04 ± 0.36[b]
AUC_{20}	7.05 ± 0.88[a]	10.28 ± 0.85[b]	13.95 ± 0.85[c]
AUC_{30}	10.0 ± 1.36[a]	15.9 ± 1.33[b]	22.1 ± 1.33[c]
AUC_{45}	13.0 ± 1.94[a]	21.8 ± 1.89[b]	31.0 ± 1.89[c]
AUC_{60}	14.9 ± 2.35[a]	25.5 ± 2.28[b]	36.8 ± 2.28[c]
AUC_{130}	18.8 ± 3.22[a]	31.4 ± 3.13[b]	47.3 ± 3.13[c]

[a-c] within row means without a common superscript differ ($P \leq 0.028$)

Basal NEFA concentrations were greater in the 10 % CP diet when compared with the 20 % and 30 % diets (Table 3.10). Similarly, the time to maximum NEFA concentration was 2.59 ± 0.90 and 3.75 ± 0.88 minutes longer for cattle in the 10 % CP diet compared with the 20 % and 30 % CP diets ($P \leq 0.03$; Table 3.10). Adrenaline challenge also influenced the time to maximum NEFA concentration, where time to maximal concentration was 3.16 ± 0.86 and 2.81 ± 0.86 minute faster in the 1.0 ug/kg LW, when compared with the 3.0 ug/kg LW and 5.0 ug/kg LW adrenaline challenges ($P \leq 0.007$). Dietary CP level influenced plasma NEFA concentrations greater for cattle in the 10 % CP diet when compared with 20 % and 30 % at all time points between AUC_{10} and AUC_{130} ($P \leq 0.03$; Table 3.10). Similarly, adrenaline challenge influenced NEFA between AUC20 and 45, where generally plasma NEFA concentrations were lower in the 1.0 ug/kg LW adrenaline challenge when compared with the 3.0 ug/kg LW and 5.0 ug/kg LW challenges ($P \leq 0.05$; Table 3.11). However, there were no diet × adrenaline challenge influenced on plasma NEFA concentrations ($P \geq 0.21$).

Table 3.10. Estimated marginal means (± SE) for non-esterified fatty acids (NEFA) lactate measures in response to adrenaline challenges for steers fed three different levels of dietary protein

NEFA Parameter	Dietary CP, %		
	10	20	30
Basal Concentration	0.023 ± 0.002[a]	0.010 ± 0.001	0.009 ± 0.001
Time to max concentration (min)	11.33 ± 0.63[a]	8.74 ± 0.62[b]	7.58 ± 0.61[b]
AUC_{10}	0.48 ± 0.04[a]	0.29 ± 0.04[b]	0.29 ± 0.04[b]
AUC_{20}	1.17 ± 0.1[a]	0.64 ± 0.1[b]	0.58 ± 0.1[b]
AUC_{30}	1.66 ± 0.15[a]	0.81 ± 0.15[b]	0.71 ± 0.14[b]
AUC_{45}	2.07 ± 0.21[a]	0.91 ± 0.21[b]	0.79 ± 0.21[b]
AUC_{60}	2.27 ± 0.26[a]	0.95 ± 0.26[b]	0.83 ± 0.25[b]
AUC_{130}	2.42 ± 0.37[a]	1.08 ± 0.37[b]	0.93 ± 0.36[b]

[a-b] within row means without a common superscript differ ($P \leq 0.03$)

Table 3.11. Estimated marginal means (± SE) for non-esterified fatty acids (NEFA) for the adrenaline challenges (ug/kg LW) in steers fed three different levels of dietary protein

NEFA Parameter	Adrenaline Challenge (ug/kg LW)		
	1.0	3.0	5.0
Basal Concentration	0.016 ± 0.002	0.013 ± 0.002	0.014 ± 0.002
AUC_{10}	0.34 ± 0.03	0.34 ± 0.03	0.38 ± 0.03
AUC_{20}	0.67 ± 0.07[a]	0.83 ± 0.07[b]	0.88 ± 0.07[b]
AUC_{30}	0.85 ± 0.11[a]	1.14 ± 0.11[b]	1.19 ± 0.11[b]
AUC_{45}	0.98 ± 0.16[a]	1.37 ± 0.16[ab]	1.43 ± 0.16[b]
AUC_{60}	1.05 ± 0.20	1.47 ± 0.20	1.52 ± 0.20
AUC_{130}	1.26 ± 0.29	1.57 ± 0.29	1.59 ± 0.29

[a-b] within row means without a common superscript differ ($P \leq 0.05$)

3.3.5 Muscle glycogen and lactate

Muscle glycogen concentrations in the LT, ST and SM were not influenced by diet ($P \geq 0.4108$). Muscle glycogen concentrations of the LT and ST were influenced by biopsy. Within the LT where muscle glycogen decreased 0.25 g/100g and 0.15 g/100g, respectively, between biopsy collection 1 and 4 ($P \leq 0.0004$; Table 3.12), although muscle glycogen concentrations of the SM were not influenced by biopsy ($P = 0.1654$; Table 3.12). Muscle lactate concentration in the LT, ST and SM decreased by 31.8 µmole/g ($P < 0.0001$), 8.9 µmole/g ($P < 0.0001$) and 10.9 µmole/g ($P < 0.0001$) between biopsy collection 1 and 4 (Table 3.13). There was no influence or diet × biopsy on muscle glycogen ($P = 0.295239$) concentrations in the LT, ST or SM (Table 3.12), nor were there diet × biopsy influenced on muscle lactate ($P = 0.1940$) in the ST or SM within this experiment (Table 3.13). However for the LT muscle lactate was influenced by diet × biopsy ($P = 0.007218$), where muscle lactate decreased by 30.6 µmole/g, 36.8 µmole/g and 28 µmole/g between biopsy 1 and 4, for the 10 %, 20 % and 30 % CP diet respectively (Table 3.13).

Table 3.12. Muscle glycogen (g/100g) concentrations for the Longissimus thoracis (LT), Semitendinosus (ST), Semimembranosus (SM) from steers fed three different levels of dietary protein across the four muscle biopsy samples

Muscle	Dietary CP, %	Biopsy			
		1	2	3	4
LT	10	1.51 ± 0.09	1.37 ± 0.09	1.48 ± 0.09	1.20 ± 0.09
	20	1.48 ± 0.09	1.31 ± 0.09	1.44 ± 0.09	1.21 ± 0.09
	30	1.62 ± 0.09	1.42 ± 0.09	1.37 ± 0.09	1.46 ± 0.09
ST	10	1.34 ± 0.08	1.30 ± 0.08	1.20 ± 0.08	1.23 ± 0.08
	20	1.31 ± 0.08	1.40 ± 0.08	1.26 ± 0.08	1.09 ± 0.08
	30	1.38 ± 0.08	1.30 ± 0.08	1.27 ± 0.08	1.26 ± 0.08
SM	10	1.58 ± 0.10	1.79 ± 0.10	1.68 ± 0.10	1.75 ± 0.10
	20	1.53 ± 0.10	1.69 ± 0.10	1.60 ± 0.10	1.70 ± 0.10
	30	1.75 ± 0.10	1.83 ± 0.10	1.76 ± 0.10	1.55 ± 0.11

Table 3.13. Muscle lactate (µmole/g) concentrations for the Longissimus thoracis, semitendinosus, semimembranosus from steers fed three different levels of dietary protein at four different biopsy times

Muscle	Dietary CP, %	Biopsy			
		1	2	3	4
LT	10	85.5 ± 3.16	41.3 ± 3.16	34.8 ± 3.16	54.9 ± 3.16
	20	90.1± 3.16	49.5 ± 3.16	49.7 ± 3.16	53.3 ± 3.16
	30	83.6 ± 3.16	43.1 ± 3.46	54.3 ± 3.16	55.6 ± 3.46
ST	10	71.5 ± 3.09	42.0 ± 3.09	51.9 ± 3.09	62.2 ± 3.09
	20	67.5 ± 3.09	43.3 ± 3.09	57.6 ± 3.09	60.3 ± 3.09
	30	78.1 ± 3.09	40.3 ± 3.09	59.3 ± 3.09	61.8 ± 3.37
SM	10	67.5 ± 3.25	42.7 ± 3.25	52.1 ± 3.25	55.3 ± 3.25
	20	69.1 ± 3.25	46.7 ± 3.25	51.6 ± 3.25	58.3 ± 3.25
	30	64.9 ± 3.25	43.5 ± 3.25	47.9 ± 3.25	61.4 ± 3.56

3.4 Discussion

Increasing dietary protein within the current experiment had no impact on insulin sensitivity, insulin responsiveness, glucose disappearance rates, adrenaline sensitivity or glycogen metabolism during and after an exercise challenge, resulting in a rejection of all of the initial hypotheses. Janes *et al.* (1985) also identified that diet (hay versus ground corn) did not influence insulin sensitivity or insulin responsiveness in sheep. Within the current experiment, the amount of glucose required to maintain euglycaemia during the HEIG clamp procedure at the lower insulin infusion rate (0.6 mU/kg/min) was not influenced by dietary protein concentrations. However, at the higher insulin infusion rate (6 mU/kg/min) the volume of glucose required to maintain euglycaemia was greater for animals within the 30 % protein diet, in comparison with the 10% protein diet.

Insulin is an important regulator of carbohydrate and lipid metabolism (Baumgard and Rhoads 2012). However, the main activity of insulin is to facilitate prompt glucose uptake (Mehla *et al.* 2014), in muscle and adipose tissues where muscle accounts for approximately 80% of insulin dependent glucose uptake (Kraegen *et al.* 1985). It is well accepted that diet can influence insulin sensitivity, particularly in monogastrics. Sternbauer and Luthman (2002) concluded that insulin sensitivity was not different in heifers fed to achieve growth rates of 400 g/day and 900 g/day. However, the authors noted that the diet composition may not have been sufficient to elicit alterations in insulin sensitivity (Sternbauer and Luthman 2002). The diet used within the current experiment was formulated to achieve an average daily gain of 1 kg/day (NRC 2016). Additionally the diets used within the current experiment were formulated to ensure that the mineral concentrations, energy and protein were comparable with lush, rapidly growing pastures. However, it is important to consider that these diets were offered to these cattle as chaff and a pelleted ration. As such, these rations had a greater dry matter content when compared with pastures, which have very low dry matter content. The high water content in pastures serves to increase both rumen passage rates (Pasha *et al.* 1994; Forbes 1995) in addition to the volume of pasture an animal must consume on a daily basis in order to meet its nutrient needs. Hatfield *et al.* (1998) reported DMI was greater in sheep consuming a diet consisting of 18% protein when compared with sheep fed a 10% protein diet. The authors also reported that VFA concentrations were also greater for sheep consuming the 18% protein diet, suggesting that this was associated with differing dietary protein concentrations (Hatfield *et al.* 1998). It is also possible that dietary concentrations influence intermediate metabolism and its endocrine control in ruminants (Sano and Terashima 2001). In addition, previous studies have identified that dietary protein concentrations have the potential to alter rumen fermentation and the passage rate of both microbial and dietary protein from the rumen (Clark *et al.* 1992). Due to the high dry matter content of the feeds fed in this experiment, the rumen passage rates would have been much slower than those observed in grazing cattle, possibly leading to increased or at least more efficient Mg absorption in these cattle. Subsequently, resulting in the limited influence of dietary protein observed within this experiment.

3.5 Conclusions

Although it was not observed in this experiment, it was anticipated that increased dietary protein intake by ruminants would reduce Mg absorption and increase adrenaline sensitivity in these cattle. The controlled conditions within this experiment, predominantly associated with diet presentation, were probably insufficient to elicit the anticipated responses due to restricted movement and consistent quality of available feed. Future studies resembling commercial conditions more closely are required to develop an understanding of the influence of protein content in grazing pastures on the mechanisms involved in glycogen deposition and utilisation, which may lead to dark cutting. However, the results presented here are suggestive that dark cutting in cattle consuming high protein diets may not be elicited by changes in insulin sensitivity or altered sensitivity to adrenaline.

4 Impact of varying dietary potassium concentrations on whole body insulin responsiveness, adrenaline sensitivity and muscle glycogen

Project Objective 2. Determine the impact of varying levels of minerals and DCAD on the Mg status of cattle and its impact on glycogen synthesis and glycogen depletion

4.1 Introduction

High dietary protein, potassium (**K**) and a high Dietary Cation-Anion Difference (**DCAD**) can reduce the efficiency of ruminal Mg absorption and as such predispose cattle to low Mg absorption. Reduced Mg has been associated with reduced feed intake, subsequent decreased growth and muscle glycogen, overall cumulating with an increasing risk of dark cutting. Low muscle glycogen levels and higher incidences of dark cutting have long been observed during periods when pasture quality is at its lowest (Pethick *et al.* 1999), for example during late spring and summer in Mediterranean climates when the pastures enter their late reproductive phases and become senescent. However, in southern Australia, the incidences of dark cutting are highest in the period between February and June (McGilchrist *et al.* 2014). A high incidence of dark cutting throughout this period does not seem to be able to be explained by a lack of metabolisable energy (McGilchrist *et al.* 2014), hence the true cause of this spike in dark cutting is currently unknown. Pasture-finished cattle are most likely consuming highly nutritious vegetative-phase pastures during the autumn and winter periods when the high incidence of dark cutting appears to occur (McGilchrist *et al.* 2014). Grass species in their vegetative phase are typically growing rapidly, have high protein levels, variable but often high K and phosphorus (**P**) levels combined with variable calcium (**Ca**) levels and moderate magnesium (**Mg**) concentrations (Metson *et al.* 1966; Metson and Saunders 1978). In addition the absorption of Mg is impacted by the K content of the pasture (Suttle 2010). Therefore, the aims of this experiment were to evaluate the impact of variations in dietary K on i) responses to exogenous insulin and glucose disappearance following an glucose tolerance test; ii) adrenaline sensitivity during an exogenous adrenaline challenge; and iii) the impact on glycogen depletion and repletion during and after an exercise challenge. It was hypothesised that increasing the dietary K will reduce insulin sensitivity, insulin responsiveness and increase adrenaline sensitivity in beef cattle.

4.2 Materials and Methods

This experiment was conducted with the approval of the University of New England's animal ethics committee (AEC17-108; Appendix 1).

4.2.1 Animals and housing

Eighteen Angus steers (441 ± 28 kg), approximately 15 months of age were enrolled into the experiment. The steers were sired by three bulls, each of which had two progeny within each treatment group. Cattle were housed in individual animal pens for a total of 60 days, where there was a 30 day acclimation period and a 30 day data collection period.

4.2.2 Nutritional management

Cattle were randomly allocated to three dietary K treatment groups consisting of: 1) low (1.5 %); 2) medium (2.7 %); and high (3.8%) dietary K levels (Table 4.1). Diets were balanced for minerals and metabolizable energy (**ME**) to allow for a comparison between diets of differing dietary K to occur. The diets were balanced according to the guidelines established by the NRC (2016), to meet the nutritional requirements to achieve an average daily gain of 1 kg/d. Cattle were fed once daily at 1600h, any refusals were removed and weighed daily with average per animal calculated.

Table 4.1. Diet composition

Ingredient, % of DM	Potassium level , %		
	1.5	**2.7**	**3.8**
Oat chaff	55.74	54.35	52.97
Wheat millrun	11.15	10.87	10.59
Wheat ground	31.22	30.44	29.66
Limestone	0.28	0.27	0.26
Calcium sulfate	1.45	1.41	1.38
Magnesium oxide	0.05	0.05	0.05
Potassium chloride	0	2.5	4.98
Mineral premix	0.11	0.11	0.11
Chemical composition			
Dry matter (%)	88.84	88.75	88.63
Crude protein (% DM)	12.29	11.98	11.71
Neutral detergent fiber (% DM)	37.42	36.69	35.75
Acid detergent fiber (% DM)	20.68	20.06	19.95
Metabolizable energy (ME MJ/kg DM)	11.62	11.65	11.71
TDN (%)	72.25	72.49	72.73
Calcium (%)	0.49	0.54	0.55
Phosphorus (%)	0.34	0.36	0.35
Magnesium (%)	0.18	0.20	0.19
Potassium (%)	1.54	2.72	3.93
Sodium (%)	0.24	0.25	0.26
Calcium to Phosphorus Ratio	1.44	1.51	1.56
Grass Tetany Index	1.00	1.60	2.32

4.2.3 Phenotypic measures and acclimation

Cattle were divided in three groups of six animals consisting of two animals from each dietary treatment. On the day prior to the first challenge, cattle were weighed (non-fasted) and real-time ultrasound measurements (3.5 MHz/180-mm linear array animal science probe, Esoate Pie Medical, Maastricht, Netherlands) of subcutaneous fat depth between the 12/13th rib (rib fat) and P8 rump (rump fat) site were determined. Cattle were then fitted with two catheters, one in each external jugular vein. The cannulation site was clipped to remove all hair and sterilized using betadine surgical scrub and ethanol swabs. The site was infiltrated with 2 ml of 2% Lignocaine into the skin (dermis). After 5 minutes an 18-gauge needle was introduced into the jugular vein through the site and a sterile teflon coated surgical wire guide passed through the needle. The needle was then removed and a treated sterile polyethylene catheter with a 2 mm outer diameter and 1 mm inner diameter (Portex, Hythe, Kent, England) was passed over the wire guide and fed into the jugular vein. The wire guide was then withdrawn through the catheter, with the catheter sutured in place at the site of protrusion on the animals' neck. Catheters were then fixed to the neck, providing access for sample collection during the challenges. Catheter were flushed and filled with a heparin/saline to remain patent.

4.2.4 Hyperinsulinaemic, euglycaemic clamp (HIEG) procedure

The day after the catheters were fitted the hyperinsulinaemic, euglycaemic clamp (**HIEG**) technique, as described by DeFronzo *et al.* (1979) for use in humans and subsequently adapted Bergman *et al.* (1989) for use in ruminants by, was implemented to determine the whole body glucose use in response to insulin. Prior to commencing insulin infusions, basal blood glucose level was determined by successive blood samples collected at 15 minutes over a 60 minute period. An 8 mL sample was collected using S-Monovette Vacutainer® tubes (Sarstedt Australia Pty. Ltd. SA Australia, Cat. No. 02.1066.001) containing ethylenediaminetetraacetic acid (**EDTA**) to prevent clot formation. From these samples, blood glucose concentration was determined immediately using portable blood glucose meters (AscensiaTM Esprit® 2, Bayer, USA). The remaining samples were

centrifuged and the harvested plasma frozen at -20°C, for later laboratory determination of glucose. The five glucose meter readings were used to estimate blood glucose concentration, prior to insulin infusion. Glucose concentration was later confirmed by laboratory analysis of plasma samples.

The insulin infusion commenced with a single dose of 6mU/kg live weight of insulin (Actrapid®, Novo Nordisk Pharmaceuticals Pty. Ltd., Baulkham Hills, NSW) administered via a singular infusion catheter. From then a continuous insulin infusion occurred at a rate of 0.6mU/kg live weight per minute using a dual channel infusion pump (LIFECARE® 5000 Plum™ Infusion system, Abbott Laboratories, USA). Concurrently, glucose (50% w/v; Baxter Healthcare, Old Toongabbie, NSW) was infused, utilizing the same infusion pump as preciously described. Initially the glucose infusion occurred at a rate of ≈ 125 ml/h. Every 5 minutes, a 2 mL blood sample was collected and blood glucose concentration was rapidly determined using the portable blood glucose meter. Depending on the blood glucose concentration, the glucose infusion rate was adjusted to establishing a constant level of blood glucose at the same level as the pre-infusion level (basal blood glucose) or euglycemia. Once euglycemia was observed, the glucose infusion rate was held constant for 60 minutes and defined as the steady state glucose infusion rate (**SSGIR**). An 8 ml blood samples was collected at 15 minutes intervals S-Monovette Vacutainer® tubes (Sarstedt Australia Pty. Ltd. SA Australia, Cat. No. 02.1066.001), with one drop used to ensure that euglycaemic glucose concentrations were being maintained. The remaining blood samples were then centrifuged and plasma harvested. Plasma samples were then frozen at -20°C until laboratory determination of glucose occurred. The SSGIR required was then used to determine whole body insulin responsiveness.

4.2.5 Insulin challenge

Throughout the 2 days following the HIEG, steers were subjected to two insulin challenges and a dextrose challenge. Therefore, a total of three different challenges given in the morning (1000h) or afternoon (1400h). Steers were randomly subjected to these insulin challenges either in the morning or afternoon. This was conducted in a way that by the second morning all six animals had been through both insulin challenges. The insulin challenges consisted of 0.5 ug/kg live weight and 2.0 ug/kg live weight, whilst the dextrose challenge was applied at a rate of 0.35 g/kg live weight. A 5 ml blood sample was taken from the catheter at -30, -15, -10, -5, 0, 2.5, 5, 10, 15, 20, 30, 45, 60, 120, 125, and 130 minutes relative to the administration of the respective challenge. Whole blood was collected into S-Monovette Vacutainer® tubes (Sarstedt Australia Pty. Ltd. SA Australia, Cat. No. 02.1066.001) containing EDTA. Immediately after collection, blood samples were stored on ice to minimize further metabolic activity of red blood cells converting glucose to lactate. Within 20 minutes of collection, blood samples were centrifuged at 3500 rpm for 15 minutes at 5 $^{\circ}$C. Plasma decanted into two separate sample tubes, where one sample was stored at -20 $^{\circ}$C for glucose and lactate analysis and second sample was stored at -80 °C for non-esterified fatty acid (**NEFA**) analysis.

4.2.6 Adrenaline challenge

After Biopsy 4 the 18 steers were scanned at the P8 rump and in between 12-13th rib with real-time ultrasound measurements (3.5 MHz/180-mm linear array animal science probe, Esoate Pie Medical, Maastricht, Netherlands) for subcutaneous fat depth. The steers were then weighed and fitted with one catheter into the external jugular vein. The site for cannulation was clipped to remove all hair and sterilised using betadine surgical scrub and ethanol swabs. A 2% Lignocaine (2ml/steer) was infiltrated into the skin (dermis) and after 5 minutes a 18 gauge needle was introduced into the jugular vein through the site and a sterile teflon coated surgical wire guide passed through the needle. The needle was then removed and a treated sterile polyethylene catheter (2mm o.d. and 1mm i.d., Portex, Hythe, Kent, England) was passed over the wire guide (at the end protruding from the animal) and fed into the jugular vein. The wire guide was then withdrawn through the catheter, with the catheter sutured in place at the site of protrusion on the

animals' neck. The exposed length of the catheter was tied behind the animals' neck for easy access during sampling. The catheter was filled with heparin/saline mixture to prevent thrombis formation.

In the following three days after the catheters were fitted the steers were subjected to three different adrenaline challenges (1.0 ug/kg LW, 3.0 ug/kg LW and 5.0 ug/kg LW) given in the morning (1000h) or afternoon (1400h). The challenges given to the steers in the morning and afternoon were randomized and in the end of the 2nd day, all six animals have had received all three challenges. Blood samples were collected, using S-Monovette Vacutainer® tubes containing EDTA to prevent clotting, from the catheter at -30, -15, -10, -5, 0, 2.5, 5, 10, 15, 20, 30, 45, 60, 120, 125, and 130 minutes relative to the administration of the challenge. Immediately after the blood collection the tubes were stored on ice to minimize further metabolic activity of red blood cells converting glucose to lactate. Within 20 minutes of collection, the samples were centrifuged at 3500 rpm for 15 minutes at 5°C and the plasma was separated into two eppendorf of 2mL where one was stored at -20°C for glucose and lactate analysis and the other tube was stored at -80°C for NEFA analysis.

4.2.7 Muscle biopsies

Muscle samples from *Longissimus thoracis* (LT), *semitendinosus* (ST) and *semimembranosus* (SM) were collected at day 0 (Biopsy 1) in order to obtain a base line of glycogen concentration within the muscle. On day three (Biopsy 2) the steers were weighed and then exercised at 65% of VO_2 max for one hour to deplete muscle glycogen concentration and a biopsy (Biopsy 2) was taken immediately after the exercise. Two more samples were taken at day six (Biopsy 3) and day nine (Biopsy 4) what mean three and six days after exercises the steers, respectively. Muscle samples were taken as described by McGilchrist *et al.* (2011b). Briefly, a purpose built 12 volt powered motorized biopsy drill were using to sampling the muscle while the steers were in a restrained crush. For sampling the SM and ST, the area between 15 and 25 cm below the anus, and over the poverty groove with both muscles accessed through the same incision; the area was prepared by clipping the hair from a 10 x 10 cm area around the point of incision. The site prepared for the LD was at the top of the 12^{th}-13^{th} rib, approximately 5 cm from the backbone.

Muscle samples were analyzed for lactate and glycogen concentration at Murdoch University. The muscle samples were homogenized in 30 mM HCl in a ratio of 1 part muscle to 10 parts acid. An enzymatic method kit (Trinder 1969; Badham and Trinder 1972) was used to analyze lactate on the homogenate. A method outlined by method (Passonneau and Lauderdale 1974) was used to hydrolyze the glycogen in the homogenate into glucose and then it was measured by the Olympus AU 400 using a glucose kit (Cat.No OSR6121). Total glycogen (g/100 g) was then determined by halving the lactate concentration and adding it to the glucose concentration.

4.2.8 Plasma analysis

Laboratory determination of plasma samples were carried out in duplicate using enzymatic methods for glucose (Kunst *et al.* 1984) and lactate (Marbach and Weil 1967) using an integrated chemistry system auto-analyser (Siemens Dimension® XPand Plus). Similarly, NEFA concentrations were analyzed in duplicate using a NEFA Kit from Siemens Dimension®. The Flex® reagent cartridge Kit was used to measure Glucose and Lactate concentration.

4.2.9 Modelling of response curves

Basal concentrations of glucose, lactate and NEFA were calculated as the mean of samples collected at -30, -15, -10, -5 and 0 minutes relative to the insulin challenges and dextrose challenge. The concentrations of each substrate were then plotted against time for each challenge administered to each animal and a derived function with multiple exponential components was fitted to the raw data (McGilchrist *et al.* 2011a, 2011b). This showed a classical increase and then tapering off in response which was modeled using the following function:

$$y(t)= A + ([–\gamma/(\beta – \alpha) + \gamma/(\beta–\alpha–\Delta) – \theta] e– \beta t + \gamma/(\beta – \alpha)e– \alpha t – \gamma/(\beta–\alpha–\Delta) e–(\alpha +\Delta)t + \theta) ; \varepsilon(0,\sigma)$$

where y(t) is the plasma metabolite concentration at time *t*; *t* is time (min); *A* is basal metabolite concentration (average of 5 samples taken in the 30 minute period pre-challenge); β,γ,α,Δ = exponential constants; θ = the adjustment from the basal metabolite concentration; and ε = error

This function was used to determine the time to maximum substrate concentration, the maximum substrate concentration and the area under the response curve (**AUC**) between 0 and 10 minutes (**AUC_{10}**) for glucose relative to administration of the dextrose challenge. For insulin challenge the function was used to determine the time to minimal substrate concentration, the minimal substrate concentration and the area under the response curve between 0 and 10 minutes (**AUC_{10}**). The function was also used to model plasma glucose concentration following the intravenous glucose challenge, with glucose area under curve between 0 and 130 minutes (**AUC_{130}**) reflecting glucose disappearance rate.

4.2.10 Statistical analysis

Data exploration and statistical analyses were generally conducted in R (R Core Team 2019). Data merging and manipulation, data visualizations and summary data were prepared using the 'dplyr' (Wickham *et al.* 2019b) and 'ggplot2' (Wickham *et al.* 2019a) packages. Data from the SSGIR, insulin challenges and dextrose were analysed using a linear mixed effects model from the 'lme4' package (Bates *et al.* 2019) and estimated marginal means were generated using the 'emmeans' package (Length 2019). Models pertaining to the insulin challenges investigated glucose disappearance, plasma lactate and plasma NEFA.

The SSGIR was not influenced by rib fat or rump fat ($P > 0.05$), thus these fat measures were excluded from the final model. The final SSGIR model incorporated diet and live weight as fixed effects. Similarly the models for glucose disappearance during the insulin challenges incorporated diet, insulin challenge (0.6 mU/kg/min and 6 mU/kg/min), live weight, a measure of fat (rib or rump), and diet × challenge as fixed effects. Models were refined to remove relevant insignificant interactions in a step-wise manner. For the dextrose challenge, the glucose clearance models incorporated diet and live weight. Animal id nested within sire was incorporated into all models described above as a random effect.

For the adrenaline challenges models the investigated plasma glucose, lactate and NEFA concentrations. These models incorporated diet, adrenaline challenge (1.0 ug/kg LW, 3.0 ug/kg LW and 5.0 ug/kg LW), live weight, and diet × challenge as fixed effects. Animal id nested within sire was incorporated into all models described above as a random effect. Models were refined to remove relevant insignificant interactions in a step-wise manner. Fat measures (rib fat or rump fat depth) were investigated within all models, but did not contribute significance ($P > 0.05$), thus, were excluded from the final models. Additionally basal glucose concentration was investigated as co-variate, however but did not contribute significance ($P > 0.05$), thus, were excluded from the final models. Additionally, muscle glycogen and lactate concentrations were analysed using a repeated measures linear mixed effects model, models incorporated diet, biopsy and diet × biopsy as fixed effects and animal id nested within sire as a random effect. Cattle live weight was investigated as co-variate, however but did not contribute significance ($P > 0.05$), thus, were excluded from the final models.

4.3 Results

4.3.1 Influence of dietary potassium on HEIG clamp

The responsiveness to insulin during the HEIG clamp was influenced by dietary K level ($P = 0.009$). There were no differences in the SSGIR between diets containing 1.5 % and 3.8 % dietary K ($P = 0.2607$), nor were there differences between diets containing 2.7 % and 3.8 % K ($P = 0.3151$). However, the SSGIR for diets contacting 2.7 % dietary K had a glucose infusion rate 132.0 ± 43.3 ml/hr

greater than diets containing 1.5 % K (P = 0.0295; Figure 4.1). The model suggested that live weight influenced SSGIR (P < 0.001), however the relationship was insignificant (r = -0.71, P = 0.53).

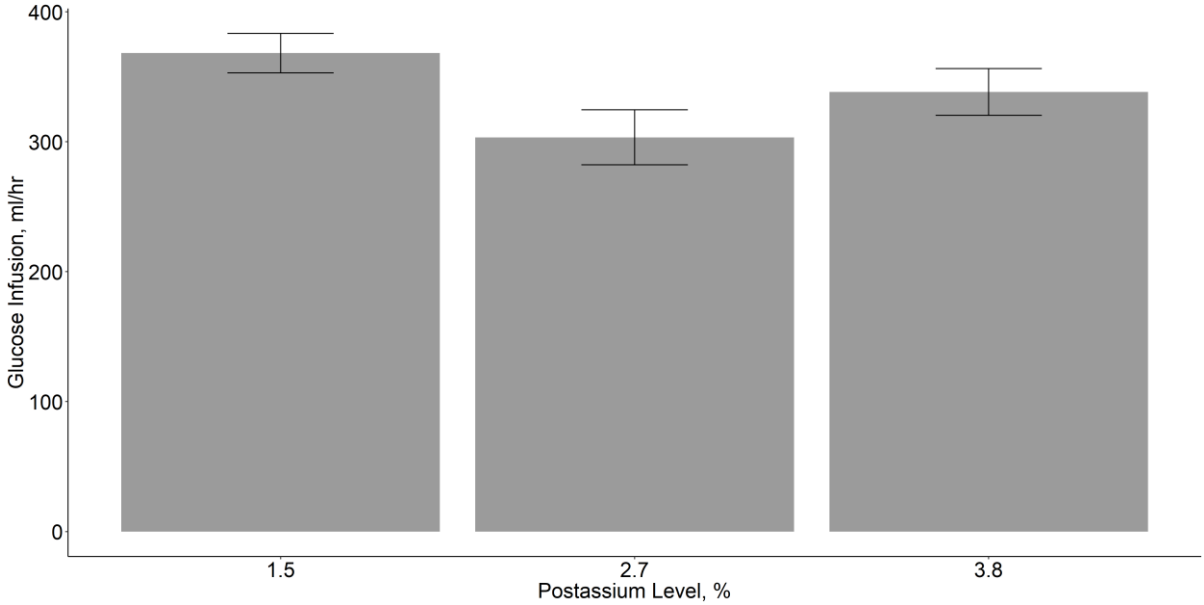

Figure 4.1. Influence of dietary potassium level (%) on steady state glucose infusion rate (SSGIR; glucose infusion, ml/hr) at an insulin infusion rateof 0.6 mU/kg/min. Values are means ± SEM

4.3.2 Glucose disappearance, plasma lactate and plasma NEFA during insulin challenges

Basal glucose concentration was higher in the 1.5 % K diet, when compared with the 2.7 % and 3.8 % K diets ($P \leq 0.0236$; Table 4.2). Additionally, basal glucose concentration was 0.29 ± 0.08 mM higher at the commencement of the 6 mU/kg/min insulin infusion rate challenge (P = 0.0039). Dietary K levels did not influence the time to maximum glucose concentration (P = 0.16; Table 4.2), however time to maximum glucose concentration during the 0.6 mU/kg/min insulin infusion rate challenge occurred 16.6 ± 1.91 min earlier when compared with the 6 mU/kg/min insulin infusion rate challenge (P < 0.0001). Diet did not influence glucose disappearance in any of the AUC investigated (Table 4.2), although steers in the 1.5 % K diet tended to have a lower glucose disappearance at AUC_{130}, when compared with the 3.8 % K diet (P = 0.0715; Table 4.2). Glucose disappearance was influenced by the insulin challenges between AUC_{20} and AUC_{130} (Table 4.3).

Table 4.2. Estimated marginal means (± SE) for glucose disappearance measures in response to insulin challenges for steers fed three different levels of dietary potassium

Glucose Parameter	Dietary K Level, %		
	1.5	**2.7**	**3.8**
Basal Concentration	5.42 ± 0.11[a]	4.91 ± 0.11	4.96 ± 0.10
AUC_{10}	28.6 ± 2.16	31.9 ± 1.88	31.7 ± 1.65
AUC_{20}	-2.71 ± 0.49	-2.75 ± 0.42	-3.03 ± 0.37
AUC_{30}	-12.1 ± 1.82	-12.4 ± 1.59	-13.6 ± 1.39
AUC_{45}	-24.8 ± 3.32	-26.3 ± 2.89	-28.6 ± 2.53
AUC_{60}	-43.7 ± 5.12	-48.6 ± 4.46	-52.6 ± 3.91
AUC_{130}	-58.9 ± 6.54	-68.6 ± 5.62	-73.4 ± 5.09

[a] within row means without a common superscript differ ($P \leq 0.0236$)

Table 4.3. Estimated marginal means (± SE) for glucose disappearance measures for the insulin challenges in steers fed three different levels of dietary potassium

Glucose Parameter	Insulin infusion rate		Significance
	0.6	6	
AUC_{10}	-2.66 ± 0.33	-3.00 ± 0.34	0.5067
AUC_{20}	-9.98 ± 1.24	-15.45 ± 1.26	0.0135
AUC_{30}	-18.0 ± 2.26	-35.2 ± 2.30	0.0003
AUC_{45}	-27.6 ± 3.49	-69.0 ± 3.55	< 0.0001
AUC_{60}	-34.0 ± 4.29	-100.0 ± 4.36	< 0.0001
AUC_{130}	-46.4 ± 6.55	-168.8 ± 6.66	< 0.0001

Basal lactate concentration was not influenced by the different dietary K levels (P = 0.3733; Table 4.4), insulin challenge (P = 0.2980), or diet × insulin challenge (P = 0.3666). There were no influences of dietary K levels (P = 0.8617; Table 4.4), insulin challenge (P = 0.6783), or diet × insulin challenge (P = 0.8662) on the time to maximum lactate concentration. Furthermore, plasma lactate concentrations between AUC_{10} and AUC_{130} were not influenced by diet ($P \geq 0.3312$; Table 4.4), insulin challenge ($P \geq 0.3394$), live weight ($P \geq 0.4437$) or diet × insulin challenge ($P \geq 0.5299$). Similarly, there were no differences in the basal NEFA concentrations, or the time to maximum NEFA concentration across the three dietary K levels (Table 4.5). Nor were there dietary K level influences on plasma lactate concentrations between AUC_{10} and AUC_{130} were not influenced by diet ($P \geq 0.2797$; Table 4.5), insulin challenge ($P \geq 0.5069$), live weight ($P \geq 0.1308$) or diet × insulin challenge ($P \geq 0.5468$).

Table 4.4. Estimated marginal means (± SE) for lactate during insulin challenges for steers fed three different levels of dietary potassium

Glucose Parameter	Dietary K Level, %		
	1.5	2.7	3.8
Basal Concentration	0.78 ± 0.28	0.31 ± 0.28	0.28 ± 0.26
AUC_{10}	14.17 ± 10.9	5.79 ± 10.9	8.76 ± 10.0
AUC_{20}	-0.75 ± 0.58	0.20 ± 0.58	0.35 ± 0.35
AUC_{30}	-1.01 ± 1.11	0.64 ± 1.11	0.73 ± 1.02
AUC_{45}	-1.95 ± 1.99	0.99 ± 1.99	0.98 ± 1.82
AUC_{60}	-4.27 ± 3.65	1.19 ± 3.65	1.15 ± 3.35
AUC_{130}	-6.99 ± 5.36	1.11 ± 5.35	1.13 ± 4.91

Table 4.5. Estimated marginal means (± SE) for non-esterified fatty acids (NEFA) during insulin challenges for steers fed three levels of dietary potassium

NEFA Parameter	Dietary potassium Level, %		
	1.5	2.7	3.8
Basal Concentration	0.091 ± 0.02	0.094 ± 0.01	0.093 ± 0.01
Time to max concentration (min)	12.5 ± 22.3	24.2 ± 21.1	34.6 ± 19.4
AUC_{10}	0.05 ± 0.08	-0.12 ± 0.07	-0.09 ± 0.07
AUC_{20}	0.01 ± 0.24	-0.36 ± 0.22	-0.26 ± 0.20
AUC_{30}	-0.01 ± 0.40	-0.57 ± 0.38	-0.41 ± 0.35
AUC_{45}	0.08 ± 0.64	-0.75 ± 0.60	-0.51 ± 0.55
AUC_{60}	0.29 ± 0.86	-0.82 ± 0.82	-0.46 ± 0.74
AUC_{130}	1.91 ± 2.03	-0.31 ± 1.93	0.76 ± 1.77

4.3.3 Dextrose challenge

There were no differences in the basal glucose concentrations across the three dietary K levels at the commencement of the dextrose challenge (P = 0.8211; Table 4.6), nor were there difference in the time to maximum concentration (P = 0.3798). Dietary K levels did not influence

glucose clearance between AUC_{10} and AUC_{130} throughout the dextrose challenge ($P \geq 0.4709$; Table 4.6).

Table 4.6. Estimated marginal means (± SE) for glucose measures during a dextrose challenge for steers fed three levels of dietary potassium

Glucose Parameter	Dietary Potassium Level, %		
	1.5	2.7	3.8
Basal Concentration	5.05 ± 0.18	4.90 ± 0.15	4.96 ± 0.15
Time to max concentration (min)	1.87 ± 0.31	1.89± 0.27	1.43 ± 0.26
AUC_{10}	127.0 ± 7.57	130.0 ± 6.45	127.0 ± 6.35
AUC_{20}	220.0 ± 12.1	223.0 ± 10.3	223.0 ± 10.2
AUC_{30}	284.0 ± 17.7	285.0 ± 15.1	292.0 ± 14.8
AUC_{45}	345.0 ± 27.7	341.0 ± 23.6	363.0 ± 23.3
AUC_{60}	380.0 ± 37.1	371.0 ± 31.6	408.0 ± 31.1
AUC_{130}	418.0 ± 59.2	389.0 ± 50.4	473.0 ± 49.7

4.3.4 Plasma glucose, plasma lactate and plasma NEFA during adrenaline challenges

Basal glucose concentration was higher in the 1.5 % K diet, when compared with the 2.7 % ($P \leq 0.0424$) and 3.8 % K diets ($P \leq 0.0234$; Table 4.7). There were no differences in basal glucose concentration between the adrenaline challenges ($P \geq 0.81$). Dietary K levels did not influence the time to maximum glucose concentration, where the time to reach maximum glucose concentration occurred between 10.4 and 11.2 minutes ($P \geq 0.72$; Table 4.7). Diet did not influence glucose availability in any of the AUC investigated (Table 1). Glucose availability was influenced by the adrenaline challenges between AUC_{10} and AUC_{130} as exogenous adrenaline increased glucose availability regardless of AUC time point (Table 4.8).

Table 4.7 Estimated marginal means (± SE) for glucose availability measures in response to adrenaline challenges for steers fed three different levels of dietary potassium

Glucose Parameter	Dietary K Level, %		
	1.5	2.7	3.8
Basal Concentration	5.47 ± 0.12[a]	4.99 ± 0.12	4.97 ± 0.11
Time to max concentration (min)	10.6 ± 0.79	11.2 ± 0.79	10.4 ± 0.72
AUC_{10}	16.2 ± 2.69	17.7 ± 2.69	20.0 ± 2.46
AUC_{20}	39.6 ± 5.56	43.3 ± 5.56	46.8 ± 5.09
AUC_{30}	58.8 ± 7.99	65.0 ± 7.98	69.0 ± 7.31
AUC_{45}	80.1 ± 10.91	89.7 ± 10.90	94.8 ± 9.99
AUC_{60}	95.4 ± 13.2	107.1± 13.2	114.1 ± 12.1
AUC_{130}	127.0 ± 20.8	142.0 ± 20.8	162.0 ± 19.1

[a] within row means without a common superscript differ ($P = 0.0376$)

Table 4.8. Estimated marginal means (± SE) for glucose availability measures for the adrenaline challenges (ug/kg LW) in steers fed three different levels of dietary potassium

Glucose Parameter	Adrenaline Challenge (ug/kg LW)		
	1.0	3.0	5.0
Basal Concentration	5.18 ± 0.10	5.15 ± 0.10	5.09 ± 0.10
AUC_{10}	10.8 ± 1.77[a]	19.0 ± 1.77[b]	24.1 ± 1.77[c]
AUC_{20}	24.1 ± 3.80[a]	45.6 ± 3.80[b]	60.0 ± 3.80[c]
AUC_{30}	34.1 ± 5.57[a]	68.1 ± 5.57[b]	90.6 ± 5.57[c]
AUC_{45}	44.4 ± 7.71[a]	93.7 ± 7.71[b]	126.5 ± 7.71[c]
AUC_{60}	51.3 ± 9.38[a]	112.2 ± 9.38[b]	153.0 ± 9.38[c]
AUC_{130}	66.0 ± 14.5[a]	157.0 ± 14.5[b]	208.0 ± 14.5[c]

[a] within row means without a common superscript differ ($P \leq 0.0095$)

Basal lactate concentration was not influenced by the different dietary K levels (*P* = 0.1936; Table 4.9), adrenaline challenge (*P* = 0.8005), or diet × insulin challenge (*P* = 0.9320). There were no influences of or dietary K concentration (*P* = 0.08449) or diet × adrenaline challenge (*P* = 0.27204) on the time to maximum lactate concentration. However, increasing exogenous adrenaline was associated increase time to maximum lactate concentration, specifically there was a 5.62 ± 1.16 min increase in time to maximum concentration between 1.0 ug/kg LW and 5.0 ug/kg LW adrenaline challenges (*P* ≤ 0.0001). The adrenaline challenge influenced plasma lactate, where plasma lactate for the 1.0 ug/kg LW challenge had lower lactate concentrations when compared with the 5.0 ug/kg LW challenge and at times 3.0 ug/kg LW (*P* ≤ 0.0226; Table 4.10). Plasma lactate concentrations between AUC_{10} and AUC_{130} were not influenced by diet (*P* ≥ 0. 21234) or diet × adrenaline challenge (*P* ≥ 0. 88257; Table 4.9). Plasma lactate was influenced by live weight at AUC_{10} (P = 0.04322), AUC_{45} (P = 0.04322) and AUC_{60} (P = 0.04106), although this shifted towards tendency for AUC_{20} (*P* = 0.09193), AUC_{30} (*P* = 0.0588) and AUC_{130} (*P* = 0.08984).

Table 4.9. Estimated marginal means (± SE) for lactate concentration (µmole/g) in response to adrenaline challenges for steers fed three different levels of dietary potassium

Lactate Parameter	Dietary K Level, %		
	1.5	2.7	3.8
Basal Concentration	0.34 ± 0.02	0.31 ± 0.02	0.29 ± 0.02
Time to max concentration (min)	13.61 ± 1.28	12.45 ± 1.28	9.85 ± 1.18
AUC_{10}	6.46 ± 1.52	6.93 ± 1.52	7.38 ± 1.40
AUC_{20}	17.6 ± 2.74	17.7 ± 2.74	16.3 ± 2.51
AUC_{30}	27.4 ± 3.71	26.5 ± 3.71	23.0 ± 3.40
AUC_{45}	37.8 ± 4.85	35.5 ± 4.85	29.6 ± 4.44
AUC_{60}	44.1 ± 5.67	41.2 ± 5.67	33.3 ± 5.19
AUC_{130}	53.8 ± 7.42	51.9 ± 7.41	37.8 ± 6.79

Table 4.10. Estimated marginal means (± SE) for plasma lactate measures for the adrenaline challenges (ug/kg LW) in steers fed three different levels of dietary potassium

Lactate Parameter	Adrenaline Challenge (ug/kg LW)		
	1.0	3.0	5.0
Basal Concentration	0.31 ± 0.02	0.33 ± 0.02	0.31 ± 0.02
AUC_{10}	4.49 ± 1.23^{a}	7.44 ± 1.23^{ab}	8.84 ± 1.23^{b}
AUC_{20}	9.61 ± 2.15^{a}	19.32 ± 2.15^{b}	22.68 ± 2.15^{b}
AUC_{30}	13.2 ± 2.90^{a}	29.0 ± 2.90^{b}	34.7 ± 2.90^{b}
AUC_{45}	16.5 ± 3.81^{a}	38.9 ± 3.81^{b}	47.5 ± 3.81^{b}
AUC_{60}	18.4 ± 4.47^{a}	44.8 ± 4.47^{b}	55.5 ± 4.47^{b}
AUC_{130}	21.9 ± 5.64^{a}	54.0 ± 5.64^{b}	67.6 ± 5.64^{b}

$^{a-b}$ within row means without a common superscript differ (*P* ≤ 0.0226)

Similarly, there were no differences in the basal NEFA concentrations, or the time to maximum NEFA concentration across the three dietary K levels (Table 4.11). Nor were there dietary K level influences on plasma NEFA concentrations between AUC_{10} and AUC_{130} were not influenced by diet (*P* ≥ 0.836; Table 4.11), live weight (*P* ≥ 0.54919) or diet × adrenaline challenge (*P* ≥ 0.28978). However adrenaline challenge influenced NEFA AUC regardless of time point (Table 4.12), where NEFA concentrations increased as the exogenous adrenaline challenge increased (Table 4.12).

Table 4.11. Estimated marginal means (± SE) for non-esterified fatty acids (NEFA) lactate measures in response to adrenaline challenges for steers fed three different levels of dietary potassium

NEFA Parameter	Dietary K Level, %		
	1.5	2.7	3.8
Basal Concentration	0.11 ± 0.02	0.12 ± 0.02	0.09 ± 0.02
Time to max concentration (min)	10.26 ± 0.71	9.03 ± 0.71	8.18 ± 0.67
AUC_{10}	1.68 ± 0.33	1.53 ± 0.33	1.75 ± 0.30

AUC_{20}	4.05 ± 0.81	3.54 ± 0.81	3.75 ± 0.75
AUC_{30}	5.42 ± 1.12	4.60 ± 1.11	4.79 ± 1.02
AUC_{45}	6.21 ± 1.33	5.16 ± 1.33	5.37 ± 1.22
AUC_{60}	6.38 ± 1.43	5.27 ± 1.43	5.52 ± 1.31
AUC_{130}	5.96 ± 1.81	4.90 ± 1.81	5.32 ± 1.66

Table 4.12 Estimated marginal means (± SE) for non-esterified fatty acids (NEFA) for the adrenaline challenges (ug/kg LW) in steers fed three different levels of dietary potassium

NEFA Parameter	Adrenaline Challenge (ug/kg LW)		
	1.0	3.0	5.0
Basal Concentration	0.09 ± 0.02	0.11 ± 0.02	0.11 ± 0.11
AUC_{10}	1.41 ± 0.23	1.61 ± 0.23	1.98 ± 0.23
AUC_{20}	2.76 ± 0.55[a]	3.74 ± 0.55[ab]	4.87 ± 0.55[b]
AUC_{30}	3.38 ± 0.76[a]	4.85 ± 0.76[ab]	6.58 ± 0.76[b]
AUC_{45}	3.71 ± 0.92[a]	5.43 ± 0.92[ab]	7.60 ± 0.92[b]
AUC_{60}	3.76 ± 1.00[a]	5.55 ± 1.00[ab]	7.86 ± 1.00[b]
AUC_{130}	3.44 ± 1.36[a]	5.20 ± 1.36[ab]	7.53 ± 1.36[b]

[a-b] within row means without a common superscript differ ($P \leq 0.05$)

4.3.5 Muscle glycogen and lactate

Muscle glycogen and lactate concentration in the LT, ST and SM were not influenced by diet ($P \geq 0.4219$) or diet × biopsy ($P \geq 0.50$). Muscle glycogen concentrations of the LT were influenced by biopsy ($P < 0.0001$), where muscle glycogen decreased by 0.35 g/100g ($P = 0.02$), 0.33 g/100g ($P = 0.02$) and 0.48 g/100g ($P = 0.0004$) between biopsy collection 1 and 4 within the 1.5 %, 2.7 % and 3.8 % K diets respectively (Table 4.13). Similarly, muscle glycogen concentrations of the ST and SM decreased between biopsy 1 and 4, regardless of dietary K ($P < 0.0001$; Table 4.13). Muscle lactate concentration in the LT, SM and ST also decreased between biopsy 1 and 4 (Table 4.14). Within the LT lactate concentration decreased by 11.67 lactate concentration µmole/g ($P = 0.0013$), 14.01 µmole/g ($P < 0.0001$) and 13.87 µmole/g ($P = 0.0001$) within the 1.5 %, 2.7 % and 3.8 % K diets respectively, between biopsy collection 1 and 4 (Table 4.14). Similar declines between biopsy 1 and 4 were also observed in the SM (12 µmole/g) and ST (11.8 µmole/g; Table 4.14).

Table 4.13 Muscle glycogen (g/100g) concentrations for the *Longissimus thoracis* (LT), *Semitendinosus* (ST), *Semimembranosus* (SM) from steers fed three different levels of dietary potassium across the four muscle biopsy samples

Muscle	Dietary K, %	Biopsy			
		1	2	3	4
LT	1.5	1.48 ± 0.08	1.43 ± 0.07	1.10 ± 0.08	1.13 ± 0.08
	2.7	1.46 ± 0.07	1.23 ± 0.07	1.09 ± 0.07	1.13 ± 0.07
	3.8	1.45 ± 0.08	1.22 ± 0.75	1.09 ± 0.08	0.97 ± 0.08
ST	1.5	1.48 ± 0.07	0.93 ± 0.07	0.95 ± 0.07	1.07 ±0.07
	2.7	1.50 ± 0.06	1.01 ± 0.06	1.03 ± 0.06	1.27 ± 0.06
	3.8	1.60 ± 0.07	0.98 ± 0.07	0.98 ± 0.07	1.13 ± 0.07
SM	1.5	1.95 ± 0.08	1.11 ± 0.08	1.23 ± 0.08	1.41 ± 0.08
	2.7	2.01 ± 0.07	1.17 ± 0.07	1.27 ± 0.07	1.52 ± 0.07
	3.8	1.96 ± 0.08	1.08 ± 0.08	1.17 ± 0.08	1.40 ± 0.08

Table 4.14. Muscle lactate (µmole/g) concentrations for the *Longissimus thoracis* (LT), *Semitendinosus* (ST), *Semimembranosus* (SM) from steers fed three different levels of dietary potassium at four different biopsy times

Muscle	Dietary K, %	Biopsy			
		1	2	3	4
LT	1.5	32.2 ± 2.01	29.2 ± 2.01	20.9 ± 2.01	20.6 ± 2.01
	2.7	29.4 ± 1.86	33.0 ± 1.86	21.3 ± 1.86	19.0 ± 1.86
	3.8	31.3 ± 2.01	30.2 ± 2.01	19.6 ± 2.01	17.4 ± 2.01

	1.5	29.7 ± 2.16	29.0 ± 2.16	34.7 ± 2.16	19.1 ± 2.16
ST	**2.7**	32.4 ± 2.00	26.7 ± 2.00	33.4 ± 2.00	18.9 ± 2.00
	3.8	32.2 ± 2.16	28.1 ± 2.16	31.3 ± 2.16	20.7 ± 2.16
	1.5	32.2 ± 2.01	29.2 ± 2.01	20.9 ± 2.01	20.6 ± 2.01
SM	**2.7**	29.4 ± 1.86	33.0 ± 1.86	21.3 ± 1.86	19.0 ± 1.86
	3.8	31.3 ± 2.01	30.2 ± 2.01	19.6 ± 2.01	17.4 ± 2.01

4.4 Discussion

Increasing dietary K had no impact on insulin sensitivity, insulin responsiveness, glucose disappearance rates, adrenaline sensitivity or glycogen metabolism during and after an exercise challenge, rejecting all the initial hypotheses. The results differ from other findings in literature where high levels of K in ruminant diets affect insulin and glucagon. Lentz *et al.* (1976) reported that cattle with elevated plasma K levels had elevated insulin levels, suggesting reduced insulin sensitivity, which will subsequently reduce glycogenesis in the post-prandial period. Animals in the study by Lentz *et al.* (1976) also experienced a drop in the plasma levels of Ca and Mg, suggesting that prolonged levels of increased dietary K could accelerate the progression of metabolic disorders, causing production losses due to altered animal behaviour, lethargy and reduced feed intake.

High K diets were used in this experiment because high K intake affects the absorption of Mg in grazing animals (Newton *et al.* 1972; Lentz *et al.* 1976; Parkinson *et al.* 2010). Potassium-rich diets cause the potential difference across the rumen mucosa to increase, decreasing the apparent absorption of Mg (Jittakhot *et al.* 2004). High K and low Mg initiates the metabolic disorder commonly called grass tetany (Metson *et al.* 1966) causing uncontrolled spasmodic muscle contractions that increase glycogenolysis, potentially reducing muscle glycogen pre-slaughter. This well known consequence of high K was not replicated in this experiment.

Varying the concentrations of K in the diets of these cattle enough to generate a grass tetany index score of 2.32 appears to have not generated subclinical hypomagnesaemia because neither glycogenesis and glycogenolysis was affected by the dietary treatment. Low Mg has been previously shown to reduce insulin secretion and peripheral tissue sensitivity to insulin (Lentz *et al.* 1976; Miller *et al.* 1980; Matsunobu *et al.* 1990; Suárez *et al.* 1995), decreasing glycogenesis in the post prandial period, however this could not be replicated using the diets formulated for this experiment. Suárez *et al.* (1995) proposed that the reduction in insulin sensitivity and glucose transport is partially due to decreased plasma Mg impairing tyrosine kinase activity thus interfering with the insulin signaling mechanism. Achmadi *et al.* (2001) also showed that cold weather (0 ^{o}C , for 7 days) also decreased plasma Mg levels when they were fed a high K, low Mg diet. This also led to lower tissue responsiveness to insulin (Achmadi *et al.* 2001). Low circulating Mg concentrations in plasma also cause an increased stress response and increased muscle contraction (Ebel and Günther 1980; Schonewille 2013) increasing the rates of glycogenolysis during the moderately stressful pre-slaughter period. Interestingly non-stress induced exercise, i.e. exercise without endogenous adrenaline, reduced muscle glycogen in locomotor muscles (SM and ST) but was not associated with decreased muscle glycogen in postural muscles (LT). This suggests that there is a systemic regulation of glycogen usage throughout the body in response to differing stimuli, thus further studies investigating the impact various stimuli and subsequent muscle glycogen usage across muscles and the relationship on the incidence of dark cutting could be beneficial.

The results from this experiment suggest that the cattle were not actually in a state of hypomagnesaemia. Hypomagnesaemia in grazing beef cattle originate because of a) low pasture mineral concentrations; b) insufficient intake of pastures to meet mineral needs; or c) reduced mineral absorption from the gastrointestinal tract due to various factors. The mineral concentrations, energy and protein levels in the formulated diets were similar to that measured in lush, rapidly growing pastures, however they were fed to the cattle via chaff and pelleted rations.

These rations had high dry matter content which differs vastly from rapidly growing pastures which have very low dry matter content. The high water content in pastures serves to increase both rumen passage rates (Pasha *et al.* 1994; Forbes 1995) and the physical bulk of pasture an animal must consume on a daily basis in order to meet its nutrient needs. Due to the high dry matter content of the feeds fed in this experiment, the rumen passage rates were much slower than those observed in pasture fed cattle, possibly leading to increased or maximised levels of magnesium absorption from the diets. This could be a possible explanation why there was no hypomagnesaemia. The other possible reason why the cattle did not have hypomagnesaemia could be because they were fed *ad-libitum*. The supply of pasture to grazing stock is variable and is influenced by stocking rates, supplementation strategies, fertiliser use and soil temperature and moisture. Low pasture availability or FOO reduces daily feed intakes of cattle and therefore reduces the total quantity of minerals that the animals consume. Additionally, in this experiment feed intakes were not limited, hence sufficient mineral quantities were possibly consumed.

The cattle for this experiment were in individual pens which is vastly different to cattle grazing. Cattle in pens expend far less energy compared to grazing cattle as they do not need to walk around foraging for feed, walking to water or generate heat energy as they are not outside in the elements. The formulated rations for this experiment were very similar in metabolisable energy (11.62 to 11.71 MJ ME/kg DM) to lush green pastures, however the maintenance energy requirements of pen animals is far lower. Hence, these levels of ME may have also been significant enough to overshadow any possible effects of high K or hypomagnesaemia.

4.5 Conclusions

This experiment has shown that under highly controlled conditions that increasing dietary K did not influence insulin sensitivity, insulin responsiveness, glucose disappearance rates or adrenaline sensitivity. In addition glycogen metabolism was not altered before or after exercising in the LD, although muscle glycogen was reduced in the SM and ST post-exercise. Therefore results reject the hypotheses where it was anticipated that increasing the dietary K will reduce insulin sensitivity, insulin responsiveness and increase adrenaline sensitivity in these cattle. Further investigation into the effect of pasture composition on hypomagnesaemia and subsequent dark cutting in grazing cattle is warranted.

5 Impact of short term Magnesium (Mg) supplementation prior to slaughter in pasture finished beef

Project Objective 3. Determine the effectiveness of commercially available Mg supplements (mineral supplements) to reduce the impact of low Mg levels on dark cutting rates and whole farm profitability in southern grass fed production systems

5.1 Introduction

Recently, Loudon *et al.* (2018) demonstrated that there was an increase in risk of dark cutting in cattle grazing perennial grass dominant pastures with magnesium (**Mg**) concentrations ≤ 0.24 % dry matter (**DM**). Cattle grazing temperate pastures during winter and early spring are at risk of hypomagnesaemia (particularly in lactating cows from 4-8 years old that are unable to mobilise Mg from bones) as the pasture composition is typically grass dominant and the sward is rapidly growing, short (< 3 leaf), with a high water content. In addition, it also frequently has a high protein and potassium (**K**) concentration, but low in Mg and calcium (**Ca**) concentrations (Schonewille 2013). Magnesium is essential for glucose metabolism as an enzymatic cofactor for all major metabolic pathways, particularly those utilising high energy phosphate bonds, and through its action as a secondary messenger for insulin (Heaton and Elie 1984; Paolisso *et al.* 1990). As the physiological antagonist to Ca, Mg regulates synaptic transmission in the central nervous system and motor end plates by reducing stimulus-secretion coupling of acetylcholine at the adrenal medulla and excitation-coupling in the peripheral nerve terminals (Douglas and Rubin 1963). Magnesium is pivotal in modulating the body's responsiveness to adrenergic stress by reducing the output of catecholamines (Douglas and Rubin 1963; Kietzmann and Jablonski 1985; Smetana *et al.* 1995) and is associated with depressing neuromuscular stimulation (Johnson 2001). Thus, Mg has an essential role in the regulation of muscle glycogenolysis. Previous studies have identified that Mg supplementation prior to slaughter improve muscle glycogen and meat quality, in pigs (D'Souza *et al.* 1998) and sheep (Gardner *et al.* 2001a). Furthermore, Mg supplementation has been shown to reduce glycogenolysis in sheep during periods of cold stress (Terashima *et al.* 1983). No hormonal mechanism directly controls Mg homeostasis, thus plasma Mg concentration is dependent on alimentary absorption, renal excretion, tissue requirements and endogenous losses, thus daily intake is required (Martens and Schweigel 2000). Therefore, the aim of this experiment was to examine i) the influence of Mg supplementation on muscle glycogen concentration; and ii) the potential role of Mg supplementation in reducing the incidence of dark cutting in grass-fed cattle.

5.2 Materials and Methods

This experiment was conducted over two periods (Season): Season 1, August to September 2016; and Season 2, May to July 2017. All cattle were part of the Australian Pasture-fed Cattle Assurance System which has a mandatory requirement that all cattle have continuous access to graze open pastures and at no point in their life are fed cereal grains or cereal by products. The experiment was conducted with the approval of the Murdoch University's animal ethics committee (R2693; Appendix 2).

5.2.1 Animals

Commercial producers were approached to source groups of finishing cattle, containing 30 animals or more, which could be split and held in two separate paddocks of similar pasture quality and quantity in the last 14 days prior to slaughter. At the commencement of the experiment cattle were randomly split from stratified weight groups into a control or a Mg supplement group (described below). For each group breed, average age and sex were recorded (Table 5.1). A total of 1075 *Bos taurus* cattle were sourced from commercial properties on King Island, Tasmania. The

cattle were ≤ 24 months of age; either heifer or steers and predominately consisted of British breeds (Angus, Hereford & Murray Grey).

Table 5.1: Cattle consignment groups identifying cohort sex, producer, slaughter group, pellet batch and number of cattle allocated to the Magnesium (Mg) supplement and control treatments

Year	Consignment group	Sex	Producer	Slaughter group	Pellet batch	Treatment	
						Magnesium (n = 563)	Control (n = 512)
2016	1	Steer	A	1	1	22	21
	2	Heifer	B	2	1	41	43
	3	Heifer	C	2	1	15	14
	4	Steer	D	2	1	24	24
	5	Mixed	E	3	1	20	19
	6	Steer	F	4	2	19	15
	7	Steer	C	4	2	16	14
	8	Heifer	B	5	2	30	32
	9	Steer	G	5	2	21	17
	10	Steer	H	5	2	21	17
2017	11	Steer	A	6	3	18	18
	12	Steer	I	6	3	21	21
	13	Heifer	I	7	3	43	44
	14	Mixed	J	7	3	19	20
	15	Heifer	K	7	3	24	20
	16	Steer	L	8	3	60	31
	17	Heifer	M	8	3	39	42
	18	Heifer	I	9	3	16	18
	19	Steer	I	9	3	22	24
	20	Steer	N	9	3	20	16
	21	Heifer	E	10	3	20	18
	22	Heifer	N	10	3	32	24

5.2.2 Pasture Evaluations

Paddock size (hectares), days in rotation, predominant pasture type, perennial ryegrass or fescue cultivar, obvious fungal infection in pasture and supplementary feed (type, duration of days fed) were recorded for all paddocks cattle were grazing within this experiment. Pasture samples were collected from each grazing paddock within 3 days of cattle consignment date.

Food on offer (**FOO**; kg DM/ha) calculated using the average of fifteen 0.1 m^2 quadrants. Pasture swards were cut at grazing height (3 cm, i.e. grazing height, above ground level). Quadrant selection was random and care taken to avoid high traffic areas, fence lines and watering areas. Samples were dried using a microwave method (Daines and Ingpen 1985). These data were then used to calculate DM per hectare using the following formula:

$$\frac{Dry\ weight,\ g\ DM}{Number\ of\ quadrants} = \frac{g/0.1m}{1000} \times 100\ 000 = kg\ DM/ha$$

Feed samples were also collected for forage quality analysis. Random grab samples of pasture and supplementary feed (hay/silage) were taken obtained and immediately placed on ice, vacuum packed and frozen before transportation to Murdoch University (Department of Agriculture and Food, Western Australia (DAFWA) Permit to Import a Potential Carrier which requires a permit (r72): Permit #002085). Feed samples were oven dried at 60 °C and dry matter recorded. Samples were then shipped to Dairy One Ithaca New York for quality analysis. Forage quality measures included ME, crude protein (**CP**), DM, acid detergent fibre (**ADF**), effective neutral detergent fibre

(**NDF**), in vitro true digestibility, macro and micro mineral concentrations (Mg, K, Ca, Sodium (**Na**), Chloride (**Cl**), Copper (**Cu**), Molybdenum (**Mo**)) using Near Infra-Red and Wet Chemistry (Dairy One, Ithaca, New York, USA). These data were then used to calculate the grass tetany index was calculated using the equation:

$$Grass\ tetany\ index = \frac{K\ (MEq)}{Ca\ (MEq) + Mg\ (MEq)}$$

Additionally the pasture Ca to P ratio was calculated using the equation:

$$Ca{:}\,P = \frac{Ca\ (\%\ DM)}{P\ (\%\ DM)}$$

5.2.3 Magnesium Supplementation

Cattle were supplemented with pellets containing Mg or the same pellet with no added Mg. The pellets were isoenergetic and isonitrogenous, the only difference being the Mg content (Table 5.2). There were palatability issues with the Mg supplementation pellet in 2016. To address this the Mg pellet was reformulated 3 times (Table 5.2). The breakdown of which group received which pellet batch is summarised in (Table 5.1). Consequently, the 2016 consignment group 1 had 30 g/head molasses added to both the Mg and control group daily ration and consignment groups 2 to 10 had 30 g/head of sucrose as white sugar. The pellets were offered to cattle 7 to 14 days prior to slaughter.

Table 5.2: Composition of each pellet batch[1]

Item	Magnesium Pellet Formulation			Control
	1	2	3	
Canola Meal[2]	825.0	814.5	904.0	926.0
Sugar	19.0	19.0	9.5	19.0
Limestone	19.0	19.0	9.5	2.0
Magnesium Oxide	19.0	19.0	9.5	0.0
Magnesium Sulphate	92.0	92.0	45.4	0.0
Ammonium Sulphate	0.0	0.0	0.0	27.0
Urea Granules	26.0	26.0	12.8	0.0
Calcium Sulphate	0.0	0.0	0.0	26.0
Used Cooking oil	0.0	10.0	10.0	0.0

[1]Values are indicative of ingredient composition within the diet used, g/kg
[2]Canola meal was certified non-genetically modified

Pellets were offered daily at an allocation of 500 g/head/day in 2016 and 1000 g/head/day in 2017. All pellets had a ME content of 12.5 MJ/kg DM and crude protein of 31 %. The pellet was dispensed in feed troughs in the paddocks (1500 cm × 55 cm) allowing for a minimum of 30 cm head space per animal utilising both sides of the feed trough. Magnesium oxide contains 54% Mg, and magnesium sulphate contains 10% Mg (McDowell 1996), therefore the total Mg supplementation was 9.83 grams/head/day.

5.2.4 Farm Departure and Slaughter

On the day of consignment, cattle were mustered and remained in their treatment group until slaughter. Transport utilised commercial stock trailers and a short sea voyage by ship from King Island to Devonport port, Tasmania. All cattle were trucked from farm gate to King Island port on a Sunday and loaded into their respective semi-trailers, which were then loaded onto the ship. Trucking distance between farm and port on King Island ranged between 15 and 90 km. The ship had an 8 to 10 hour transit time to Devonport port where they docked overnight, then unloaded and subsequently transport to Longford, Tasmania. Trucking distance from Devonport port to Longford

was approximately 100 km (approximately 1 hour transit time). At the abattoir cattle that were transported on the same day were slaughtered in successive lots. All cattle were slaughtered within 36 hours after departing from farm gate, therefore complying with MSA pathway requirements.

5.2.5 Carcass Evaluations

Carcasses were dressed to AUS-MEAT carcass standards and hot standard carcass weight (**HSCW**, kg) was recorded on abattoirs processing chain. Carcasses were then corralled into 'chillers' until grading. Approximately 20 hours post-slaughter carcasses were MSA and AUS-MEAT graded by qualified MSA graders (AUS-MEAT Limited 2005; Meat Standards Australia 2007). AUS-MEAT Limited (2005) carcass traits evaluated at grading included:

- Gender, steer (castrated male) versus heifer (young female);
- Eye muscle area (**EMA**, cm^3), of the *m. longissimus thoracic* using a standardised grid;
- Ultimate pH, of the *m. longissimus thoracic et lumborum* (Loin eye);
- Rib fat depth, subcutaneous fat measurement (mm) using a graduated metal ruler of the *m. longissimus thoracic et lumborum* at the quartering site (12^{th}/13^{th} rib) ;
- Ossification, ossification score is the amount of spinous process calcification of the thoracic, lumbar and sacral vertebrae. Ossification is measured on a scale between 100 and 590;
- MSA marbling score is the amount of fat between muscle fibres in the m. *longissimus thoracic et lumborum* at the quartering site;

Other measures included temperature and colour of the m. *longissimus thoracic et lumborum.* Ultimate pH was measured using a TPS WP-80 pH meter. Both pH and temperature probes were inserted into the centre of the *longissimus thoracic et lumborum* and the carcass deemed as eligible for grading if muscle temperature was < 12 °C.

The MSA grading system specifies that for MSA carcass compliance the *m. longissimus thoracic et lumborum* must have a rib fat depth of ≥ 3 mm and an pHu ≤ 5.7. Carcasses which did not comply with pHu specifications were classified as dark cutting. Dark cutting percentage was calculated for each group using the following equation:

$$\%\, DFD = \frac{number\ DFD\ carcases\ per\ group}{total\ number\ of\ cattle\ in\ group}$$

5.2.6 Plasma Magnesium Concentration

During exsanguination blood samples were collected from all animals (n = 1075) into a clean, dry stainless steel cup. A 9 mL blood sample was then transferred into a lithium heparin Vacuette® tubes (Greiner bio-one, Kremsmuenster, Austria). Each Vacuette® was inverted seven times then placed into shaved ice in a cooler box, until centrifuging. Within 2-4 hours of collection, blood samples were centrifuged at 2000 RPM for 15 min, plasma was then separated into 3 × 2mL sample vials and frozen at -20 °C until further analysis.

Plasma samples were analysed at Murdoch University for Mg concentrations. The Mg assay was performed using commercially available assay kits (Beckman, OSR6189) designed for use by an Olympus AU400 Automated Chemistry Analyser (Olympus Optical CO. Ltd, Melville, NY). The analysis was performed according to kit protocols with calibration and quality control strictly adhered to. No plasma Mg data available for animals in slaughter groups 9 and 10.

5.2.7 Muscle Biopsies and Muscle Glycogen

Approximately 40 min after slaughter a small (≈ 10 g) core sample of the *m. longissiumus thoracis* was obtained at the quartering site, the division of the carcass between the 12^{th} and 13^{th} ribs. A stainless steel drill bit (10 cm × 1.5 cm) on a hand-held electric drill was used to obtain the core sample. Core samples were denuded of fat and then placed into a 5 mL sample tube. These

samples were placed into shaved ice until all carcasses for each kill group were collected. Core samples were then stored at -20 °C for later analysis.

Core samples were then used to determine muscle glycogen, free glucose and lactate at Murdoch University on an Olympus AU 400 auto analyser (Olympus Diagnostics, Tokyo, Japan). The enzymatic method of (Chan and Exton 1976) excluding the filter paper step was used to determine muscle glycogen concentration. The glycogen homogenates were used to determine lactate concentration in muscle by the method described by (Noll 1985). To account for degradation of glycogen between exsanguination and core sampling, total muscle glycogen was calculated by the following equation:

$$Total\ Glycogen, \frac{g}{100g} = glycogen + free\ glucose + lactate$$

5.2.8 Statistical Analysis

Muscle glycogen was analysed using a linear fixed effects model within the 'lme4' package in R (Bates *et al.* 2014; R Core Team 2019). Initially a base model was established with fixed effects including supplementation group (Mg or control), Mg pellet batch (1, 2, 3), producer identification and their interactions. Random effects included consignment group, year, slaughter number and supplementation group. Pellet batch was removed as it was not significant. Within this base model, plasma Mg was then included as a covariate along with its interaction with supplementation group.

Pasture variables were also added individually as covariates to the base model. These included FOO, DM, CP, ME, ADF, NDF, dietary cation anion difference (**DCAD**), Mg, K, Ca, Na, grass tetany index and Ca:P ratio. Pasture variables were tested in the model one at a time as well as collectively. A dichotomous transformation was used to further evaluate pasture Mg, of > 0.24 % or ≤ 0.24 % (Loudon *et al.* 2018). For the Ca:P ratio a series of dichotomous levels were explored testing at intervals of 0.1 starting at 0.6 to 2.0. The grass tetany index was explored as a fixed effect, of ≤ 2.2 or >2.2.

Plasma Mg was also analysed as the dependent variable using the same model described above. Pasture variables were tested as covariates against plasma Mg singularly and simultaneously. The pasture variables were tested in the same method previously described, including continuous variables as well as the dichotomous transformation of pasture Mg, Ca:P ratio and grass tetany index.

After assessing the interaction between muscle glycogen and producer, Producer I was identified to have a positive interaction with the Mg supplementation. Producer I contributed four consignment groups, all in 2017. Therefore, data from Producer I was investigated using the same models described above.

To assess the risk of dark cutting the total number of carcasses ineligible for MSA grading (pHu >5.7) was used to give the total number of dark cutting carcasses and percent incidence of dark cutting within each consignment group and treatment. A random effects logistic regression analysis model was used to explore the effects of pasture variables on percentage of dark cutting. The dependent variable was the total number of dark cutting carcasses. Continuous variables in the model included FOO, DM, CP, ME, ADF, NDF, DCAD, Mg, K, Ca and Na. As above the dichotomous transformations of pasture Mg, Ca:P and grass tetany index was evaluated. The model included the same random effects as previously described for the muscle glycogen and plasma Mg models.

5.3 Results

From the 22 groups of cattle included in the analysis 11 groups were steers, 9 groups were heifers and two groups were mixed sex. The cattle were all spring born and age at slaughter was ≤ 24 months. All groups were rotationally grazed on *Lolium perenne* (perennial ryegrass) with *Trifolium repens* (white clover) and *Trifolium pratense* (red clover), with the exception of one group (2016-7) which was grazed on *Medicago sativa* (lucerne) and white clover. The cattle consumed all of the pellet supplementation, after the palatability issues were solved, on a daily basis with the trough refilled daily.

5.3.1 Carcass Evaluations

A total of 1068 carcasses were MSA graded (Mg supplemented, n = 556; control, n = 512). A total of 71 carcasses failed to meet MSA pHu requirements, of these 31 were from the Mg group and 40 were from the control group. This is reflected in a higher mean group percentage non-compliance in the control animals (Table 5.3). Individual group compliance data are summarised in Table 5.4. Overall, rib fat compliance was good with only four carcasses failing to meet minimum rib fat depth (≥ 3 mm), all of these carcasses were from the same consignment group (Table 5.3).

Table 5.3: Meat Standards Australia (MSA) carcass compliance based on ultimate pH (pHu) and rib fat depth for cattle supplemented with Magnesium (Mg) and not supplemented (Control)

Item	Treatment	
	Control (n = 512)	Magnesium (n = 556)
pH compliant (pHu ≤ 5.7)	472 (92.2%)	525 (94.4%)
pH non-compliant (pHu >5.7)	40 (7.8%)	31 (5.6%)
Rib fat compliant (≥3mm)	511 (99.8%)	553 (99.5%)
Rib fat non-compliant (<3mm)	1 (0.2%)	3 (0.5%)

Table 5.4: Summary of the number of cattle, food on offer (kg DM/ha) and incidence of dark cutting per consignment group

Year	Consignment group	Number of cattle per consignment group		Food on offer (kg DM/ha)		Dark Cutting (pHu >5.7)	
		Magnesium (n = 561)	Control (n = 512)	Magnesium (n = 512)	Control (n = 512)	Magnesium (n = 561)	Control (n = 512)
2016	1	22	21	650	600	0.0	0.0
	2	41	43	1300	1300	0.0	2.3
	3	15	14	1150	1100	6.7	7.1
	4	24	24	1000	1000	0.0	0.0
	5	20	19	1800	1850	0.0	15.8
	6	19	15	1500	1500	5.3	0.0
	7	16	14	1100	1000	0.0	7.1
	8	30	32	1200	1250	6.7	6.25
	9	21	17	800	850	0.0	5.9
	10	21	17	900	800	0.0	4.8
2017	11	18	18	442	325	0.0	0.0
	12	21	21	495	489	9.5	28.6
	13	43	44	486	362	4.7	11.4
	14	19	20	440	445	0.0	0.0
	15	24	20	380	338	5.0	0.0
	16	60	31	410	390	15.0	6.5
	17	39	42	405	481	10.3	2.4
	18	16	18	442	428	0.0	16.7
	19	20	24	N/A	N/A	6.3	15.0
	20	20	16	423	440	20.0	16.7

21	20	18	835	1035	10.0	27.8
22	32	24	472	439	6.25	4.2

A summary of the descriptive statistics for the MSA carcass grading data, plasma Mg and muscle glycogen for the treatment groups is provided below in Table 5.5. Carcass characteristics were similar for the treatment groups, however rib fat depth was greater in the control group (p = 0.016) although had a smaller range in the Mg supplemented cattle (Table 5.5). Additionally, EMA and P8 fat depth tended to be lower in Mg supplemented cattle ($p \leq 0.103$).

There were no statistically significant differences in mean muscle glycogen between the two treatment groups ($p = 0.584$). A total of 804 carcasses had plasma Mg concentrations (Mg supplemented, n = 423; control, n = 381). Plasma Mg concentrations were greater in cattle supplemented with Mg ($p = 0.006$).

Table 5.5: MSA grading characteristics, plasma magnesium and muscle glycogen for cattle supplemented with Magnesium (Mg) and not supplemented (Control)

Item	Control (n=512)			Magnesium (n=556)			Significance[1]
	Range	Mean ± SD	Median	Range	Mean ± SD	Median	
Carcass weight, kg	214 - 428	294 ± 35	295	209 - 396	295 ± 35	295	0.728
P8 Fat depth, mm	4.0 – 30.0	10.7 ± 4.6	10	4.0 – 41.0	10.3 ± 4.7	10.0	0.103
Eye Muscle Area, cm^3	40.0 – 86.0	75.2 ± 3.5	76.0	60.0 – 81.0	74.8 ± 3.7	76.0	0.101
Rib fat depth, mm	2.0 – 55.0	9.2 ± 4.7	8.0	2.0 – 40.0	8.6 ± 4.2	8.0	0.016
Ossification score	110 – 590	160 ± 32	160	120 – 500	158 ± 28	160	0.143
MSA Marble Score	150 – 680	345 ± 87	330	160 – 680	344 ± 87	320	0.810
Plasma Mg[2], mmol/L	0.28 – 1.42	0.93 ± 0.14	0.93	0.41 – 1.61	0.96 ± 0.15	0.95	0.006
Muscle glycogen, g/100g	0.38 – 2.07	1.15 ± 0.24	1.14	0.44 – 1.84	1.15 ± 0.23	1.15	0.584

[1]The p-values for the numeric variables are (two tailed) two sample t-tests.

[2]Plasma Mg had the following missing samples from each group: Control, n = 131 (25.6%); Magnesium, n = 133 (23.9%)

5.3.2 Pasture Evaluations

Pasture data were available for 21 of the 22 consignment groups. There were no pasture data available for consignment group 19. There was no significant difference between FOO between Mg and control treatment groups (Table 5.6). However, there was a large range of availability among groups particularly between the years where FOO was markedly lower in 2017 than 2016 (Table 5.4). The pasture feed quality analysis demonstrated that Mg treatment and control cattle were grazing pastures similar pasture in quality composition (Table 5.6). In addition, there were no differences between the pasture mineral content for cattle in the Mg or control treatment groups (Table 5.6). Overall 25 of 42 pastures (59.5%) returned Ca concentrations less than 0.43% DM. On average the Ca:P for both groups was 0.88, however 30 of 42 pastures (71.4%) had inverted < 1:1 ratios of Ca:P. Grass Tetany Index means were less than 2.2 (MEq), however 13 of 42 pastures (31.0%) had levels greater than 2.2.

Table 5.6: The range, mean, standard deviation and median of pasture quality and mineral concentration

Variable	Control groups (n=21)			Magnesium groups (n=21)			Significance
	Range	Mean ± SD	Median	Range	Mean ± SD	Median	
Food on offer (kg DM/ha)	325 – 1800	787 ± 435	600	380 – 1850	790 ± 426	650	0.984
Metabolisable Energy (% DM)	8.8 – 11.5	10.5 ± 0.6	10.6	9.0 – 11.8	10.5 ± 0.6	10.5	0.796
Crude Protein (% DM)	16.9 – 29.8	24.8 ± 3.3	25.3	16.9 – 34.4	25.0 ± 3.8	24.8	0.825
Dry Matter (% DM)	12.1 – 21.6	17.7 ± 2.6	18.5	12.1 – 21.5	17.5 ± 2.6	17.5	0.772
Acid Detergent Fibre (% DM)	23.7 – 39.0	29.0 ± 3.4	29.3	23.4 – 36.9	29.4 ± 3.1	29.6	0.680
aNeutral Detergent Fibre (% DM)	39.8 – 61.6	47.2 ± 4.7	46.6	40.5 – 60.0	47.5 ± 4.4	47.0	0.850
Magnesium (% DM)	0.14 – 0.30	0.20 ± 0.03	0.20	0.15 – 0.30	0.21 ± 0.03	0.20	0.415
Calcium (% DM)	0.20 – 0.64	0.41 ± 0.12	0.40	0.20 – 0.91	0.43 ± 0.17	0.40	0.589
Potassium (% DM)	1.48 – 3.60	2.79 ± 0.51	2.80	1.52 – 4.40	2.92 ± 0.59	3.02	0.436
Sodium (% DM)	0.12 – 0.90	0.41 ± 0.15	0.40	1.52 – 4.40	2.92 ± 0.59	3.02	0.818
Phosphorus (% DM)	0.21 – 0.60	0.49 ± 0.11	0.50	0.26 – 0.66	0.51 ± 0.10	0.50	0.624
Iron (PPM)	87 – 1570	335 ± 368	155	92 – 1440	356 ± 361	194	0.856
Zinc (PPM)	11 – 31	23 ± 4	25	16 – 43	24 ± 5	23	0.690
Copper (PPM)	3 – 123	15 ± 27	6	4 – 141	18 ± 35	8	0.765
Manganese (PPM)	38 – 149	80 ± 28	73	35 – 148	68 ± 27	62	0.197
Molybdenum (PPM)	0.8 – 5.1	2.0 ± 1.2	1.7	0.3 – 5.2	2.3 ± 1.3	1.9	0.456
Sulphur (%DM)	0.11 – 0.54	0.34 ± 0.09	0.33	0.23 – 0.50	0.36 ± 0.07	0.34	0.567
Chloride (%DM)	1.31 – 2.60	1.80 ± 0.32	1.79	1.49 – 2.30	1.86 ± 0.25	1.90	0.532
Grass Tetany Index (MEq)	1.24 – 2.60	1.97 ± 0.26	2.05	0.62 – 3.40	2.03 ± 0.53	2.07	0.684
Calcium:Phosphorus Ratio (% DM)	0.50 – 1.60	0.88 ± 0.30	0.80	0.30 – 2.50	0.88 ± 0.48	0.80	1.000
Dietary cation anion difference	-67.40 – 384.39	166.36 ± 98.91	166.00	-18.10 – 343.58	183.65 ± 80.50	181.00	0.589

5.3.3 Multivariate Analysis

Magnesium supplementation increased muscle glycogen on some producer farms (p = 0.032). A summary of the influence of Mg supplementation on muscle glycogen for each producer is described below (Table 5.7). There were two producers (producer E and producer I) where cattle supplemented with Mg had an increased muscle glycogen ($p \leq 0.01$; Table 5.7). Magnesium supplemented group for Producer E was associated with a 0.119 g/100g increase in muscle glycogen (p = 0.011). For Producer I, Mg supplementation increased muscle glycogen by 0.09 g/100g when compared with the control cattle ($p < 0.001$; Figure 1). Pasture data was only available for 3 of the 4 consignment groups for Producer I, however the pasture mineral results demonstrated deficiencies (Table 5.8) as defined by NRC (2016).

Table 5.7: Pairwise contrast table of the effect of magnesium supplementation on muscle glycogen by producer. Values in bold represent significant differences to the 95% confidence level.

Producer	Number of Consignments	muscle glycogen (g/100g)	SE	t. ratio	*P* value
A	2	-0.066	0.046	1.433	0.152
B	2	-0.031	0.034	0.916	0.360
C	2	0.072	0.053	-1.357	0.170
D	1	0.006	0.046	-0.124	0.901
E	**2**	**0.119**	**0.047**	**-2.546**	**0.011**
F	1	-0.091	0.071	1.270	0.204
G	1	0.000	0.067	0.002	0.998
H	1	0.016	0.067	-0.243	0.808
I	**4**	**0.093**	**0.029**	**-3.237**	**0.001**
J	1	-0.079	0.066	1.206	0.228
K	1	-0.040	0.064	0.625	0.532
L	1	-0.010	0.047	0.209	0.834
M	1	0.028	0.059	-0.470	0.639
N	2	0.015	0.041	-0.362	0.717

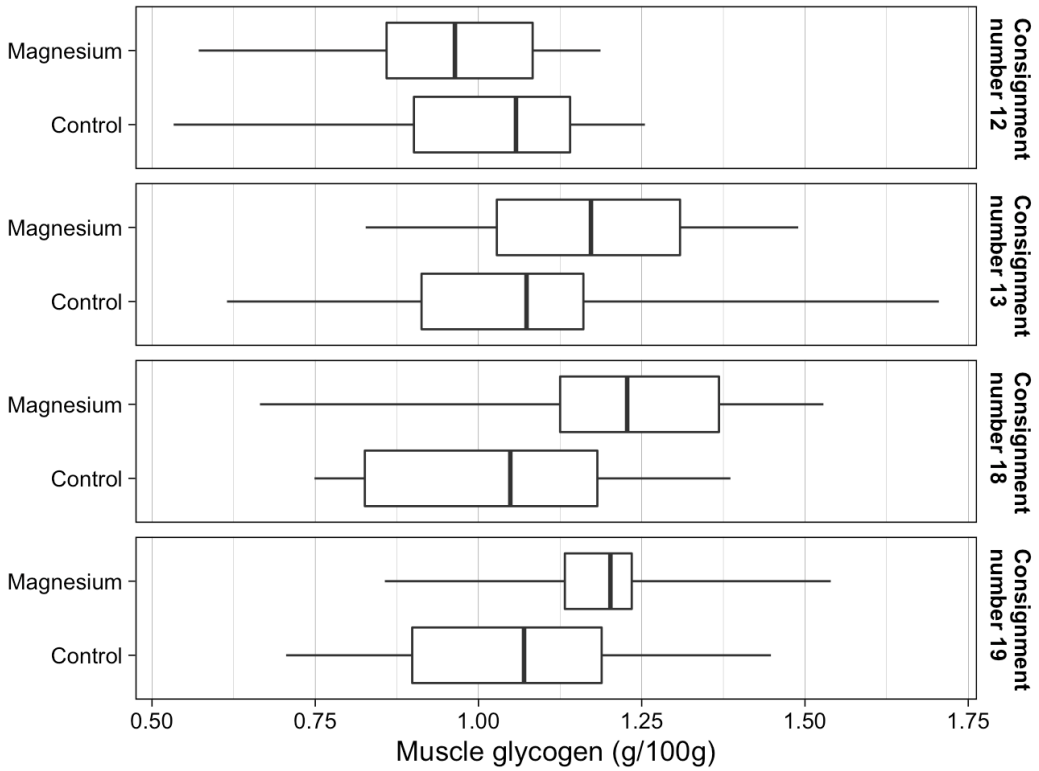

Figure 5.1: Box plot depicting difference in muscle glycogen concentration (g/100g) of +Magnesium and Control consignments for Producer I

Table 5.8: Pasture mineral concentrations for Producer I

Consignment Group	Treatment	FOO	DM %	Magnesium (%DM)	Potassium (%DM)	Ca:P	Grass Tetany Index (MEq)
12	Magnesium	495	21.5	0.2	2.5	1.0	1.9
	Control	489	19.1	0.2	3.1	0.6	2.3
13	Magnesium	486	17.3	0.2	3.1	0.6	2.1
	Control	362	19.6	0.2	2.6	0.6	2.2
18	Magnesium	442	20.1	0.2	3.2	0.6	2.6
	Control	428	20.1	0.2	1.5	1.2	1.5

Magnesium supplementation increased plasma Mg concentration for four producers: producer E, producer I, producer B and producer L ($p \leq 0.02$; Table 5.9). Plasma Mg data was not available for cattle in consignment groups 9 and 10. The Mg supplementation was associated with plasma Mg increase of 0.078 mmol/L, 0.080 mmol/L, 0.060 mmol/L and 0.093 mmol/L for Producer B, Producer E, Producer I, and Producer L, respectively. Pasture quality and mineral components did not influence plasma Mg, even when adjusting for paddock and producer. The FOO had a tendency to increase muscle glycogen by 0.014 g/100g for each 100 kg increase in DM/hectare ($p = 0.06$).

Table 5.9: Pairwise contrast of the effect of Magnesium supplementation on plasma magnesium (mmol/L). Values in bold represent significant differences to the 95% confidence level

Producer	Number of Consignments	Plasma Mg (mmol/L)	SE	t. ratio	P value
A	2	0.000	0.030	0.015	0.988
B	**2**	**0.078**	**0.019**	**-4.125**	**<0.001**
C	2	0.041	0.030	-1.358	0.175
D	1	-0.006	0.033	0.177	0.860
E	**1**	**0.080**	**0.037**	**-2.194**	**0.029**
F	1	-0.052	0.040	1.305	0.192
G	1	-0.003	0.037	0.083	0.934
H	1	-0.057	0.037	1.529	0.127
I	**2**	**0.060**	**0.022**	**-2.719**	**0.007**
J	1	0.026	0.037	-0.708	0.479
K	1	-0.073	0.040	1.832	0.067
L	**1**	**0.093**	**0.026**	**-3.543**	**<0.001**
M	1	-0.030	0.026	1.159	0.247

5.4 Discussion

Supplementing cattle with increased dietary Mg did not influence overall muscle glycogen concentration or incidence of dark cutting. The lack of improvement in pHu and dark cutting in this experiment differs from previous Mg supplementation studies in other species. Specifically, Gardner *et al.* (2001b) demonstrated that pasture raised Merino sheep supplemented with a concentrate ration containing added magnesium oxide at 1 % DM during the four days prior to slaughter, increased muscle glycogen concentration and decreased pHu. However, Gardner *et al.* (2001b) had no recorded pasture quality or mineral data, thus the hypomagnesaemia status of these animals cannot be established. Pigs supplemented with Mg for five days prior to slaughter has been associated with improved pHu, meat quality and plasma Mg concentrations (D'Souza *et al.* 1998; Lahucky *et al.* 2004a; Lahucky *et al.* 2004b) and decreased plasma adrenaline (D'Souza *et al.* 1998). However, it has also been suggested that the apparent digestibility of Mg is lower in cattle compared with sheep (Greene *et al.* 1983a; Greene *et al.* 1983b; Schonewille 2013), which in part may explain some of the differences the supplementation results presented here and those in sheep (Gardner *et al.* 2001b). It is unclear as to the mechanism of action behind the contrasting response of Mg supplementation and meat quality traits across studies. There may be differing genetic sensitivity between and within species. Gardner *et al.* (1999) suggested that Merinos have a greater stress sensitivity, as there was a large difference in the muscle glycogen depot between Merino and second

cross Merino lambs when subjected to pre-slaughter stress. Additionally, magnesium oxide supplementation was only able to offset approximately half the glycogen loss in the second cross lambs (Gardner *et al.* 2001a). Whilst the cattle within this experiment had similar backgrounds there may have been differing genetic lines that may contribute to poor temperaments, thus increasing the sensitivity to stress in these animals. Furthermore, there is the potential that the contrasting results across studies may be due to differing apparent availability of Mg offered. Other factors that could influence the response include the high K concentrations in pasture and the markedly inverted Ca:P ratio of the pastures in this experiment.

The intrinsic Mg concentration in the diet does not provide an accurate indication of an animals risk of developing hypomagnesaemia, as there are several factors that influence rumen Mg solubility and transport across the ruminal epithelium (Martens and Rayssiguier 1980). There are numerous reviews that discuss the regulation of Mg homeostasis in cattle (Martens and Rayssiguier 1980; Martens and Schweigel 2000; Schonewille 2013; Martens *et al.* 2018). However, these reviews suggest that DM availability and concentrations of Mg and K are of major importance for pasture fed cattle, particularly during winter and early spring. Dietary Mg absorption rates are variable with ranges as low as 12.1 % in 100 % grass-fed diets, increasing to 21.5 % for diets containing 40 % grass and 60 % concentrate (Schonewille *et al.* 2002). Magnesium absorption across the rumen mucosa can occur via two transport mechanisms, 1) is sensitive to ruminal K concentration, and 2) is insensitive to K concentration (Martens and Rayssiguier 1980; Leonhard *et al.* 1989; Suttle 2010). Within the K sensitive pathway, or potential difference dependent pathway, Mg uptake is driven by the electrochemical gradient across the rumen mucosa apical membrane (Martens *et al.* 1991; Suttle 2010). Increasing ruminal K concentration depolarises the apical membrane potential, increasing the transmural potential difference, thus decreasing Mg uptake (Leonhard *et al.* 1989). This pathway has the ability to absorb Mg from the rumen when luminal concentrations are low, therefore becomes critical at low dietary Mg intakes (Goff 2014). The second transport mechanism for Mg is the potential difference independent pathway, which is reliant on the gradients of ions and is insensitive to K concentration (Martens *et al.* 1978; Martens *et al.* 1991; Suttle 2010). With the ability to exchange one Mg^{2+} for two H^{+} ions, this pathway can become saturated at ruminal Mg concentrations greater than 4 mM (Martens *et al.* 1978; Martens *et al.* 1991; Suttle 2010). Of the pastures tested in this experiment, 92.9 % had Mg concentrations below < 0.25 % DM, thus it is anticipated that absorption occurred through the K sensitive pathway.

In feeding trials utilising rumen-fistulated sheep, Ram *et al.* (1998) demonstrated the importance of Mg level on the K inhibitory effect, where Mg absorption was reduced by 54 % at low Mg (1.3 g/kg DM) intake compared with 27 % at high Mg intake levels (3.1 g/kg DM). From a practical supplementation perspective, increasing Mg does not affect the inhibition of K, rather it increases Mg availability for absorption by the K insensitive pathway, highlighting the importance of this dual Mg absorption uptake mechanism (Ram *et al.* 1998). The pasture mineral concentrations in this experiment suggest that some of these cattle were at risk of hypomagnesaemia. However, only one Mg concentration was supplemented within this experiment and it is unclear if the concentration was sufficient to overcome dietary factors inhibiting Mg absorption. Furthermore, as Mg supplementation was not consistently associated with increased muscle glycogen, further studies to understand the prevalence pastures predisposing cattle to hypomagnesaemia and appropriate Mg supplementation rates are required. However, a supplementation rate of 9.83 grams/head/day is consistent with supplemental guidelines for beef cattle during high risk periods of 8 to 10 grams per day (Grunes and Mayland 1984).

Magnesium supplementation was associated with increased plasma Mg concentration for 4 of the 14 producers ($p \leq 0.02$; Table 5.9). The results from the univariate analysis, indicate that mean plasma Mg concentrations between the two groups were significantly different, suggesting that Mg

supplementation resulted in a higher plasma Mg concentration in these cattle. However, it is important to consider that the blood sampling for Mg concentrations occurred 36 to 48 hours after cattle had left the pasture supplement. Given the lability of Mg in the body, the difference in Mg concentration may have been higher if tested during the period of supplementation. There is a normal variation in plasma Mg concentration that exists and the severity of nervous disturbances that can effect this, make it difficult to interpret plasma Mg concentrations (Martens *et al.* 2018). However, reported reference ranges for plasma Mg range between 0.6 and 1.10 mmol/L, where levels < 0.8mmol/L often classified as subclinical hypomagnesaemia (Parkinson *et al.* 2010; Martens *et al.* 2018). The plasma Mg concentrations observed within this experiment suggests that some of these cattle were hypomagnesaemic. The lack of association between plasma Mg and muscle glycogen or pasture parameters on plasma Mg may reflect the inadequacies of interpreting plasma Mg, as circulating concentrations are in part genetically determined (Field and Suttle 1979). Furthermore plasma Mg concentrations have a curvi-linear response with dietary Mg uptake, thus plasma Mg does not typically change in moderate deficiencies (Martens and Schweigel 2000). Creatinine corrected urinary Mg concentration is considered a more sensitive predictor of dietary Mg uptake as it has been identified to have a linear relationship with dietary intake (Martens and Schweigel 2000; Parkinson *et al.* 2010).

The influence of FOO on muscle glycogen concentration was anticipated, as it is associated with an increased DMI in grazing animals. Pethick and Rowe (1996) demonstrated that the main factor influencing muscle glycogen concentration was ME intake. The pattern of dark cutting in pasture fed cattle in southern Australia reflects the pasture growing season and quality of feed available (Knee *et al.* 2004). Dry matter intake for grazing animals can be constrained by FOO as well as the physical and quality parameters of the pasture (Weston 2002). High water content of forage has been associated with decreased DMI, particularly when DM is < 16 % (Waghorn and Clark 2004). Reviews on dietary Mg availability have suggested that high rumen outflow rates impede Mg absorption (Goff 2014) and hypomagnesaemia prevention strategies, such as feeding hay, have been shown to reduce the risk (Harris *et al.* 1983). In addition, high K in the pasture may further increase rumen transit times. Rumen outflow in fistulated sheep was increased with a high K diet, however the high K diet did not influence the Mg concentration in the ruminal liquid phase (Ram *et al.* 1998). There appears to have been no direct studies evaluating rumen outflow rates and risk of hypomagnesaemia, therefore drawing conclusions on rumen transit times becomes difficult.

Rib fat depth was greater in the control group on univariable analysis, however was not higher on multivariable analysis. This was an unexpected finding that the control group would have less fat as the expected response was that cattle experiencing Mg deficiency would have increased rates of catecholamine driven lipolysis (Rayssiguier 1977). Increased levels of plasma non-esterified fatty acids (**NEFA**) have been demonstrated to bind plasma Mg creating a negative feedback loop (Rayssiguier 1977). The impact of Mg supplementation on carcass fatness in cattle has been variable across studies. Zinn *et al.* (1996) concluded that magnesium oxide supplementation had no effect on carcass weight, fat thickness, rib eye area or American marbling score in feedlot steers supplemented for 56 days. Whereas, Coffey and Brazle (1996) demonstrated that magnesium-mica fed to feedlot steers for 141 days markedly improved American marbling score but resulted in no difference in carcass weight or fat thickness. However, neither of these studies reported pHu or muscle glycogen data. These studies may indicate that the number of days cattle are supplemented with Mg may influence carcass fatness, particularly in feedlot cattle, however further studies would need to quantify this.

The pasture mineral analysis suggest that these cattle were at risk of Ca deficiency, which may have increased the risk of hypomagnesaemia. Subclinical hypocalcaemia in cattle results in increased plasma NEFA levels (Reinhardt *et al.* 2011; Martinez *et al.* 2014) which directly bind Mg in the blood

resulting in decreased circulating free Mg (Rayssiguier 1977; Kurstjens *et al.* 2019). The relationship of Ca and Mg is cyclical in nature with hypomagnesaemia reducing tissue responsiveness to parathyroid hormone, thus impairing Ca mobilisation and increasing risk of hypocalcaemia (Contreras *et al.* 1982; Reinhardt *et al.* 1988; Goff 2008). While there was no association of Ca levels or Ca:P ratio on muscle glycogen or risk of dark cutting, there may be wider reaching implications for meat quality with low Ca. Calcium is an essential cofactor in the calpain proteolytic system which is essential for post-mortem aging and improved tenderness (Matarneh *et al.* 2017). Pre-slaughter oral Ca supplementation to beef cattle had higher muscle Ca concentrations, increased calpain activity and improved consumer sensory scores for meat tenderness, although no effect on pHu (Duckett *et al.* 2001). Further research is required to quantify the risk of pasture mineral deficiencies on meat quality independent of pHu. Regardless, the incidence of dark cutting in this experiment was 7.1%, which is similar to reported average rates in grass fed beef cattle (Jose *et al.* 2015).

5.5 Conclusions

Short-term Mg supplementation prior to slaughter was inconsistent at improving muscle glycogen concentration and plasma Mg in groups of cattle supplied from 14 participating producers. A challenge of grazing studies is the difficulty in replicating exact forage mineral and quality parameters, thus making a comparison with other trials difficult. In addition, the complexities of Mg absorption in the ruminant and varying dietary factors impeding absorption are likely confounding these results. Further research investigating an objective measurement of detecting risk of hypomagnesaemia to finishing stock would be beneficial to industry, alongside mechanistic studies looking at mineral excretion in cattle grazing pasture with a high water content.

6 The influence of supplementing a mycotoxin binder on animal growth rates and dark cutting

Project Objective 4. Determine the ability for commercial mycotoxin binder products to reduce the impact of mycotoxins originating from pastures on animal growth rates and dark cutting rates, in addition to whole farm profitability

6.1 Introduction

It is well established that perennial pastures that are prominent throughout southern Australia have a symbiotic relationship with endophytes (Henry *et al.* 2019). These endophytes are also of commercial importance, as they produce alkaloids particularly when stressed. Subsequently, when these alkaloids are in high concentrations, they can have toxicity effects on animals when grazed (Henry *et al.* 2019). The toxic effects from endophyte toxicosis has been estimated to have an economic impact of over $ 72 million (AUD) annually, across the Australian sheep and beef industries (Sackett and Francis 2006). The toxic effects of these alkaloids is typically referred to as ryegrass staggers and can present as: increased temperamental or flighty behaviour; tremors and twitching of muscles; spasmodic movement of the distal limbs; swaying and staggering; increased stiffness and rigid tetanic extension of legs (Reed *et al.* 2011). A recent study conducted on King Island reported that there was a high prevalence of mycotoxins detected in pastures across all farms evaluated (Loudon *et al.* 2018). This is unsurprising given that the predominant pasture is perennial ryegrass that has not experienced pasture renovation within the last decade (Loudon *et al.* 2018). The authors also identified that all cattle within the study were exposed to three or more mycotoxin families and subsequently that cattle grazing pastures containing ergot alkaloid and Fumonisin-B1 increased the relative risk of dark cutting by 45% and 58%, respectively. Whilst dark cutting, is a complex multifactorial problem that is influenced by numerous pre-slaughter factors, it is predominantly associated with low muscle glycogen reserves at slaughter (Tarrant 1989). Reduced muscle glycogen at slaughter is a function of the relationship between available muscle glycogen on-farm minus the quantity used throughout the pre-slaughter period including mustering, transport and lairage (McGilchrist *et al.* 2014). Given the association between dark cutting risk and mycotoxins described by Loudon *et al.* (2018), it was anticipated that supplementing cattle with mycotoxin binders may negate the impact of mycotoxins on dark cutting prevalence via a reduced absorption in the digestive tract (Binder 2007; Vanhoutte *et al.* 2016). As such, feeding a commercially available mycotoxin binder may be a cost-effective management strategy to reduce the incidence of dark cutting from cattle grazing high risk pastures. Therefore the objectives of this experiment were to evaluate the influence of mycotoxin binders on ii) the growth performance of cattle; ii) glycogen storage and metabolism[2] and iii) the incidence of dark cutting in cattle grazing pastures containing endophytes.

6.2 Materials and Methods

This experiment was conducted with the approval of the University of New England's (UNE) animal ethics committee (AEC 19-071; Appendix 3). The experiment was undertaken on King Island, Australia.

[2] Due to the impact of COVID-19 on travel, an on farm muscle sample was unable to be collected from these cattle. A muscle sample from slaughter has been collected, however samples remain in storage in Tasmania due to logistical constraints. Pasture samples also remain on King Island due to logistic challenges of frozen transport

6.2.1 Animals

One hundred and one Black Angus, Hereford and Hereford × Angus steers (577.1 ± 28.6 kg) were used in this experiment. Upon enrolment into the experiment cattle were allocated to one of two treatments: 1) Control (n = 51) or 2) supplemented with a commercially available mycotoxin binder for 50 days prior to slaughter (binder, n = 50). Cattle in the binder cohort were offered 50 g/head/day mycotoxin binder (Mycofix ® plus, Biomin Holding GmbH, Austria). All cattle were offered 500g/hd/day of pellets (Feed Beef Hi Pro 9mm pellets), to ensure binder consumption. Control cattle were offered the same pellets, but were not supplemented with the mycotoxin binder. Supplementary feeding occurred once per day and any refusals were weighed daily. Cattle in the binder and control treatments were grazed on 34 ha and 37.5 ha of perennial pastures, respectively.

6.2.2 Transport and Slaughter

On the day of consignment, cattle were mustered and remained within their respective treatment cohorts for the duration of transport, lairage and slaughter. All cattle departing King Island were required to travel ≈ 250 km through the Bass Straight via sea voyage, therefore transporting cattle from farm to abattoir consisted of both road and sea (ship) stages. Cattle were loaded into stock crates, hauled to the port on King Island, then the crates are loaded onto the ship, then unloaded again after crossing Bass strait. All transport was conducted in accordance with the Australian Animal Welfare Standards and Guidelines - Land Transport of Livestsock (2012) and Animal Welfare Guidelines – Transport of Livestock on Bass Strait (2017). The sea voyage had an 8-10 hour transit time to Devonport port. From the port cattle were trucked approximately 100 km to Longford. Cattle were slaughtered within 36 hours after farm departure complying with MSA pathway guidelines. At the abattoir cattle were slaughtered on a single day.

6.2.3 MSA carcass characteristics

Post-chilling, carcasses were evaluated by a single accredited MSA grader 20 hours post-slaughter (Meat Standards Australia 2007). The grading data compromised of: eye muscle area (EMA, cm^2) of the *longissimus thoracic* measured at the $12/13^{th}$ rib, carcass quartering site; pH at time of grading; rib fat depth (mm) measured from the subcutaneous fat depth adjacent to the *longissimus thoracic* at the quartering site; ossification score (scale between 100 and 590) that is evaluated but the amount of spinous process calcification of the thoracic, lumbar and sacral vertebrae; and MSA marbling score (scored between100 and 1190 in increments of 10), evaluating the presence of intramuscular fat present on the surface of the *longissimus thoracic* at the quartering site. These data were then used to determine the MSA index as described by (McGilchrist *et al.* 2019) as a prediction of eating quality. Data from MSA carcass grading was used to determine dark cutting carcasses are defined as those carcass that have an ultimate pH (**pHu**) > 5.70, as per the MSA specifications.

6.2.4 Statistical analysis

All data exploration and statistical analyses were conducted in R (R Core Team 2019). Data merging and manipulation, data visualisations and summary data were conducted using the 'dplyr' (Wickham *et al.* 2019b), 'ggplot2' (Wickham *et al.* 2019a) and 'table1' (Rich 2018) packages respectively. Data were analyzed using a linear model in the 'lme4' package (Bates *et al.* 2019) and estimated marginal means were generated using the 'emmeans' package (Length 2019). Statistical significance was set at $P < 0.05$.

6.3 Results

6.3.1 Growth performance

There were no differences in the starting live weight ($P = 0.13$) between the treatments, nor did supplementing cattle with the binder influence final live weight ($P = 0.52$; Table 6.1). However, at final weighing it was determined that the control cattle had put on 34.1 ± 1.5 kg ($P < 0.0001$), at an average daily gain (ADG) of 0.68 ± 0.03 kg/day ($P < 0.0001$). In comparison, the binder cattle put on 21.6 ± 1.5 kg at an ADG of 0.43 ± 0.03 (Table 6.1).

Table 6.1. Estimated marginal means (± SE) for starting and final liveweights, change in liveweight and average daily gain for control cattle and cattle supplemented with a mycotoxin binder

Treatment	Starting live weight	Final live weight	Change in live weight	ADG
Control	573 ± 4	607 ± 4	34.1 ± 1.5	0.68 ± 0.03
Binder	581 ± 4	603 ± 4	21.6 ± 1.5*	0.43 ± 0.03*

* within column denotes significance ($P < 0.0001$)

6.3.2 Carcass characteristics

Three carcasses were classified as dark cutting (pH ≥ 5.79), two of these were from the control treatment and one was from the binder treatment. Supplementing these cattle with a mycotoxin binder did not influence carcass characteristics (P ≥ 0.21; Table 6.2).

Table 6.2. Estimated marginal means (± SE) for carcass characteristics as per the Meat Standards Australia (MSA) beef carcass grading

Item	Control	Binder
HSCW	320 ± 2.3	322 ± 2.4
EMA	75.5 ± 0.57	74.8 ± 0.58
Ossification	135 ± 1.27	135 ± 1.28
MSA Marble Score	343 ± 7	330 ± 7
Rib Fat	11.1 ± 0.42	10.3 ± 0.42
p8 fat	13.1 ± 0.5	12.4 ± 0.5
pH @ grading	5.62 ± 0.01	5.61 ± 0.01
MSA Index	62.1 ± 0.15	61.8 ± 0.15

6.4 Discussion

The initial findings from this experiment suggest that the mycotoxin binder did not influence the performance of these cattle, in fact it was associated with reduced growth rates. Specifically, the control cattle put on more weight during the experiment (an additional 12.1 ± 2.1 kg; $P < 0.0001$) due to a higher ADG (an additional 0.25 ± 0.04; $P \leq 0.0001$) when compared with cattle in the binder treatment. Similar findings have been reported for sheep (Coufal-Majewski *et al.* 2017; Stanford *et al.* 2018) and growing dairy calves (Schumann *et al.* 2007). It could be that the higher ADG may be a reflection of increased feed intake in the control cattle when compared with the binder cattle in this experiment. Alternatively, these cattle may have had a greater internal fat deposition. However, this is unable to be quantified here.

The incidence of dark cutting in these cattle was also minimal (2.9%), particularly when compared with previous studies evaluating the incidence of dark cutting in Tasmania (McGilchrist *et al.* 2014) and in cattle supplied from King Island (Loudon *et al.* 2018; Loudon *et al.* 2019). As such, it is difficult to make any assumptions about the ability of the mycotoxin binder to reduce toxicity effects and the subsequent relationship with dark cutting. Furthermore, it is difficult from these results to define if the cattle were consuming sufficient quantities of the binder to negate the

negative effects of mycotoxins. Muscle glycogen determination may reveal some additional insights into this, once the samples are able to be analysed. Regardless, mycotoxin binders were developed to negate mycotoxin absorption in the gastro-intestinal tract, thus mitigating toxicity effects (Binder 2007; Vanhoutte *et al.* 2016). However, the efficacy of mycotoxin binders may be influenced by the mycotoxin families being consumed (Stanford *et al.* 2018). Pasture samples will need to be evaluated to confirm the presence and identify specific mycotoxin families that were present during this experiment, or if in fact a mycotoxin challenge existed for these cattle. This is particularly important, as supplementing cattle with a mycotoxin binder will only be beneficial for commercial producers when grazing pastures that contain endophytes that are producing mycotoxins at the time. However, further studies evaluating mycotoxin binders are likely needed as the growth performance data from this experiment and other studies in both sheep (Coufal-Majewski *et al.* 2017; Stanford *et al.* 2018) and growing dairy calves (Schumann *et al.* 2007), suggest that binder supplementation may have a negative impact on productivity. This reduction in on farm productivity compounds the estimated economic impact of mycotoxins across the Australian sheep and beef industries (Sackett and Francis 2006). Regardless, the results from this experiment are inconclusive and as such it is difficult to make specific recommendations regarding mycotoxin binder supplementation.

7 Conclusions/recommendations

Although not completely resolved in this project, hypomagnesaemia may still be associated with dark cutting under grazing conditions, as these are difficult to replicate in experimental pen studies and as such makes it difficult to define the underlying mechanisms that are responsible for dark cutting in grazing cattle. The pasture based mechanisms associated with hypomagnesaemia in cattle grazing pasture are increased water content, which can reduce Mg absorption due to an increased rumen passage rate, coupled with low pasture availability and an inability for the cattle to get their fill. Thus the dietary protein and potassium (**K**) studies conducted within this project may not have been capable of replicating the issues due to the increased dry matter content of the rations offered, ad-libitum feed availability and subsequent reduction in rumen passage rates. However, from previous research and results from the current project, the valuable conclusions can be summarised as:

- Diets containing 30 % protein were associated with an increased insulin sensitivity, but were also less sensitive to the adrenaline challenges. Interestingly cattle consuming the 20% protein diet were more insulin sensitive and had a greater dextrose clearance. Collectively, these results indicate that dark cutting in cattle consuming high protein diets is not associated with increased adrenaline sensitivity nor alterations in insulin sensitivity. This could be due to 10% protein diets being formulated to encourage fat deposition, whereas the 20% and 30% protein diets may not observe the same level of fat deposition. Furthermore, the lipogenic pathways may be influenced by dietary protein. However further studies would be needed to understand the influences of dietary protein on these metabolic pathways.
- Dietary K concentration was not associated with alterations in insulin or adrenaline sensitivity. However, within this experiment non-stress induced exercise, i.e. exercise without endogenous adrenaline, reduced muscle glycogen in locomotor muscles (SM and ST) but was not associated with decreased muscle glycogen in postural muscles (LT). This suggests that there is a systemic regulation of glycogen usage throughout the body in response to differing stimuli, thus further studies investigating the impact various stimuli and subsequent muscle glycogen usage across muscles and the relationship on the incidence of dark cutting could be beneficial
- The most beneficial strategy to reduce hypomagnesaemia in cattle grazing ryegrass pastures is to avoid grazing these pastures during early stages of pasture growth (≥ 3 leaf stage). In addition graziers should endure that there is enough FOO (≥ 1500 kg/ha) to ensure that cattle consumption rates are adequate to avoid hypomagnesaemia. Finally, providing cattle with a high fibre roughage will support reduced rumen passage rates thus increasing Mg absorption. Where possible, avoidance of young rapidly growing pastures by finishing stock is recommended. However, supplementing cattle with a Mg supplement, via a loose lick or lick block, during high risk periods may potentially mitigate hypomagnesaemia risk.
- Results from the mycotoxin supplementation experiment were inconclusive and as such it is difficult to make specific recommendations regarding the benefits/non-benefits of using these supplements. However, the presence of endophytes in these pastures is yet to be quantified.

8 Key message/s

This project has provided additional knowledge towards understanding of the nutritional factors and the mechanisms in which dietary factors influence the incidence of dark cutting in grazing beef cattle. Developing a greater understanding of the on farm factors increasing the risk of dark cutting will allow for the development of effective management strategies to reduce the incidence of dark cutting in the Australian grass fed industry.

- Continual education and extension for producers in southern grass fed production systems of the risks associated with grazing young improved pastures in autumn and winter is needed. The risks that need to be extended are those of subclinical grass tetany (caused by mild hypomagnesaemia) and subclinical ryegrass staggers (caused by consumption of mycotoxins)
- Greater extension of research that was carried out on these issues in grazing stock in the 60's to 90's needs consideration.
- Grazing systems are very complex and there is no easy, cost-effective and reliable way to test if cattle are at risk of hypomagnesaemia and subclinical ryegrass staggers.
- The mycotoxin content of pastures is unpredictable, costly to measure, and difficult to evaluate in commercial production system environments. The knowledge of producers about which paddocks potentially cause ryegrass staggers needs empowering so that these pastures are not used in the final months for finishing slaughter stock.
- Producers should ensure that the pastures which are grazed in the final months of life for slaughter stock are more mature (3 leaf stage for ryegrass) to allow for a more balanced mineral profile, higher dry matter percentages and greater availability. A balanced pasture with grasses and legumes are less risky for cattle than monoculture pastures.

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10 Appendix

10.1 Animal Ethics Approval – Dietary protein and potassium experiments

une
University of New England

AUTHORITY No.: AEC17-108

ANIMAL ETHICS COMMITTEE

ANIMAL RESEARCH AUTHORITY
And Approval for Animal Experimentation

RESEARCH TEAM: Dr Peter McGilchrist, Dr Flavio Pereira Alvarenga, Mr Aaron van den Heuvel & Ms Xuemei Han

EMERGENCY CONTACT Dr Peter McGilchrist
0419986056

Are authorised to conduct the following research:

TITLE: Impact of dietary protein and DCAD intake by ruminants on muscle glycogen metabolism, adrenaline and insulin sensitivity and magnesium absorption

LOCATION(S): Large Animal Facility CART Ring Road UNE

ANIMALS:

Species	Strain	No's Required	Procedure Details
09 - Domestic Cattle	Angus	18	3 & 4

This authority remains in force from **10/12/2017** to **10/12/2018** unless suspended, cancelled or surrendered.

This statement must be read in conjunction with the Conditions for Animal Experimentation at UNE as stated on the reverse.

Jo-Ann Sozou
AEC Secretary and UNE Research

05/12/2017 A17/92

10.2 Animal Ethics Approval – Magnesium Supplementation

Murdoch UNIVERSITY

Research and Innovation
Research Ethics and Integrity Office

Animal Ethics
Room 1.006, Chancellery, South Street
MURDOCH WA 6150
Telephone: 08 9360 7366
Facsimile: 08 9360 6686
animal.ethics@murdoch.edu.au
www.murdoch.edu.au

Dr Peter McGilchrist
School of Veterinary and Life Sciences
Murdoch University

Wednesday, 14 September 2016

Dear Pete,

ANIMAL ETHICS - Amendment

Protocol ID.	125
Permit No.	**R2693/14**
Protocol Title	Nutritional intervention in preventing dark cutting beef carcasses at the end of season

At its meeting on the 6th September 2016, the Animal Ethics Committee, Murdoch University, considered the below amendment.

Amendment details:

a. Reopen recently closed permit.
b. Additional use of 1800 cattle in King Island, Tasmania

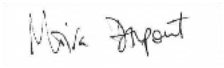
It has been granted **OUTRIGHT** approval. Amended work using animals may commence.

The approval of this project amendment requires you to adhere to the conditions outlined in this letter and to comply with the Animal Welfare Act (2002) and the *Australian code for the care and use of animals for scientific purposes* (8th edition, 2013).

Investigators must maintain records of the care and use of animals and Chief Investigators must provide to the AEC an Annual Report which is due in January each year.

Location	Impact	Species Code	Animal Species	Number Approved	Number Remaining
WA	3. Minor conscious intervention	11	Cattle	1800	1100
TAS	3. Minor conscious intervention	11	Cattle	1800	1800

The Research Ethics and Integrity Office wish you every success for your research.



10.3 Animal Ethics Approval – Mycotoxin binder experiment

une
University of New England

AUTHORITY No.: AEC19-071

ANIMAL ETHICS COMMITTEE

ANIMAL RESEARCH AUTHORITY
And Approval for Animal Experimentation

RESEARCH TEAM: Dr Peter McGilchrist, Dr Angela Lees, Dr Flavio Pereira Alvarenga, Dr Jarrod Lees, Mr Cameron Steel, Ms Ashleigh Barnett, Ms Xuemei Han & Miss Ella Palmer

EMERGENCY CONTACT Dr Peter McGilchrist & Dr Angela Lees
0419986056, 6773 1845, 0407 570 373, 6773 5332

Are authorised to conduct the following research:

TITLE: The influence of mycotoxin binders on glycogen metabolism and incidence of dark cutting in grass-fed cattle

LOCATION(S): 233 Yarra Creek Road
Lymwood King Island
TAS 7256

ANIMALS:

Species	Strain	No's Required	Procedure Details
S9 - Domestic Cattle	Bos Taurus	160	1, P2, P3 & F

This authority remains in force from **25/02/2020** to **25/02/2021** unless suspended, cancelled or surrendered.

This statement must be read in conjunction with the Conditions for Animal Experimentation at UNE as stated on the reverse.

Sarah Model
AEC Secretary

06/04/2020 A19/26