THE SAFETY AND EFFICACY OF NITRATE N SUPPLEMENTATION TO *BOS INDICUS* CATTLE

Thesis submitted by

Imanuel BENU, S.Pt

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ABSTRACT

The aims of this study were to determine if nitrate (NO_3) can safely replace urea as a source of nitrogen (N) and deliver an effective dose to reduce the enteric methane emissions of cattle consuming low quality forages typically in northern Australia without any negative impact on growth rates and grazing activity.

A series of four experimental studies were conducted to test the safety of nitrate supplementation in *Bos indicus* steers.

The first experimental study was designed to compare two methods used for the analysis of methaemoglobin (MetHb). These methods were compared to each other for use with three different species of ruminant. The accuracy, precision and correlation of a co-oximetry method for measuring MetHb concentrations in whole blood was compared to the gold standard, Evelyn and Malloy (1938) method. This study indicated that the values obtained using co-oximetry for MetHb were in good agreement with the MetHb values obtained from the Evelyn and Malloy (1938) method. Therefore, the co-oximetry method was a safer and more robust method, and could be utilised for MetHb measurement in further studies.

The second experiment examined daily nitrate dose and feeding frequency on MetHb, carboxyhaemoglobin, oxyhaemoglobin, total haemoglobin, and haematocrit concentrations in the blood of twelve *Bos indicus* steers. This was also correlated with the dry matter intake (DMI) of the cattle to assess feeding preference for supplemented cattle. Indwelling venous jugular catheters were fitted to the animals and blood samples were collected at two hour intervals, over a period of seven days. Increasing dose rate of nitrate increased MetHb concentration in the blood of steers. For once a day nitrate feeding, the dose rates of 40 and 50 g nitrate/day had greater impact on MetHb values compared to 0 or 30 g nitrate/day.

Increasing dose rates of nitrate also affected the daily peak MetHb values in the blood of cattle. However, no significant effects of increasing dose rate of nitrate were observed in haemoglobin, deoxyhaemoglobin, carboxyhaemoglobin, haematocrit concentrations or DMI. Twice a day feeding of nitrate decreased the formation of MetHb in the blood of *Bos indicus* steers. Therefore, caution should be exercised when feeding nitrate, as an NPN source to cattle grazing on low quality pastures in northern Australia.

The animals were penned individually under low physiological stress in the second experiment and this, therefore, led to the question of would happen if exercise was imposed on the animals when feeding nitrate. Twelve *Bos indicus* steers were used in the third study to investigate the effects of nitrate dose rates on arterial blood gas, MetHb, carboxyhaemoglobin, oxyhaemoglobin, total haemoglobin, haematocrit, heart rate, and respiration rate after exercise. Increasing dose rate of nitrate decreased pO_2 in the blood of cattle. Steers treated with 50 g nitrate/day had a decrease in oxyhaemoglobin and a concomitant increase in MetHb and carboxyhaemoglobin compared with steers treated with 0 or 30 nitrate/day. Steers dosed with 50 g nitrate/day exhibited greater increases in heart rate immediately after the exercise regimen compared to those animals dosed with 0 or 30 g nitrate/day. However, no difference was detected in respiration rate, or rectal temperature between treatments, after the exercise regimen. Therefore, feeding nitrate to *Bos indicus* cattle resulted in a decrease in the oxygen carrying capacity of their blood.

In the last fourth experiment, ten *Bos indicus* steers were used to determine the effect of nitrate, or no nitrate, supplementation on MetHb, carboxyhaemoglobin, total haemoglobin, oxyhaemoglobin concentrations, DMI and body weight (BW) in cattle over a 70 day period. Blood samples were collected from the jugular vein of the animals before and at two hours intervals after treatments dosages were applied at three times (1100, 1300 and 1500h), on days 10, 30, 50 and 70. Nitrate treatment caused a greater increase of mean MetHb, peak

MetHb and carboxyhaemoglobin concentrations in the blood compared to the other group which received no nitrate. Nitrate supplementation at a rate of 7.1 g/kg DMI produced a consistent MetHb profile that appeared to return to normal concentrations within 24 h. These results suggest that there was no apparent adaptation to nitrate feeding by rumen microbes. However, there was no significant effect of nitrate on the total haemoglobin, oxyhaemoglobin concentrations, DMI and BW of cattle. Consequently, there was no production advantage to feeding nitrate compared with feeding an equivalent amount of urea to steers.

Overall findings, suggest that nitrate supplementation at the rate of 50 g/day to *Bos indicus* cattle, consuming low quality forage in northern Australia, increased MetHb concentration in the blood and could have detrimental effects on their health. These studies found that another dosage regime could be implemented of twice daily dosing that would reduce toxicity of nitrate supplementation.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
BE	base excess
BGA	blood gas analyser
BW	body weight
$CaNO_3$	calcium nitrate
CI	coefficient interval
CO	carbon monoxide
CV	coefficient variation
CSIRO	the commonwealth scientific and industrial research organisation
DOM	digestible organic matter
DM	dry matter
DMI	dry matter intake
eMCP	efficiency of microbial protein
FHHB	fractions of deoxyhaemoglobin
FO_2HB	fractions of oxyhaemoglobin
FCO_2HB	fractions of carbon dioxide haemoglobin
FMetHb	fractions of Methamoglobin
g	gram
GPS	global positioning system
h	hour
Hb	haemoglobin

HCO_3	bicarbonate
Hct	hematocrit
kg	kilogram
L	liter
LOA	limits of agreement
LSD	least significant difference
LW	life weight
MDCP	mono-dicalcium phosphate
MetHb	MetHb
MetHbCN	cyanMetHb
m	meter
mL	milliliter
µL	microliter
N	nitrogen
NPN	non-protein-nitrogen
Na	sodium
NO_2	nitrite
NO_3	nitrate
NH_3-N	ammonia nitrogen
NRC	national research council
OMD	organic matter digestibility
OXYHB	oxyhaemoglobin
P	phosphate

pH	power of hydrogen
pO_2	partial pressure of oxygen
pCO_2	partial pressure of carbon dioxide
Rc	coefficient values
RDN	rumen degradable nitrogen
REML	restricted estimated mixed linear
S	sulphur
SD	standard deviation
SED	standard error difference
SEM	standard error mean
tHb	total haemoglobin

LIST OF PUBLICATIONS

Benu I, Callaghan MJ, Tomkins N, Hepworth L, Fitzpatrick LA and Parker AJ (2015). The effect of feeding frequency and dose rate of nitrate supplements on blood haemoglobin fractions in *Bos indicus* cattle fed Flinders grass (*Iseilemia spp.*) hay. *Animal Production Science.* *http://dx.doi.org/10.1071/AN14886*

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Callaghan MJ, Tomkins NW, Benu I and Parker AJ (2014). How feasible is to replace urea with nitrates to mitigate greenhouse gas emissions from extensively managed beef cattle? *Animal Production Science. 54, 1300-1304.* *http://dx.doi.org/10.1071/AN14270*

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CHAPTER 1 GENERAL INTRODUCTION

1.1 Background

Livestock animals are considered to be the largest contributor to greenhouse gas emissions, accounting for 15% of total global methane emissions, due to their enteric methane production (Bruinsma, 2003). In Australia, methane accounts for about 11% of greenhouse gas emissions (Commonwealth of Australia, 2014), and beef cattle are the largest emitters. It has been recorded that about 60 % of Australia's national beef herd is located in northern Australia, which is predominately based in tropical pastures (Callaghan et al., 2014). In addition, cattle consuming dry season pastures produce greater amounts of methane than intensively fed cattle (Charmley et al., 2008). Despite the large contribution of cattle to global methane emission, global demand of total animal products is projected to be more than doubled by 2030, particularly in developing countries, causing methane production to be as high as 60% of global methane emission (Bruinsma, 2003)

The low quality and quantity of nutrients from tropical pastures in the dry season are believed to be limiting factors in animal production in the tropics, including northern Australia. Under these circumstances, the major diet management intervention most commonly practiced is to provide the animal with nitrogen supplementation, which accounted for more than 90% of total nitrogen consumed (Bortolussi et al., 2005). Provision of nitrogen in the diet of an animal is mainly via urea, which is offered in the form of free choice, low-intake loose licks and lick blocks, using molasses as a carrier to minimize live weight losses and reduce mortality (Callaghan et al., 2014). A previous study reported that body weight of cattle was increased from 0.14, 0.19 and 0.23 kg/day for the low, medium and high levels of urea, respectively, compared with control animals that lost 0.01 kg/day when urea mixed molasses was provided to the animal (Winks et al., 1972). In addition, provision of urea

supplementation on *Bos indicus* cattle has been reported to significantly reduce live weight loss during dry seasons (Coates and Dixon, 2008).

Nitrate has been proposed as a means of mitigating methane production from ruminants (Leng, 2008). This has led to the Australian Government advocating for the feeding of nitrate to ruminants in a bid to decrease methane production. Nitrate compounds, however, can be toxic to the animal. Nitrate is reduced by the rumen microflora to nitrite and then to ammonia. In some feeding scenarios, rumen nitrate may increase to concentrations that exceed the conversion of nitrate to ammonia. In this situation, nitrite level in the blood can increase to a point where it oxidises haemoglobin to form methaemoglobin (MetHb). Methaemoglobin is unable to carry and transport oxygen around the body and tissues and can cause hypoxia induced fatality in the animal (Holtenius, 1957).

The Australian Government's confidence in nitrate to reduce methane is shadowed by the compound's toxicity. Most studies investigating the ability of nitrate compounds to reduce methane emissions from ruminants are based on high digestibility diets of grain and temperate forages (Nolan et al., 2010, Li et al., 2012). Highly digestible diets provide a degree of protection against nitrate toxicity (Burrows et al., 1987). Such a diet does not reflect the forages and supplements found in tropical regions. In addition, supplement delivery systems in northern Australia provide for *ad libitum* with minimal human control over the supplement consumption resulting in variable intakes of the supplement by the animal (Eggington et al.1990; Dixon et al. 2003). Furthermore, a single feeding event could result in the prescribed consumption of the daily dose of nitrogen. Consequently, the supplement delivery systems in northern Australia increase the risk of nitrite toxicity in beef cattle if nitrate salts make up the dominant supply of NPN to cattle (Benu et al. 2017). However, the risk of toxicity can be controlled with a dose of < 7.5 g NO_3/kg DMI over a 7 day period (Benu et al. 2017). Recent study reported that dose rate and feeding frequency of

nitrate affected the development of nitrate toxicity in *Bos indicus* steers consuming low quality native pasture hay over seven days (Benu et al., 2015). The exercise tolerance of beef cattle is also compromised when nitrate has been fed as a supplement (Benu et al, 2017). If exercise tolerance of cattle is compromised, then the animals may not graze out from watering points, resulting in a decrease in DMI over time. This, in itself would also decrease production by loss of live weight and milk yield. Therefore, further studies on the long-term use of nitrate salts as an NPN supplement are required (Lee and Beauchemin, 2014).

1.2 Hypothesis

The hypothesis tested in this research project was that nitrate feeding will affect the development of MetHb in the blood of cattle consuming low quality pasture grass.

1.3 Aims and objectives

The aims and objectives of this study were:

- To determine intra species and inter assay variability using two MetHb assays Evelyn and Malloy (1938) and blood co-oximetry from cattle, goats and sheep.
- To assess the effect of dose rate and frequency of nitrate feeding (once or twice a day) on MetHb in the blood of *Bos indicus* steers consuming low quality hay.
- To determine the exercise tolerance of *Bos indicus* steers supplemented with nitrate, compared to no nitrate supplementation.
- To assess long term forage intake and growth rates when *Bos indicus* steers consume low quality forage and are supplemented with either nitrate or no nitrate.

The ultimate completion of these aims will help improve our understanding of the issue of feeding nitrate salts to beef cattle grazing on low quality forages in northern Australia. This

thesis provides a literature review, four separate experiments, which have a common theme, and a general discussion and conclusion.

This literature review discusses the impact of nitrate feeding on cattle based on grazing pastures in relation to mitigating greenhouse gas emissions. It focusses on methane production from cattle, the ability of nitrate compounds to reduce methane emissions from cattle, and nitrate feeding and its constraints.

Initially, it was important to determine the most appropriate and efficient method of measuring MetHb concentrations in the blood of ruminants. In the first experimental chapter (Chapter three), two MetHb assay methods were compared; the results provided the basis for future MetHb sampling methods used throughout the subsequent studies presented in the thesis.

The remaining three experimental chapters (Chapters four to six) relate to the safety threshold and feeding frequency, exercise tolerance, and production responses of cattle when supplemented with nitrate salts and fed low quality pasture grass. Each chapter consists of an abstract, an introduction, material and methods, results, discussion, and conclusion. The general discussion chapter provides a summary of the main findings and conclusions obtained from each experimental study. Finally, at the end of the main part of the thesis appendices are provided, which support the data presented in the thesis.

CHAPTER 2 LITERATURE REVIEW

Much of enteric methane production from livestock animals that contributed to global greenhouse gases have been well studied (Moss et al., 2000, Boadi et al., 2004, Johnson et al., 2007, Beauchemin et al., 2009, Grainger and Beauchemin, 2011). Yet, there is an increased requirement for protein production to feed an ever-growing global population (Bruinsma, 2003). In recognition of this, and in order to reduce the economic burden on cattle producers, the Australian Government recently released guidelines on the use of supplemental nitrate feeding for cattle. While this recommendation was based on evidence provided by a number of studies (Alaboudi and Jones, 1985, Nolan et al.2010, van Zijderveld et al., 2010, Li et al., 2012), this work was undertaken under limited environmental conditions, in which a total mixed ration comprising concentrates are often offered to the animals and may not reflect the actual conditions under which the bulk of Australian meat production is undertaken.

The purpose of this literature review was, therefore, to examine the impact of nitrate feeding on cattle based on grazing pastures in relation to mitigating greenhouse gas emissions. The following will be discussed: the digestive system in ruminants; cattle and methane production; the ability of nitrate compounds to reduce methane emissions; nitrate feeding and its constraints; and frequency and dose of nitrate intake involved in cattle supplementation.

2.1 Effect of digestive system in ruminant and methane production

Ruminant animals have a unique digestive system for utilizing low-quality forage, or plant materials containing high cellulose, hemicellulose and non-protein-nitrogen (Van Nevel and Demeyer, 1996), as sources of energy. This has benefits for the animal's' survival but more importantly, this forage is converted into products, such as milk, meat, wool and labour. Forage is high in fiber and requires energy to be digested and converted into energy. The subsequent process, known as anaerobic fermentation, is mainly carried out by the microbes

present in the rumen (bacteria, fungi and protozoa) and produces short-chain fatty acids (VFAs), including acetate, propionate and butyrate (Buddle et al., 2011). These VFAs provide as much as 80% of metabolized energy (Krehbiel, 2014) absorbed by the host animal through the rumen wall into the blood stream (Callaghan et al., 2014). In addition, the components of VFAs also control hydrogen availability in the rumen for use by methane producing microbes (Alemu et al., 2011). Moreover, this microbial activity is also responsible for the conversion of protein and NPN to amino acids, oligopeptides, and ammonia secretions. This serves as a source of nitrogen for microbial protein production, which can then be utilized by the animal (Janssen, 2010). Figure 2.1 shows some of the rumen processes and highlights the process of feed fermentation by the microbes to produce VFAs (acetate, propionate, and butyric acids), hydrogen and carbon dioxide.

The main end-product of fermentation is VFAs, however other products such as formate, ethanol, lactate, succinate, branched-chain VFAs, as well as ammonia, carbon dioxide and hydrogen are also formed (Janssen, 2010, Krehbiel, 2014). Although, hydrogen is not stored in the rumen, it is one of the main end-products of fermentation, and instead it is utilized by methanogen bacteria (Boadi et al., 2004). In addition, hydrogen is the most important energy source for methanogen bacteria in the rumen (Buddle et al, 2011) as its utilized to reduce CO_2 to CH_4, resulting in maintaining low hydrogen pressure in the rumen (Boadi et al., 2004).

Methanogenic *archaea* are responsible for enteric methane production by utilizing carbon dioxide and hydrogen (Eckard et al., 2010). These bacteria do not have peptidoglycan polymers in their cell walls and also have a different composition of intracellular lipids compared to other bacteria (Moss et al., 2000). Both methane and carbon dioxide are released into the atmosphere through eructation and respiration of ruminants, resulting in greenhouse gas emissions. Both the rate and the amount of hydrogen that passes through the

pool determines the rate of methane formation and the amount of methane generated, respectively ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$; Janssen, 2010). In addition, methane production also represents a loss of energy from cattle of about 6% of feed consumed through eructation (Johnson and Johnson, 1995). Recently, a study recorded the energy lost as methane from cattle accounted for 6.1%, 6.7% and 5.4% of gross energy (GE) intake of early grass silage, late grass silage and maize silage, respectively (Brask et al., 2013). It has been noted that methane emissions from cattle are affected by several factors, such as the level of feed intake, type of carbohydrate in the diet, feed processing, supplementation of lipids or ionophores, alteration in the ruminal microflora (Johnson and Johnson, 1995), and ambient temperature (McAllister et al., 1996). Therefore, manipulation of these factors, ultimately, can reduce methane emission from cattle.

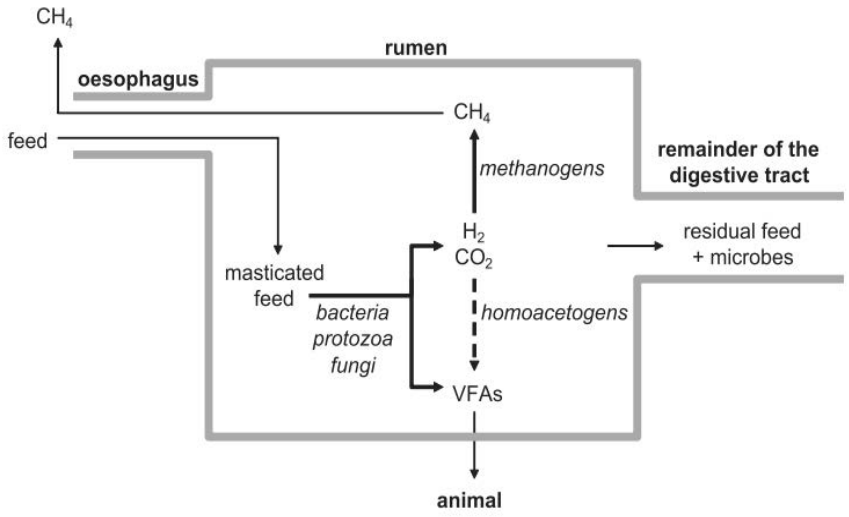

Figure 2.1. The process of feed fermentation in the rumen by the microbes to volatile fatty acids (VFAs: acetate, propionate, and butyric acids), hydrogen and carbon dioxide. (Buddle et al, 2011).

2.2 Seasonal rainfall and pasture grass production on methane yield from ruminants

It is well documented that fluctuations of seasonal rainfall affect tropical grass production across tropical countries, including Australia. Dixon and Coates (2010) indicated that quality and quantity of grass is higher during the wet season, but then declines rapidly as it reaches

maturity, coupled with the dry season. This eventually, results in higher fibrous contents and lower nitrogen, as well as metabolizable energy contents and, thus, causes low growth and productivity of cattle when cattle are fed the grass. Under these circumstances, the major management intervention commonly practiced is to supplement the animal with urea in the form of free choice, low-intake loose licks and lick blocks, and use molasses as a carrier to minimize live weight losses and reduce mortality (Callaghan et al., 2014). It has been recorded that most of the supplementation to beef cattle across the northern region was nitrogen, which accounted for more than 90% of nitrogen (Bortolussi et al., 2005). The use of urea as non-protein-nitrogen supplementation, with molasses as a carrier, has been reported to increase body weight of cattle by about 0.14, 0.19 and 0.23 kg/day for the low, medium and high levels of urea, respectively, compared with control animals that lost 0.01 kg/day (Winks et al., 1972). Provision of urea supplementation in *Bos indicus* cattle has been reported to significantly reduce live weight loss during dry seasons (Coates and Dixon, 2008).

Evidence from studies concerned with methane mitigation suggests that the type of diet offered to the animal is potentially associated with high methane production. However, cattle consuming pastures in the dry season, produce greater amounts of methane than those fed intensively (Charmley et al., 2008). In addition, ruminants fed forages high in fibrous material tended to produce more methane than those fed a high quality diet, such as concentrates (Beauchemin et al, 2008), due to the related alteration in substrate derived fermentation from fibre to starch and the decrease in ruminal pH. Furthermore, high cell wall components, such as cellulose and hemicellulose, in forages, which are less degradable than grains also contributed to methane production (Johnson and Johnson, 1995). In contrast, supplementation of readily fermentable carbohydrate degraded in the rumen, results in low methane production per unit feed (Moss et al., 2000), which is perhaps due to a higher rumen turnover resulting in a lower diet digestibility (Buddle et al., 2011).

2.3 Strategies of methane mitigation and its application

Since methane emission represents energy losses from animals, as well as contributing to greenhouse gas emission, any strategy of mitigating methane yield in the rumen aims to increase utilization of feed efficiency and results in animal production. Several strategies regarding methane mitigation have been considered (McAllister et al., 1996, Moss et al., 2000, Beauchemin et al., 2008). Although there are some promising strategies in dietary supplementation with plant secondary compounds (Chung et al., 2013), such as yeast cultures and enzymes, extensive research is still required (Beauchemin et al., 2008). Recently, an *in vitro* study evaluated the combination of several essential oils in mitigating methane and recorded that a combination of essential oils significantly reduced archaea, protozoa and select species of rumen bacteria, to different extents (Cobellis et al., 2016).

The use of nitrate as a feed additive and its ability to suppress methane production from ruminants has been reported from a number of *in vivo* studies (Nolan et al., 2010, van Zijderveld et al., 2011, Li et al., 2012). However, most data supporting the capability of nitrate to lessen methane yield without any adverse effect to the animals, have been from studies with cattle fed total mixed rations (TMR) comprising of concentrates, forage and nitrate (Callaghan et al., 2014). Such methods of feeding a diet as TMR (concentrates and forage) or even nitrate that are mixed in solution and presented to the animals often result in low MetHb concentrations in the blood of ruminants (Benu et al., 2015). This is in contrast with the northern Australia grazing system, where cattle are consuming low quality pasture grass during the dry season, therefore, these strategies are unlikely to be adopted in extensively grazed livestock in northern Australia. This is, perhaps largely due to how extensive the beef production system is, with minimal labour input, low levels of infrastructure, large paddock sizes, and expensive energy supplementation (Callaghan et al.,

2014). Alternatively, intensification of grazing management, can be achieved by allowing cattle to graze pastures at early stages of maturity, which in turn can improve feed intake and digestibility, and, consequently, lower methane yield per unit of dry matter (DM) (Hegarty et al., 2010). Another consideration for methane reduction strategies is genotype manipulation, by selecting animals with higher production performance but lower methane yields. For example, Chagunda et al. (2009) demonstrated that genotype and feeding system type were associated with a decline of methane emission per liter of milk.

2.4 Effect of nitrate supplementation and methane reduction expectation from ruminants

It has been proposed that nitrate can potentially replace urea as a source of NPN supplementation (Nolan et al., 2010), as well as provide a means to reduce enteric methane emission (Leng, 2008). Addition of nitrate in the diet of ruminants, to mitigate ruminal methane yield (McAllister et al., 1996), is promising due to the characteristic of nitrate as alternate electron acceptor in the rumen against methanogenesis (Figure 2.2) and its tendency to function effectively and persistently over time (van Zijderveld et al., 2011). Furthermore, consideration of the ability of nitrate to suppress methane production through the hydrogen sink mechanism invites future studies to be developed, in order to ensure that the perceived mechanism is accurate (Yang et al., 2016). When residence time of feed in the rumen is reduced, there would be the expectation that there will be a decrease in methane yield, due to a decline of ruminal digestion and the inability of methanogenic bacteria to compete in such circumstances (Moss et al., 2000). In addition, the expected methane reduction from nitrate supplementation can be calculated by using stoichiometry, where one mole of nitrate (100 g) produces ammonia with four moles of hydrogen, and reduces methane by one mole (26 g) (Lee and Beauchemin, 2014).

Measurement of methane reduction, in relation to nitrate supplementation is often achieved by utilising a calorimetry. For example, Hulshof et al. (2012) reported that daily methane yield of steers was reduced by 32% when cattle were fed 85 g/d of calcium nitrate. Li et al. (2012) also reported a reduction of 35.4% methane yield from sheep treated with 3% calcium nitrate in their diet. Another study reported a 23% reduction of methane production when sheep were fed 4% potassium nitrate (Nolan et al., 2010).

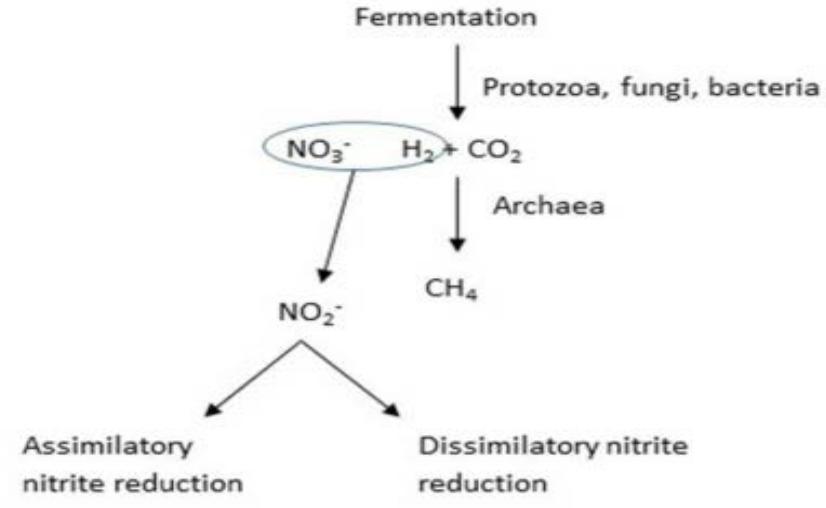

Figure 2.2. Nitrate supplementation provides an alternative hydrogen sink (Yang et al, 2016).

2.5 Effect of nitrogen metabolism in the rumen by ruminal microorganisms

It is well established that microorganisms reside in the rumen of ruminants can assimilate nitrogen not only from dietary protein and nucleic acids, but also from non-protein-nitrogen (NPN) (Walt, 1993). About 15-20% of dietary nitrogen comes from NPN sources such as free amino acids, nucleic acids, purine and pyrimidine bases, ammonia, ammonium salts, urea, nitrates, and nitrites as well as urea from saliva (Rossi et al, 2013). In addition, protein play an important role in providing nitrogen requirement for ruminant as well as providing skeletons for energy metabolism (Walt, 1993). The quality of protein consumed by ruminants and their amino acids profile may or may not beneficial for the ruminal microbial (Valente et

al, 2016). Attempt to increase the nitrogen balances in livestock fed protein-rich forages is still constrained by the imbalance between the degradative and biosynthetic activities of the rumen microbiota (Morrison and Mackie, 1996). Urea nitrogen (N) in ruminants is physiologically produced by the liver and recycled to the gastrointestinal tract (GIT) as a source of N for microbial growth (Eisemann and Tedeschi, 2016). The synthesized urea in the liver is functioning to detoxify ammonia due to the catabolism of endogenous or exogenous amino acids, where the excess ammonia is excreted through the urine (Lobley and Milano, 2007; Rossi et al, 2013).

Ammonia is essential metabolite in rumen N metabolism (Walker et al, 2005; Rossi et al, 2013), particularly for the growth of rumen microorganisms. One of the main source for ammonia which can be provided to the ruminants is urea. However, the problem with urea feeding is that rumen microbial does not effectively utilize it due to its rapidly release to ammonia, and therefore loss of nitrogen available for microbial protein synthesis (Chalupa, 1969). An increase in microbial protein synthesis is important in both improved efficiency of carbohydrate degradation and ensure and increased intake of amino acids (Rossi et al, 2013). Because microbial growth and carbohydrate fermentation affect the extent of NH_3 absorption and urea recycling and excretion (Reynolds et al, 2008). Ruminal NH_3-N is reported as a crude predictor for the efficient conversion of dietary N into microbial N, however when the concentration is below 5 mg/dL, blood urea N transfer into the rumen provides an increasing buffer against excessively low NH_3-N concentration (Firkins et al, 2007). Huntington et al (2006) reported that plasma concentrations of ammonia N in steers was reduced when urea-calcium diets fed to steers, and tended to reduce arterial glucose concentrations compare to urea alone. The researcher concluded that urea-calcium was effective in reducing rapid ammonia release in the rumen and subsequent effects on glucose and lactate metabolism.

2.6 Effect of nitrate intake and mechanism of nitrate toxicity in animals

Despite the ability of nitrate to reduce methane yield from ruminants, elevated levels of nitrate in the diet of the animal are associated with nitrate poisoning. Nitrate (NO_3) poisoning mainly occurs as a result of nitrite (NO_2) oxidation in the blood, where nitrite binds to red blood cells and changes ferrous ions (Fe^{2+}) to ferric (Fe^{3+}) ions, overwhelming the protective ferri-reductase system and inducing methaemoglobinemia (Haymond et al., 2005). Methaemoglobinemia is a condition where blood is unable to carry and transfer oxygen to active tissues of the animal, resulting in hypoxemia (Holtenius, 1957, Vermunt and Visser, 1987). However, under some circumstances, the amount of nitrite formed might be overwhelmed by the amount of nitrite reduction to ammonia, when nitrite levels are increased (Marais et al., 1988). Ruminants have been reported to be more susceptible to NO_3 toxicity when compared to monogastric animals (Yaremcio, 1991), since the microbes present in the rumen readily break down NO_3 to NO_2 (Leng, 2008). There are several factors that contribute to NO_3 toxicity, such as the amount of NO_3 consumption, the type of feed, the amount of carbohydrate in the feed, and the stage of adaptation to NO_3 in the diet (Vermunt and Visser, 1987). Figure 2.3 demonstrates how nitrate is utilised by rumen microorganisms.

Clinical signs of NO_3 toxicity generally appear within four hours of the animal ingesting NO_3 (Hixon, 1991). The symptoms appear when levels of MetHb reach 30%, and it may cause fatality in the animal when the MetHb reaches up to 80%, of total haemoglobin. The characteristics of symptoms are generally related to anoxia (lack of oxygen), such as a rapid pulse, difficulty in breathing, shivering, and brown discoloration of the mucous membrane of the vulva, vagina, and conjunctive tissue (Hixon, 1991, Al-Qudah et al., 2009). In addition, dark brown carcasses and non-clotted blood in the dilated vessels were reported in post-mortem examinations (Al-Qudah et al., 2009). Treatment with methylene blue solution is

recommended for animals suffering from NO_3 intoxication (Burrows et al., 1987). Methylene blue is applied through intravenous injection and it acts as an elector carrier via which MetHb is converted to haemoglobin (Zijlstra and Buursma, 1997). Although there is variability in dose application, Vermunt and Visser (1987) recommended 6-7 mg/kg body weight as a safe dosage of methylene blue.

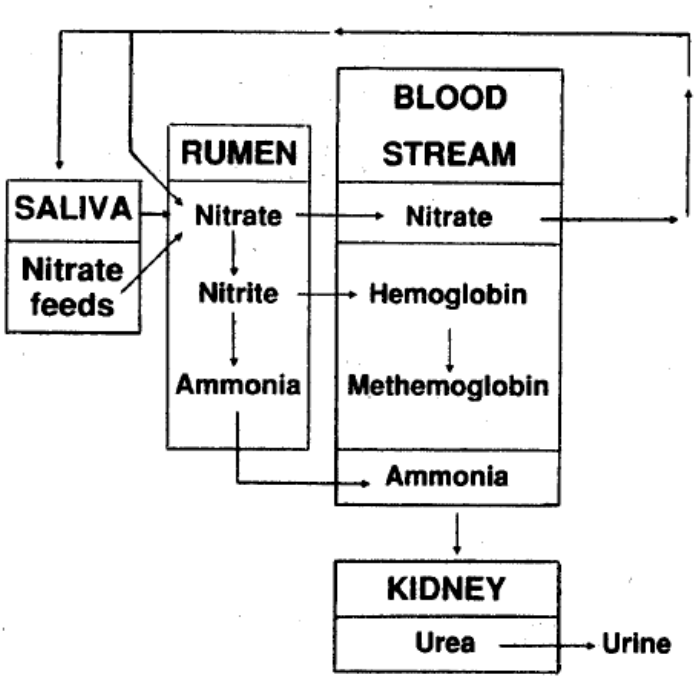

Figure 2.3. Nitrate pathway in ruminants (Yaremcio, 1991).

2.7 Effect of basal diet and nitrate supplementation on methaemoglobin formation in ruminants

Accumulation of NO_3 in the rumen appears to be positively correlated with the feed compositions offered to the animal in relation to methaemoglobin formation in the blood. For example, early work confirmed that sheep under adequate feed (0.5 kg hay, 200 g crushed oats, 50 g molasses and free access to oat straw) were more capable of breaking down NO_3 in the rumen, than those groups offered inadequate feed (0.2 kg hay and free access to oat straw) (Holtenius, 1957). Other studies in which sheep were fed with high concentrates of feed (total

mixed ration) or pellets, and offered small meals of NO_3 within a day, demonstrated low levels of MetHb production in the blood (Alaboudi and Jones, 1985, van Zijderveld et al., 2010, Li et al., 2012), with MetHb levels of < 2%, ≤ 7% and < 2.8%, respectively. Furthermore, when a 4% solution of potassium nitrate was sprayed onto oaten hay every hour, Nolan et al. (2010) found that blood MetHb of sheep was less than 2.8%. Low concentrations of MetHb formation in the blood is mainly due to highly digestible diets being offered to the animal, which provide a degree of protection against nitrate toxicity (Burrows et al., 1987). In contrast, when cattle are fed low quality rangeland hay, the production of MetHb in the blood was reported to be greater over seven days (Benu et al., 2015).

Interestingly, most studies reporting low MetHb concentration in the blood of ruminants which were fed NO_3 did not quantify the amount of NO_3 consumed before the blood was sampled (Alaboudi and Jones, 1985, van Zijderveld et al., 2010, Li et al., 2012). Therefore, the timing of blood sampling may or may not align with the peak MetHb concentration for the day, as peak MetHb concentration determines the amount of time the animal is placed in a hypoxic state (Benu et al., 2015).

The dose of nitrate feeding offered to the animal may also contribute to NO_3 poisoning; therefore, seeking management strategies, in terms of NO_3 feeding in animals is crucial. Callaghan et al. (2014) reviewed the strategy of NO_3 being used in mitigating greenhouse gas emission in northern Australia. The researchers speculated that NO_3 could be safely applied as a replacement for urea. However, the cost of NO_3 can be as much as twice the price of urea. There is an argument that feeding NO_3 to the animal in a single dose results in greater toxicity than a similar level divided and offered several times a day (Parkinson et al. 2010, A.J. Parker, pers. comm). The main reason behind this is that the concentration of MetHb formation, resulting from NO_3 intake, is apparently higher in a single dose than from feeding two or three times a day. Figure 2.4 shows the formation of MetHb in the blood of two steers

consuming 30 g of nitrate daily (~ 7.5 g NO_3/kg DM) on low quality Flinders grass hay. Recently, a study by Benu et al. (2015) demonstrated that increasing the dose rate of nitrate increased the proportion of MetHb in the blood when steers consumed low quality pasture hay and were treated with 40 or 50 g of nitrate per day, in once a day feeding, than for 0 or 30 g. In contrast, twice a day feeding decreased MetHb development in the blood of steers (Benu et al., 2015).

The animals being introduced to nitrate feeding are often required to undergo a certain adaptation period to ensure that the rumen microorganisms are acclimated to the nitrate diet (Lee and Beauchemin, 2014). It has been claimed that microorganisms can maximise the activity of nitrate reductase in the rumen by about three to five times higher than the normal levels, as long as the animal is allowed an adaptation period of three to five days (Yaremcio, 1991). Moreover, Yaremcio, (1991) indicated that, for the first three days of nitrate introduction, activity of rumen microorganisms will remain normal. However, this digestion ability will level off between days four and seven, particularly if the animals are on a forage diet and the blood MetHb levels are found to be between 20 and 30%.

Feeding nitrate to ruminants is often associated with negative impacts on animal performance, such as low feed intake, weight gain, or milk production (Lee and Beauchemin, 2014). Nevertheless, some studies have reported no negative effects of nitrate feeding on animal performance. For example, Nolan et al. (2010) and Li et al. (2012) showed that feed intake and weight gain of sheep were not affected by nitrate treatment. In addition, Benu et al. (2015) also reported that DMI by *Bos indicus* steers was not influenced by nitrate dose. However, all of these studies were conducted over a short period of time in laboratory setting and under human control. Therefore, if nitrate feeding has to be applied to cattle in grazing systems, such as those in northern Australia, where variability of individual consumption of

nitrogen supplementation is huge, then some animals will likely be placed at a high risk due to MetHb formation in their blood (Callaghan et al., 2014).

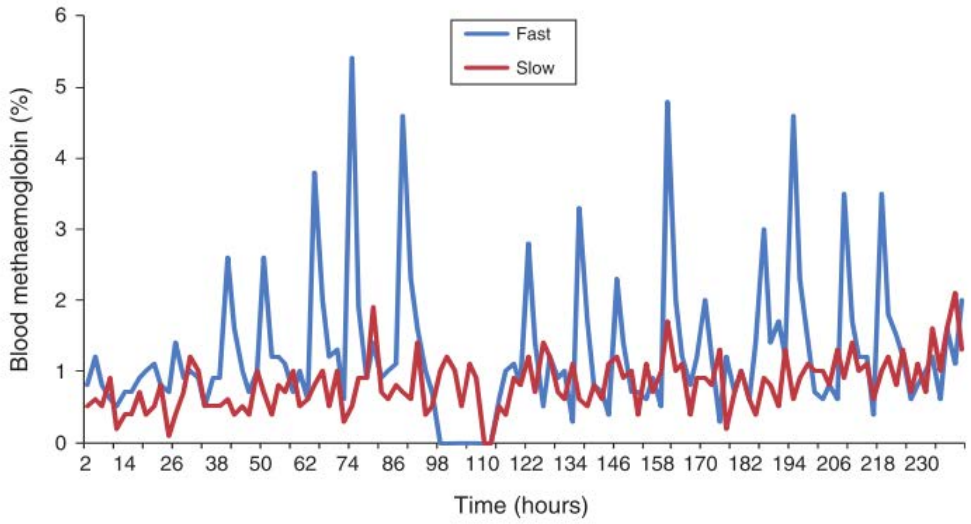

Figure 2.4. The effect of feeding 7.5 g nitrate/kg DM, fed as calcium nitrate once daily to two steers with different rates of consumption; either consuming the dose within 5 min (Fast) or 45 min (Slow) on the formation of methaemoglobin in the blood (Taken from Callaghan et al, (2014); A. J. Parker, unpubl. data).

2.8 Methaemoglobin

Increasing MetHb levels in the blood of the animal is an indication of nitrate presence. In the blood of a normal animal, the MetHb level is usually found to be less than 2% of total haemoglobin. Therefore, consideration should be taken to avoid nitrate toxicity in animals particularly when the MetHb is above the normal levels. Figure 2.5 shows blood MetHb from a cow treated with no nitrate (control) and an animal treated with calcium nitrate.

Despite the fact that feed composition is playing a crucial role in MetHb accumulation in the blood, the amount and rate of nitrate consumed by the animal also largely determines the severity of nitrate poisoning (Jainudeen et al., 1964, Vermunt and Visser, 1987). For example, studies by van Zijderveld et al. (2010) and Li et al. (2012) demonstrated that sheep produce low MetHb in the blood; ≤7% and <2.8%, respectively. In both of these experiments, the animals were offered high quality protein comprising of concentrates. However, the

researchers did not describe the amount of nitrate that the animal consumed during the 24 hours prior to blood sampling. The peak of MetHb is an important consideration in nitrate feeding for ruminant animals. An early study by Holtenius (1957) demonstrated that the peak of MetHb usually occurs between three and five hours after the animal has consumed nitrate.

Figure 2.5. Blood methaemoglobin between normal (1.1%) as a control and (15%) treated with calcium nitrate (Photo credit: Matthew Callaghan).

2.9 Conclusion

In an effort to the offset of greenhouse gas emissions from cattle, the Australian Government recently published guidelines which recommend nitrate supplementation for cattle. However, nitrate supplementation to cattle based on low quality forages and grass resulting in nitrate toxicity. Therefore, if nitrate supplementation has to be applied in northern Australia, where beef cattle are grazing on low quality pastures, then some of the animals are more likely to be challenged by greater MetHb formation in their blood.

CHAPTER 3 A COMPARISON BETWEEN METHAEMOGLOBIN METHODS IN CATTLE, SHEEP, AND GOATS

This chapter is in preparation for submission to Veterinary Clinical Pathology

Authors: I. Benu, D.M. Rudd, M.J. Callaghan, and A.J. Parker

Abstract

Methaemoglobin concentrations in the blood of ruminants are measured with a standard method (Evelyn-Malloy, 1938). However, the use of cyanide placed a number of safety constraints in this method. In particular, the need for a permit which took up to six months to attain. Co-oximetry is used in human medicine as a care diagnostic aid; however, it has not been validated in ruminant species. Due to the improved utility of the co-oximetry method, there is a need to compare the methods for agreement. Blood samples were collected from the jugular vein of 10 cattle (n =14), 9 sheep (n =22) and 10 goats (n = 21) at the College of Public Health, Medicine and Veterinary Sciences of James Cook University. The Evelyn-Malloy and co-oximetry methods were used to determine MetHb concentrations. There was no difference in MetHb concentrations between the two methods for cattle, sheep and goat samples ($P = 0.059$, $P = 0.122$, $P = 0.206$), respectively. A highly significant Pearson correlation coefficient between methods for the $\log_{10}$ MetHb concentrations was demonstrated in the blood samples from cattle ($P < 0.0001$; $r^2 = 0.8311$), sheep ($P < 0.0001$; $r^2 = 0.9850$) and goats ($P < 0.0001$; $r^2 = 0.9812$). The Bland-Altman analysis of $\log_{10}$ MetHb concentrations showed a good agreement in cattle (LOA: 0.239 – 2.511), sheep (LOA: 0.338 – 3.019) and goats (LOA: 0.524 – 1.621) respectively. The Lin's concordance coefficient values were demonstrated for the cattle ($r_c > 0.870$ are regarded as poor); however, for sheep ($r_c > 0.988$;) and goats ($r_c > 0.988$) the values are regarded as substantial and, therefore,

represents a very close agreement between the two methods. The co-oximetry values for MetHb agree with the MetHb values obtained from the Evelyn-Malloy (1938) method.

3.1 Introduction

Methaemoglobin is produced via the oxidation of ferrous ions (Fe^{2+}) to a ferric (Fe^{3+}) state in the haem group of the hemoglobin molecule, rendering it unable to bind, carry and transfer oxygen to tissue, which may lead to hypoxia and death (Holtenius, 1957, Shihana et al., 2011). The presence of MetHb shifts the oxygen dissociation curve to the left and interferes with oxygen delivery to the tissues, resulting in tissue hypoxia in the absence of cyanosis. Causes of increased MetHb may be hereditary, idiopathic, or drug or toxin induced. Nitrates and nitrites are well-documented causes of elevation of MetHb in ruminants (Holtenius, 1957, Vermunt and Visser, 1987, Takahashi et al., 1998, Sar et al., 2004). Methaemoglobinemia, caused by nitrate toxicosis, can develop rapidly in cattle and sheep resulting in death. Detection of MetHb in emergency wards is critical as patients may succumb to MetHb disruption of cellular oxidative respiration (Olson et al., 1997). Therefore, human medicine has seen the advent of co-oximetry methods for the rapid and point of care detection of abnormal haemoglobins in critical care wards. Advances in the detection of MetHb in animals affected by nitrate poisoning are also critical to prevent death, particularly in the presence of effective treatments (Holtenius, 1957, Shihana et al., 2011). To date, the standard method for the analysis of MetHb levels in the blood is using the spectrophotometric assay of Evelyn-Malloy (Evelyn and Malloy, 1938). This method is characterised by measurement of the absorbance of the maximum MetHb peak at 630 nm, where the MetHb disappears upon the addition of sodium cyanide. However, this standard method has some limitations, such as: the cyanide used to convert MetHb to cyanmethaemoglobin (MetHbCN) is dangerous; the sample must be analyzed immediately to prevent pre-analytical error; and

lab error in handling samples, such as pipetting, may cause inaccuracy of results. The Evelyn and Malloy (1938) method is also a labor intensive and time consuming procedure. The co-oximetry method, on the other hand, offers a rapid process for measuring MetHb concentrations in the whole blood; it is pre-calibrated, multiple-wavelengths are measured simultaneously, and it has internal reference standard. However, co-oximetry has not been validated for measurement of MetHb in ruminants.

In the present study, MetHb levels of cattle, sheep and goats were compared using the Evelyn and Malloy (1938) method with co-oximetry determination was performed using the co-oximetry chamber on a point of care blood gas analyser. We hypothesised that the two different methods would show good correlation.

3.2 Materials and methods

Fourteen blood samples were collected from 10 two-year-old *Bos indicus* steers, 22 blood samples from 9 two-year-old Merino rams and 21 blood samples from 10 mature female Boer goats. All animals were from healthy resident cattle, sheep and goat populations at the College of Public Health, Medicine and Veterinary Sciences of James Cook University, Townsville, Queensland. The blood samples were taken from the jugular vein following restraint, either in a cattle chute for the steers, or by an attendant in the case of sheep or goats. For each species, a 21 gauge, one inch vacutainer needle was used for the collection of 10 mL of blood into a lithium heparin containing vacutainer (Becton Dickinson). Blood samples were immediately placed on ice and transferred to the laboratory for subsequent analysis. All analyses were performed within 4 h. Samples of pooled blood from each different species (cattle, sheep and goats) were used in each method for determination of MetHb concentrations. Ethics approval was obtained from the Animal Ethics Committee of James Cook University (No.A1929).

3.2.1 Methaemoglobin measurement

In order to assess the agreement between the spectrophotometric method of Evelyn and Malloy (1938), which was undertaken on a spectrophotometer (Thermo scientific, Genesys 10S UV-VIS), and co-oximetry from a blood gas analyser (Siemens, Rapid Lab 1265, Sydney, NSW) for measuring MetHb concentration, blood samples were pooled and then gently mixed for each species. Each pooled blood sample was divided into replicate aliquots before analysis with either method (cattle 2 replicates, 7 concentrations; goats 3 replicates, 7 concentrations; and sheep 4 replicates, 5 concentrations). Aliquots of the pooled blood (2.5 mL) were treated with 0, 10, 20, 40, 60, 80 or 100 µL of 1% sodium nitrite, dissolved in isotonic saline to produce seven concentrations of MetHb. A buffer was added to the samples to maintain a constant final volume, as outlined in Table 3.1. Untreated and treated samples were analysed in triplicate and the mean of the replicates was taken as the value of MetHb for that sample. The average of the replicates was then used for further calculation against co-oximetry results.

Table 3.1. Composition of untreated or treated blood samples with sodium nitrate and phosphate buffer to produce a MetHb standard curve for the in vitro determination of MetHb concentration by two methods.

Sample no	Blood (mL)	1% NaNO2 (µL)	Phosphate Buffer (µL)
1	2.5	0	400
2	2.5	10	390
3	2.5	20	380
4	2.5	40	360
5	2.5	60	340
6	2.5	80	320
7	2.5	100	300

3.2.2 Methaemoglobin measurement using co-oximetry

The untreated and nitrite treated blood samples were aspirated into 1 mL blood gas syringes for immediate measurement of MetHb on a blood gas analyser (BGA) with co-oximetry chamber (Siemens, Rapid Lab 1265, Sydney, NSW). At the same time, the Evelyn and Malloy (1938) method was commenced.

3.2.3 Precision

Calculation of coefficient variation (CV) between triplicates of MetHb measurements was undertaken in order to assess the precision of each method.

3.2.4 Statistical analysis

Data obtained from this study were log transformed to stabilize the variance and allow determination of limits of agreement, since the variability of the differences was not constant.

Data were analysed using Graphpad Prism Version 6.04 for Windows (GraphPad Software, La Jolla California USA). For each species, mean values for the replicates at each MetHb concentration were calculated for those analysed using the Evelyn and Malloy (1938) and the co-oximetry methods, and were compared using a paired Student's t-test. Linear relationships between MetHb concentrations from the Evelyn and Malloy (1938) and the co-oximetry methods were determined from Pearson correlation coefficients by linear regression analysis. To examine how close the values for MetHb % from both methods were, a Bland-Altman scatterplot of the mean values of the two methods, versus the difference between the two methods, was produced (Bland and Altman, 1986). Lin's concordance correlation coefficient was calculated as an index of agreement between the two methods. Lin's concordance coefficient (http://gjyp.nl/marta/) was performed using SPSS (IBM SPSS Statistics 22). The strength of agreement for Lin's concordance coefficient was interpreted using the following

scale rc > 0.99: almost perfect; rc > 0.95-0.99: substantial; rc < 0.90-0.95: moderate; < 0.90: poor (McBride, 2005).

3.3 Results

An unpaired t-test indicated that there was no systematic difference in MetHb values between the Evelyn and Malloy (1938) method and the co-oximetry method, for cattle, sheep and goat samples (P = 0.059, P = 0.122, P = 0.206). There was a highly significant Pearson correlation coefficient for the $\log_{10}$ MetHb concentrations in the blood samples from cattle (P < 0.0001; $r^2 = 0.8311$), sheep (P < 0.0001; $r^2 = 0.9850$) and goats (P < 0.0001; $r^2 = 0.9812$; Table 3 2).

The Bland-Altman analysis of $\log_{10}$ MetHb concentrations generated using the Evelyn-Malloy and co-oximetry methods in cattle, sheep and goats produced very similar results (Figure 3.1, 3.2 and 3.3). The mean differences were -0.1364 on the log scale with the 95% confidence interval of 0.7380 - 0.9720 for cattle; -0.03779 with 95% confidence interval of 0.9816 - 0.9969 for sheep; and -0.02871 with 95% confidence interval of 0.9764 - 0.9962 for goats, respectively (Table 3.2). Furthermore, the back transformed limits of agreement (LOA) for MetHb in cattle, sheep and goats were 0.239 - 2.511, 0.338 - 3.019, and 0.524 - 1.621, respectively (Table 3.2). Most of the differences in values between the two methods were within the interval between the upper and lower limits of the 95% agreement (d-2s and d+2s). However, there was a difference of bias in the sample of cattle (-0.1364) compared to the samples of sheep (-0.03779) and goats (-0.02871).

The Lin's concordance coefficient values (rc) for the cattle, sheep and goat samples were 0.870, 0.988 and 0.988, respectively (Table 3.4). The rc for the cattle would be regarded as poor according to the pre-determined categorization of the rc values in this study. However, the rc values for the sheep and goat samples would be regarded as substantial, and therefore, represent a very close agreement between the two methods.

Table 3.2. Pearson correlation of log10 MetHb values produced from cattle, sheep and goat blood samples using the Evelyn-Malloy (1938) and co-oximetry methods.

	Cattle (n = 14)	Sheep (n = 22)	Goats (n = 21)
R	0.9116	0.9925	0.9906
95% CI	0.7380 – 0.9720	0.9816 – 0.9969	0.9764 – 0.9962
R^2	0.8311	0.9850	0.9812
P (two-tailed)	<0.0001	<0.0001	<0.0001
Significant at alpha 0.05	Yes	Yes	Yes

Table 3.3. Bland and Altman analysis of log10 MetHb values for the Evelyn and Malloy (1938) and co-oximetry methods with bias and 95% limits of agreement.

	Cattle (n = 14)	Sheep (n = 22)	Goats (n = 21)
Bias	-0.1364	-0.03779	-0.02871
SD of bias	0.2468	0.1115	0.1011
95% LOA	-0.6200 to 0.3473	-0.2564 to 0.1808	-0.2268 to 0.1694

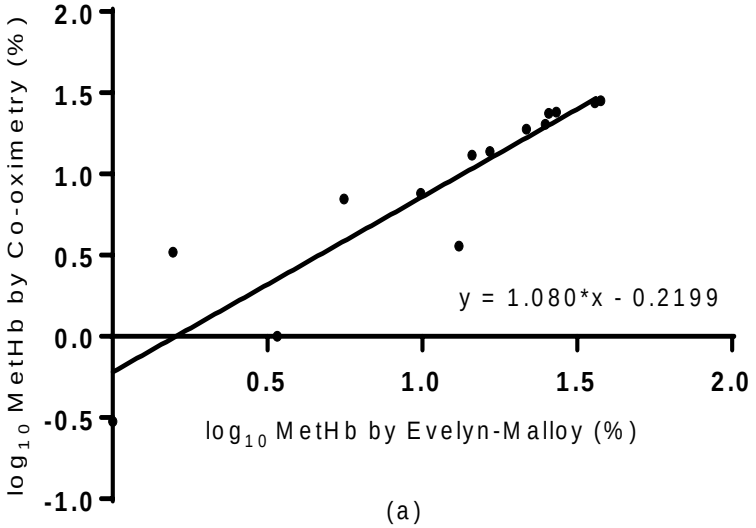

Figure 3.1. (a) Comparison of log10 MetHb values for cattle produced by the Evelyn-Malloy and co-oximetry methods (P < 0.0001; R2= 0.8311; Y=1.080*X-0.2199).

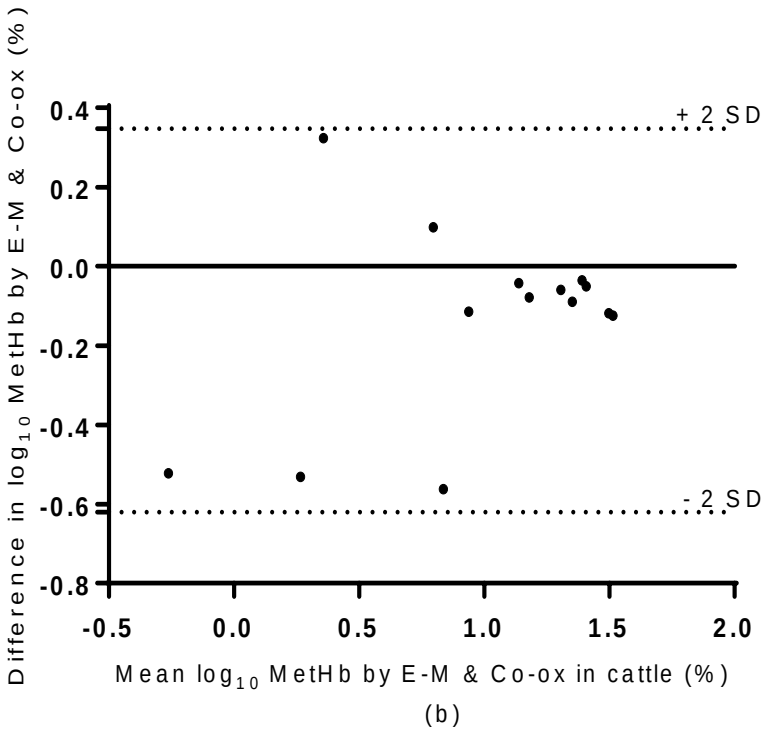

Figure 3.2. (b) Bland-Altman plot agreement between log10 MetHb values from cattle blood. The lower and upper agreement limits (bias ± 2 SD) are -0.62 and 0.34%, respectively.

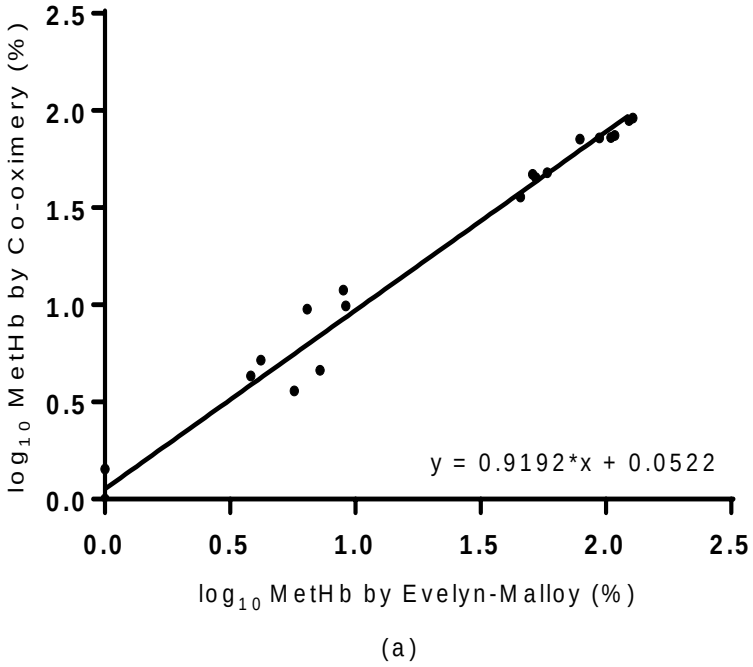

Figure 3.3. (a) Comparison of the of log10 MetHb values for sheep produced by the Evelyn-Malloy and co-oximetry methods (P < 0.0001; R2= 0.9850; Y=0.9192*X + 0.05227).

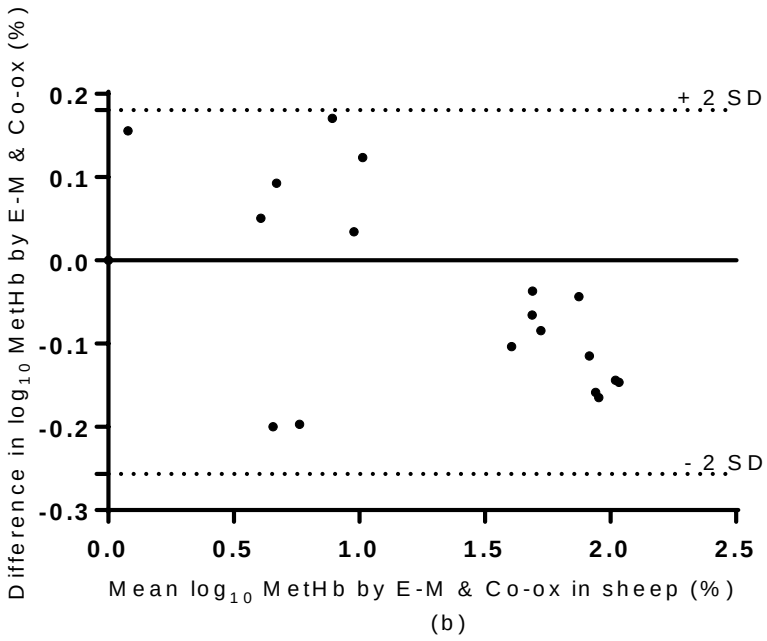

Figure 3.4. (b) Bland-Altman plot agreement between log10 MetHb values from sheep blood. The lower and upper agreement limits (bias ± 2 SD) are -0.25 and 0.18%, respectively.

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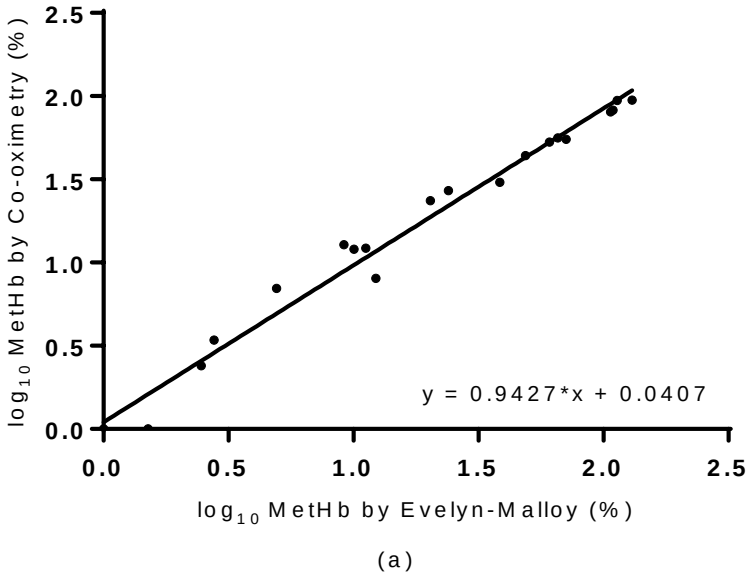

Figure 3.5. (a) Comparison of the of log10 MetHb values for goat produced by the Evelyn-Malloy and co-oximetry methods (P < 0.0001; R2 = 0.9812; Y= 0.9427*X + 0.04077).

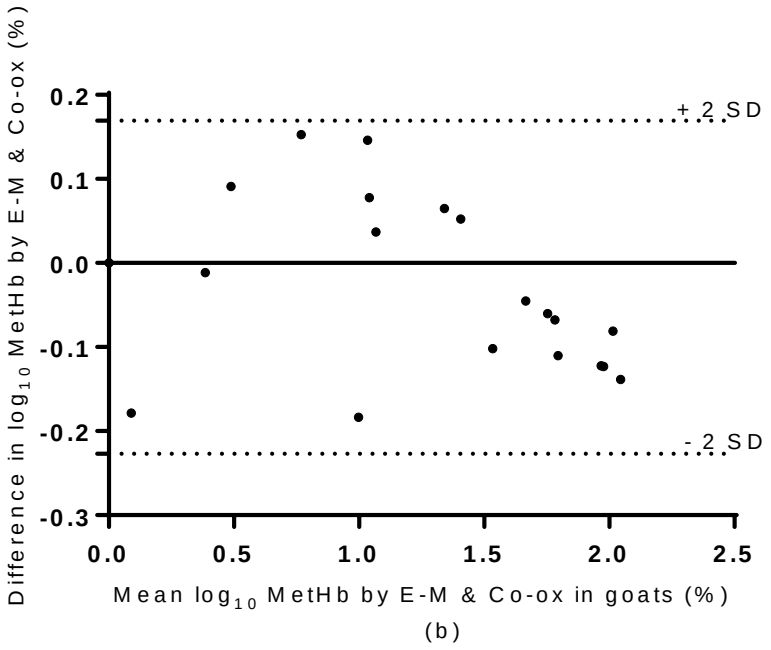

Figure 3.6. (b) Bland-Altman plot agreement between log10 MetHb values from goat blood. The lower and upper agreement limits (bias ± 2 SD) are -0.22 and 0.16%, respectively.

Table 3.4. Lin's concordance correlation coefficient (Rc) for the log10 MetHb values produced from cattle, sheep and goat blood samples and measured with the Evelyn-Malloy (1938) and co-oximetry methods.

	Cattle (n = 14)	Sheep (n = 22)	Goats (n = 21)
Rc	0.870	0.988	0.988
95% CI lower	0.679	0.975	0.973
95% CI upper	0.951	0.994	0.995
Lower one-sided 95% CL	0.720	0.978	0.977

3.4 Discussion

Before use in different species analytical methods should be validated to ensure clinical utility, particularly methods involving red cell lysis as is the case with co-oximetry. The present study, determined that there was significant correlation between the old Evelyn and Malloy (1938) method and the new co-oximetry method for the detection of MetHb concentration in cattle, sheep and goats. Pearson correlation coefficients were highly significant for all species. However, while still showing a strong correlation, the coefficient for cattle ($r^2 = 0.83$) was less than that determined for sheep ($r^2 = 0.98$) and goat ($r^2 = 0.98$) samples. The Bland-Altman and Lin's concordance coefficient analysis, after $\log_{10}$ transformation of the MetHb data, demonstrated that the results obtained from the sheep and goat samples were in substantial agreement between the Evelyn and Malloy (1938) and the co-oximetry methods. However, for the cattle samples, there was a greater discrepancy between the results obtained from the two methods. These findings invite the question as to why cattle samples were not in agreement to the same degree as the sheep and goat samples tested. This discrepancy may be due to technical issues, as the cattle samples were the first samples taken to assess agreement between the two methods and may have been subjected to

pipetting error within the Evelyn and Malloy (1938) method. This error influenced the 95% limits of agreement for Lin's concordance coefficient for the cattle samples with a much greater interval between the lower and upper limits compared with sheep and goats samples. In addition, the bias demonstrated in the Bland–Altman analysis was also greater for the cattle samples. After identification and correction of the pipetting error, the sheep and goat data significantly improved in all indices of agreement between the Evelyn and Malloy (1938) and co-oximetry methods. The pipetting error could have been avoided if the author had demonstrated competency in pipetting before undertaking the study.

In conclusion, the co-oximetry values for MetHb demonstrated significant correlation between the MetHb values obtained from the Evelyn and Malloy (1938) method for pooled sheep and goat blood. Co-oximetry may provide a more efficient and effective method to provide MetHb analysis in ruminants. Furthermore, co-oximetry derived MetHb values from cattle, sheep and goats are obtained by a direct measurement of blood and, thus, eliminate human error in the process. Co-oximetry is a safe, alternative method to the Evelyn and Malloy (1938) method for the measurement of MetHb.

CHAPTER 4 THE EFFECT OF FEEDING FREQUENCY AND DOSE RATE OF NITRATE SUPPLEMENTS ON BOS INDICUS CATTLE FED FLINDERS GRASS (*ISEILEMA* SPP.) HAY

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Authors: I. Benu, M.J. Callaghan, N. Tomkins, G. Hepworth, L.A. Fitzpatrick and A.J. Parker

Abstract

Twelve *Bos indicus* steers (liveweight ± SD., 317.8 ± 28.5 kg) were used in an experiment to examine the effect of two factors: daily nitrate dose (0, 30, 40 or 50 g of nitrate/day) and feeding frequency (once or twice a day) on MetHb concentration, daily peak MetHb concentration, rate of incline for MetHb concentration, carboxyhaemoglobin concentration, oxyhaemoglobin concentration, tHb, Hct, and DMI of Flinders grass hay. Increasing the dose rate of nitrate increased the fraction of MetHb in the blood of steers ($P = 0.014$). A highly significant effect was demonstrated for the interaction of dose rate x day ($P < 0.001$). For once a day intake of nitrate, the dose rates of 40 and 50 g per day showed a greater increase in mean MetHb values than for the 0 and 30 g of nitrate per day. Increasing the dose rate of nitrate also increased the daily peak MetHb fraction and the rate of incline to peak MetHb values for both once and twice a day feeding of the nitrate supplements. However, increasing the dose of nitrate had no significant overall effect on total haemoglobin, deoxyhaemoglobin, carboxyhaemoglobin, haematocrit, or dry matter intake. Twice a day feeding of nitrate decreased the development of MetHb in the blood of *Bos indicus* steers. This study demonstrates that caution should be exercised when feeding nitrates as a non-protein nitrogen source to cattle grazing low quality pastures in northern Australia.

4.1 Introduction

It is well accepted that nitrate can replace urea as a NPN source for ruminants (Nolan et al., 2010). Feeding nitrate has also been proposed as a means to reduce enteric methane production from cattle and sheep (Leng, 2008). The Australian Government has released a method for feeding nitrates to cattle to allow beef cattle producers to claim carbon credits for the offset in carbon emissions (Australian Government, 2014). This carbon offset methodology for cattle producers'states that nitrate can be fed safely to ruminants with several caveats. However, data supporting this methodology are largely based on either total mixed rations that include, forage, concentrates and nitrate, evenly mixed and presented to the animals, or as forage or hay sprayed with a known amount of nitrate and water. Both experimental methods allow for multiple meals containing a small amount of nitrate within a day for individual animals. This is in contrast to grazing systems in northern Australia where cattle graze low quality pasture grass and forage during the dry season when NPN supplements are usually fed. Nitrogen supplements are fed in an *ad libitum* manner with minimal human control over supplement consumption, resulting in variable intake patterns by animals (Eggington et al., 1990, Dixon et al., 2003). In addition, an animal's daily supplement consumption may occur at one feeding event in a 24 hour period (Cockwill et al., 2000). Although nitrate can replace urea as an NPN supplement, the capacity of the rumen microbial population to detoxify nitrate, or the nitrite derived from it, is believed to be energy-dependent and, therefore, progressively reduced under conditions of poor feed quality (Burrows et al., 1987, Valli, 2008). Furthermore, the rate of nitrate intake is a governing factor in the development of nitrate toxicity (Kemp et al., 1977). Therefore, it was hypothesised that the feeding frequency and quantity of nitrate consumed would affect the development of nitrate toxicity in *Bos indicus* cattle fed low quality pasture grass.

4.2 Materials and methods

4.2.1 Animals and management

All the experimental procedures were reviewed and approved by the James Cook University Animal Ethics Committee (No. A1929). Twelve two-year-old fistulated *Bos indicus* steers, with an initial body weight 271-357 kg, were used in this study. Throughout the experiment, the animals were allocated to individual pens (4.28m x 1.26m) within a covered cattle housing facility. The animals were given chaffed Flinders grass (*Iseilema* spp.) hay at three times (0800, 1200, and 1800 h) each day, in approximately equal amounts to ensure *ad libitum* intakes. The amount of hay given to individual animals was calculated from the previous day's actual intake + 20% extra hay (w:w). Samples of Flinders grass hay offered were taken, bulked over the course of the experiment and sub-sampled for analysis by a private feed analysis laboratory (Table 4.1; Symbio Laboratories, Brisbane, Queensland). Water was available to the animals *ad libitum* in 25 L containers. Hay refusals were recorded once daily at 0800 h. The animals were familiarized with handling and sampling procedures before the start of the experiment.

Table 4.1. Composition[a] of bulked Flinders grass (*Iseilema* spp.) hay throughout the experiment.

Test	Flinders Grass Hay
Protein, g/kg	29
Fat, g/kg	12
Moisture, g/kg	83
Ash, g/kg	86
Crude Fibre, g/kg	391
Dry Matter, g/kg	917
Nitrogen Free Extract DWB, g/kg	481
Acid Detergent Fibre, g/kg	524
Neutral Detergent Fibre, g/kg	721
Calcium, g/kg	3. 20
Phosphorous, g/kg	0. 64
Sodium, g/kg	0. 25
Sulphur, g/kg	0. 91

Source: Symbio Laboratories, Brisbane, Queensland

[a] All tests results except moisture are reported on a dry matter (DM) basis; moisture is reported on an as fed basis.

Preliminary chemical analysis of the Flinders grass hay demonstrated the forage comprised [g/kg dry matter (DM)] N: 0.88; P: 0.1; Na: 0.14 and S: 1.0. Feeding standards indicate that when cattle consuming low quality forages are supplemented with NPN, the efficiency of microbial protein production will peak at ~130 g microbial production/kg total digestible nutrients or digestible organic matter (NRC, 1996, CSIRO, 2007). Thus, all treatments were supplied with 15 g N daily (Table 4.2), which was the calculated amount to ensure adequate supply of rumen-degradable N. These calculations were reliant upon the preliminary chemical analysis of forage and assumed daily individual forage consumption at 5 kg DM. Further assumptions included 45% organic matter digestibility (Panjaitan et al., 2015) and

rumen degradability of forage protein was 0.8 (Bowen et al., 2008). The calculation ignored urea recycling to ensure there was an over-supply, rather than undersupply, of rumen-degradable N. The doses of 30, 40 and 50 g daily nitrate were achieved by blending urea and calcium nitrate decahydrate (Swancorp, Rocklea, Queensland, Australia). In order to correct P, Na and S deficiencies in the forage, 25 g of MDCP (Biofos®) and 25 g of sodium sulphate were added to all nitrogen supplements daily. Supplements containing urea and/or nitrate, MDCP, and sodium sulphate were premixed and stored in desiccators until required for daily dosing.

Table 4.2. Composition of raw materials and formulated analysis of nitrogen (N), nitrate (NO_3), phosphate (P), sodium (Na) and sulphate (S) in supplement treatments.

Composition	Treatments (g NO_3/day)			
	0	30	40	50
Urea (g/day)	32.5	16.5	11	5.5
Calcium Nitrate decahydrate (g/day)	0	48	64	80
Sodium Sulphate(g/day)	25	25	25	25
MDCP(g/day)	25	25	25	25
N (g/day)	15.2	15.2	15.1	15.0
NO_3 (g/day)	0	30.3	40.4	50.5
S (g/day)	5.6	5.6	5.6	5.6
P (g/day)	5.3	5.3	5.3	5.3
Na (g/day)	8.1	8.1	8.1	8.1

4.2.2 Experimental Design

The experimental design had an incomplete block structure with the 12 animals as blocks. Two treatment factors were examined: daily nitrate dose and feeding frequency. There were four levels of nitrate dose: 0 (Urea; U), 30, 40 and 50 g of nitrate daily. The nitrate treatments were dosed either once a day at 0700 h (1), or divided equally and dosed twice daily at 0700 and 1700 h (2). Due to a limitation in the number of individual pens (n = 12), the experiment was conducted over two time periods, with a clearance time of 14 days between experimental

periods. The two periods resulted in there being 24 experimental units in total, which gave three replicates of each of the eight treatment combinations. The design was as balanced as possible, with no combination occurring together with another combination more than once in the same block. Each experimental period consisted of a 6 day preliminary feeding period where steers were fed hay only, followed by a 7 day treatment period. Previous work at CSIRO Lansdowne Research Station has demonstrated that 10 days is sufficient to return the animal to a normal concentration of MetHb (Tomkins *pers. comm*., 2013).

4.2.3 Measurement and Sampling

Indwelling venous jugular catheters were fitted to steers using a modified technique adapted from Parker et al. (2009) on day 6 of feeding the adaptation period diets. On day 0, blood samples were obtained at 2 hourly intervals starting at 0600 hours, and continued for a period of 7 days. Blood was drawn from tubing using 3 mL heparinised pre-set syringes (Becton Dickinson), placed in crushed ice and analysed for total haemoglobin, oxyhaemoglobin, deoxyhaemoglobin, MetHb, and carboxyhaemoglobin in blood using a blood gas analyser (Siemens, Rapid Lab 1265, Sydney, NSW, Australia). After each blood sample was obtained 10 mL heparinised saline (15000 IU heparin) was flushed through the line to prevent clotting. The steers were randomly allocated to treatments and individual pens within the cattle house facility. Steers remained in the same pens throughout the experiment. On day 2, one steer in the 40 g of nitrate dosed once a day treatment was found to have a MetHb concentration greater than 75% and was treated with a methylene blue solution and removed from the study, in accordance with animal ethics protocols.

4.2.4 Statistical analysis

The data were analysed by fitting linear mixed models, using the REML algorithm of the GENSTAT statistical package. The analysis considered the effects of dose rate (0, 30, 40, and

50 g of nitrate/day), feeding frequency (once or twice a day), day (1, 2, 3, 4, 5, 6, and 7) and their interactions. Day was also fitted as a continuous variable or covariate (instead of a factor) in the model to estimate trends. Time of day was also included for variables which were not aggregated over time. Separate analyses for once and twice a day feeding frequencies were also conducted because they provided illumination and clearer interpretation. Adjusted means were calculated from the REML algorithm for comparison in the fitted models.

The total haemoglobin and carboxyhaemoglobin data were log-transformed before analysis. Plots of residuals versus fitted values for the other variables were consistent with the assumption of constant variance and, therefore, the data were not transformed. The peak MetHb values were defined by the following rules: when frequency equalled 1 or 2, the greatest reading between 0600 and 1400 hours inclusive was selected. The rate of incline for MetHb in the model was defined as the peak reading, minus the reading 4 h before the peak. The reported means and slopes for rate of incline were divided by four to present an hourly rate.

4.3 Results

4.3.1 Total methaemoglobin

The total mean MetHb concentration (%) in the blood of steers increased when nitrate concentration in the diet increased and was given as a singular bolus ($P = 0.014$; Figure 4.1), or given as two equal portions throughout the day ($P < 0.001$; Figure 4.2 and 4.3). The response was also time-dependent with increasing MetHb values over the 7 days of the experiment when steers were dosed once ($P < 0.001$; Figure 4.1 and 4.3) and twice ($P < 0.001$; Figure 4.2 and 4.3) daily.

A highly significant effect was demonstrated for the interaction of dose rate x day ($P < 0.001$) when nitrate treatments were dosed once a day. Steers treated with 40 or 50 g of nitrate per day showed a greater increase in mean MetHb values than for the 0 and 30 g of nitrate per day (Figure 4.3). However, the interaction of dose x rate day was not significant for twice a day feeding of the nitrate treatments.

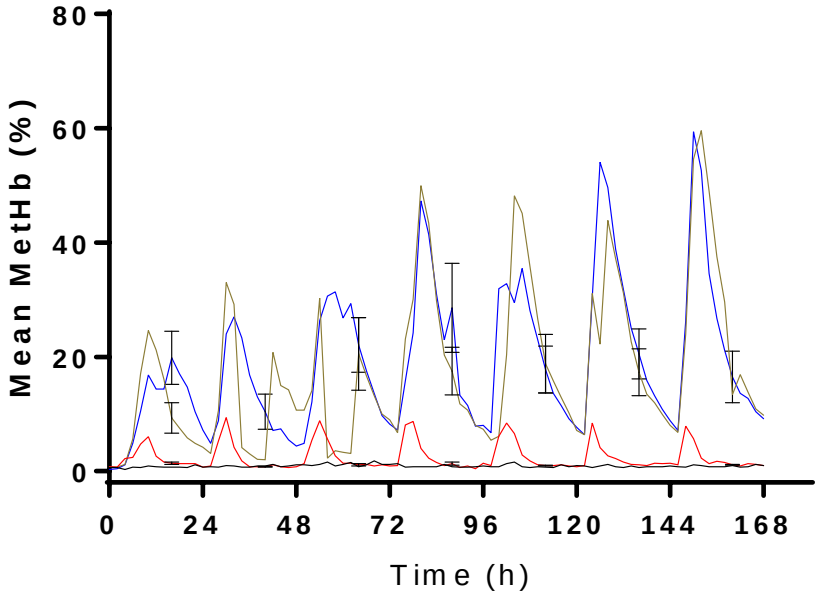

Figure 4.1. Actual mean ± SEM MetHb concentration (%) from venous blood of *Bos indicus* steers treated with 0 (—), 30 (—), 40 (—) or 50 (—) g of nitrate as a non-protein nitrogen supplement once a day in a single dose at 0700 h for seven days.

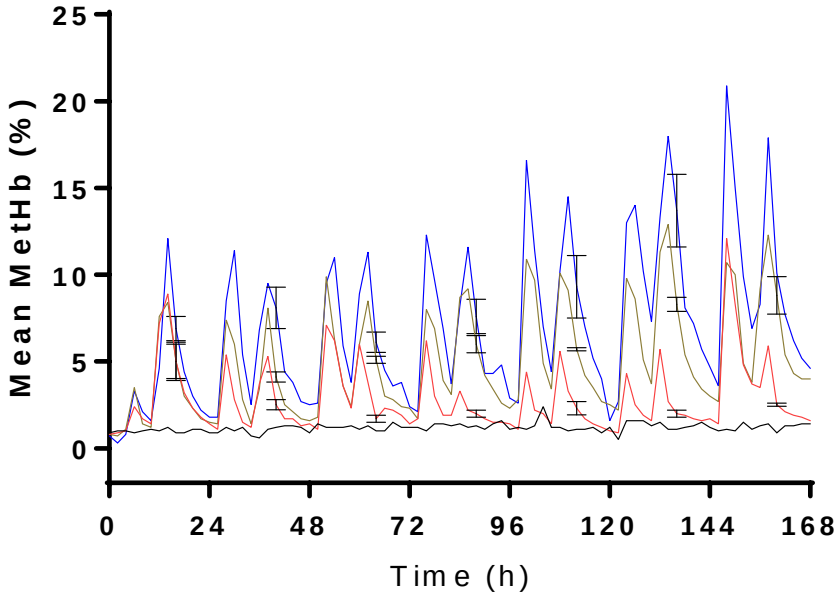

Figure 4.2. Actual mean ± SEM MetHb concentration (%) from venous blood of *Bos indicus* steers treated with 0 (—), 30 (—), 40 (—) or 50 (—) g of nitrate as a non-protein nitrogen supplement divided into two equal portions and given at 0700 and 1700 h for seven days.

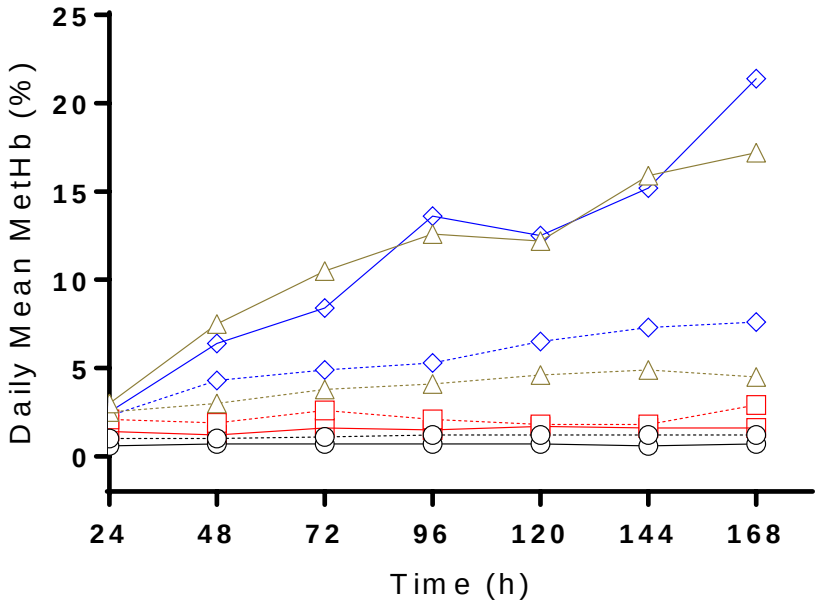

Figure 4.3. Back transformed adjusted means for MetHb (%) in venous blood of *Bos indicus* steers dosed with 0 (—○—), 30 (—□—), 40 (—Δ—) or 50 (—◊—) g of nitrate once a day at 0700 hours or dosed with 0 (--○--), 30 (--□--), 40 (--Δ--) or 50 (--◊--) g of nitrate divided into equal portions and administered at 0700 hours and 1700 h for seven days.

4.3.2 Methaemoglobin Peaks

The daily peak MetHb values increased when the nitrate concentrations in the diet increased and was given as a single bolus (P = 0.011; Figure 4.1 and. 4.4) or was divided equally into two portions and given twice daily (P = 0.002; Figure 4.2 and.4.4). The daily peak MetHb concentrations demonstrated time-dependent effects when nitrate was administered once (P < 0.001; Figure 4.1 and. 4.4) and twice (P < 0.001; Figure 4.2 and 4.4) a day. The dose rate x day interaction was highly significant (P < 0.001, average s.e.d. = 12.5%; Figure 4.4) for daily peak MetHb concentrations in the steers given nitrate once a day. Although daily peak MetHb values were non-existent for the zero nitrate-treated animals and the dose rate of 30 g of nitrate produced relatively stable adjusted mean daily peak values of between 7% and 11%

MetHb from day 1 to day 7. The dose rates of 40 and 50 g of nitrate per day demonstrated adjusted mean daily peak MetHb concentrations that increased over time, from 21.2% and 28.5% on day 1 to 53.3% and 59.4% on day 7, respectively.

A dose rate x day interaction for daily MetHb peak values was also demonstrated when nitrate treatments were dosed twice a day (P = 0.018, average s.e.d. 3.34; Figure. 4.4). As expected, the adjusted means for daily peak MetHb concentrations in the zero nitrate-treated steers was not remarkable. However, the adjusted means for the daily peak MetHb values from the steers treated with 30 g of nitrate varied daily, at 7%, 5.7%, 7.8%, 4.5%, 4.7%, 4.7% and 11% on days 1, 2, 3, 4, 5, 6 and 7, respectively. Moreover, the adjusted means for the daily peak MetHb values from the steers treated with 50 g of nitrate increased progressively each day from 9.2% on day 1 to 19.4% on day 7.

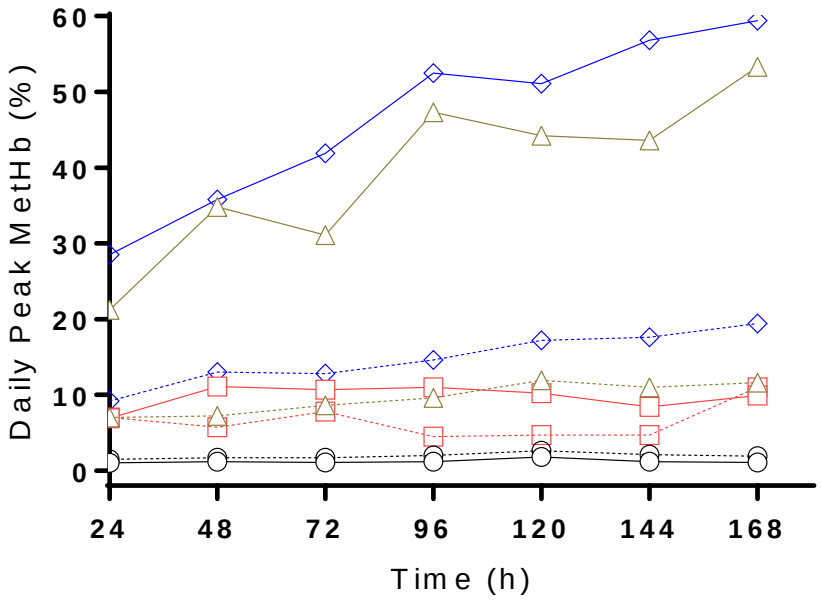

Figure 4.4. Adjusted means for daily peak MetHb (%) in venous blood of *Bos indicus* steers dosed with 0 (—○—), 30 (—□—), 40 (—Δ—) or 50 (—◊—) g of nitrate once a day at 0700 h (Ave SED = 12.5) or dosed 0 (--○--), 30 (--□--), 40 (--Δ--) or 50 (--◊--) g of nitrate.

4.3.3 Methaemoglobin rate of incline

The adjusted means for the rate of incline in MetHb per hour for the 4 h before the daily peak MetHb value increased with dose rate ($P = 0.014$) and day ($P < 0.001$) for the steers dosed once a day. Furthermore, the dose rate x day interaction was evident for once a day dosing of treatments ($P = 0.029$, s.e.d 2.5%). Specifically, when steers were administered with 40 and 50 g of nitrate, the adjusted means for the rate of incline for MetHb concentrations demonstrated an increase from 4.3% and 6.1% /h on day 1 to 10.1% /h on day 7, respectively. The dose rate of 30 g of nitrate per day resulted in adjusted means for the rate of incline for the zero nitrate treatment, and were non-existent at a range of 0.1% to 0.3%.

Similarly, twice a day dosing of nitrate treatments increased the rate of incline for MetHb concentration in the blood of steers with increasing dose rate ($P = 0.001$). The adjusted means for the hourly rate of incline for MetHb for the 0, 30, 40 and 50 g of nitrate treatments were 0.16%, 1.2%, 1.7% and 2.8% per hour (average s.e.d. =0.32%) for the 4 h immediately before the peak daily MetHb concentration. There was no effect of day, or the dose rate x day interaction, when the nitrate treatments were provided twice a day.

4.3.4 Oxyhaemoglobin

The oxyhaemoglobin concentration in the blood decreased when the nitrate concentration of the steer's diet increased and they was given as a single dose (Figure 4.5). This response was both dose-dependent ($P = 0.040$) and time-dependent ($P < 0.001$). In addition, a significant dose rate x day interaction was demonstrated for oxyhaemoglobin ($P = 0.006$, average s.e.d. = 5.5%). Oxyhaemoglobin remained consistent for the non-nitrate-treated animals, and decreased by 7.3% between day 1 and day 7 in the animals dosed with 30 g of nitrate per day. Furthermore, oxyhaemoglobin decreased by 18% between day 1 and day 7 in the animals

dosed with 40 and 50 g of nitrate per day (Figure 4.5). However, with the twice daily dosing there was no effect of dose rate, although there was a time effect ($P < 0.001$).

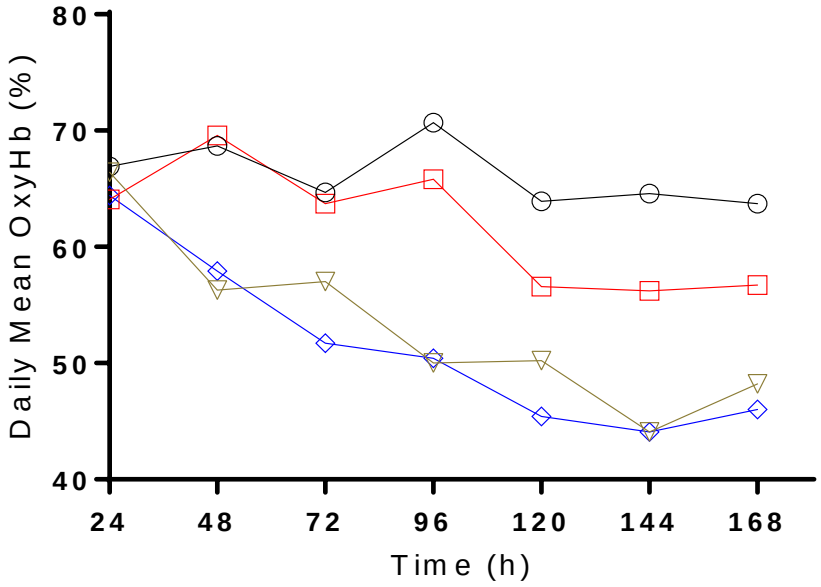

Figure 4.5. Adjusted means for daily oxyhaemoglobin (%) in venous blood of *Bos indicus* steers treated with 0 (—○—), 30 (—□—) 40 (—Δ—) or 50 (—◊—) g of nitrate as a non-protein nitrogen supplement once a day in a single dose at 0700 h for seven days (Ave SED = 5.5).

4.3.5 Total Haemoglobin

Neither dose rate nor day had a significant overall effect on tHb concentration in steers dosed once a day. However, the day x dose rate interaction was highly significant for steers dosed once a day ($P < 0.001$). The steers treated with 50 g of nitrate had the lowest tHb concentration on day 4, compared to the other treatments. The mean tHb values tended to decrease from day 1 to day 4 for all treatments. In addition, the estimated slopes for each dose rate in the model were 0.10g tHb/L.day for 0 g, -1.64g tHb/L.day for 30 g, -1.44g tHb/L.day for 40 g and 1.099g tHb/L.day for 50 g.

Dose rate had no significant effect on tHb concentrations when dosed twice a day. An effect of day was demonstrated for twice a day dosing of treatments ($P < 0.001$). The predicted means in the model for total haemoglobin for days 1, 2, 3, 4, 5, 6, and 7 were 95, 91, 89, 85, 88, 88, and 86 g tHb/L (s.e.d. = 2 g tHb/L). The overall estimated slope for day in the model was a decrease in tHb of 1.2 g/L.day. The day x dose rate interaction was not significant for twice a day dosing of the treatments.

4.3.6 Deoxyhaemoglobin

Overall deoxyhaemoglobin increased over the 7 days of the experiment for the steers dosed once a day ($P < 0.001$) and twice a day ($P = 0.023$). The predicted means for day in the model for deoxyhaemoglobin for days 1, 2, 3, 4, 5, 6, and 7 were 28, 28, 29, 28, 33, 34, and 32% (s.e.d. = 2%), respectively, for the steers dosed once a day. However, there was no significant effect of dose rate or the interaction between dose rate and day for either single or twice a day dosing.

4.3.7 Carboxyhaemoglobin

Carboxyhaemoglobin was log-transformed to better satisfy the assumption of constant variance. A significant effect day was demonstrated for once a day dosing ($P = 0.013$). The back transformed means for days 1, 2, 3, 4, 5, 6 and 7 were 1.87%, 1.6%, 1.8%, 1.95%, 2.6%, 2.7% and 2.2%, respectively. However, there was no significant effect of day demonstrated for twice a day dosing. Dose rate had no significant effect on the carboxyhaemoglobin fraction in the blood of steers dosed once or twice a day. The dose rate x day interaction was not significant for either the once or twice a day treatments.

4.3.8 Haematocrit

Dose rate had no significant effect on haematocrit values when treatments were dosed once per day. A significant effect of day was observed for haematocrit values ($P = 0.004$) and twice ($P < 0.001$) a day dosing. The steers dosed once a day demonstrated a decreased in haematocrit values until day 4. A significant effect of day x dose rate interaction for haematocrit values from steers dosed once per day was demonstrated ($P = 0.016$) but not for the steers dosed twice a day. The estimated effect of day in the model indicated an overall decrease in haematocrit values of 0.017% per day when nitrate treatments were dosed once a day. The predicted means in the model for days 1, 2, 3, 4, 5, 6, and 7 were 28, 27, 26, 25, 25, 26, and 26%, respectively (average s.e.d. = 0.47).

4.3.9 Dry Matter Intake

The DM intake (DMI) of Flinders grass hay was not affected by dose rate or day for both frequencies of nitrate intake. The mean DMI of the hay for the 0, 30, 40 and 50 g of nitrate treatments was 4.2 kg, 4.4 kg, 4.1kg and 4.6 kg, respectively (average s.e.d. 0.52 kg).

4.4 Discussion

Thresholds for nitrate toxicity are often reported on a body weight (g NO_3/kg BW) (Alaboudi and Jones, 1985, Takahashi et al., 1998, Nagy et al., 2012) or dry matter intake (g NO_3/kg DMI) (Li et al., 2012) basis. However, nitrate poisoning in cattle is principally governed by the amount and rate at which nitrate is consumed by the animal (Jainudeen et al., 1964, Vermunt and Visser, 1987). In the present study, a single dose of 30, 40 or 50 g of nitrate into the rumen of *Bos indicus* steers caused a greater increase in the MetHb concentration of their blood than the equivalent dose divided into two equal portions and administered in the morning and afternoon. Studies that have used a total mixed ration comprising concentrates

whereby small meals of nitrate are consumed over the course of 24 hours report low concentrations of MetHb in the blood of ruminants (van Zijderveld et al., 2010; ≤7% MetHb; Alaboudi and Jones, 1985; < 2% MetHb, Li et al., 2012; < 2.8% MetHb). In contrast, a single dose of nitrate administered to cattle through a rumen cannula in this experiment resulted in greater concentrations of MetHb in the blood of cattle.

Another important consideration of studies reporting low concentrations of MetHb in the blood of ruminants fed nitrate compounds is that the authors of previous articles do not quantify the amount of nitrate consumed before the blood sample was taken (Alaboudi and Jones, 1985, van Zijderveld et al., 2010, Li et al., 2012). Therefore, the timing of blood sampling may or may not align with the peak MetHb concentration for the day. The peak MetHb concentration is important because it determines the amount of time the animal is placed in a hypoxic state. The peak MetHb concentration usually occurs in cattle at three to five hours after dosing, with a single dose of nitrate (Holtenius, 1957, Kemp et al., 1977). Clinical signs of hypoxia develop when 20 to 30% of an animal's haemoglobin is converted to MetHb (Parkinson et al., 2010). The steers receiving 40 or 50 g of nitrate once a day demonstrated a peak of over 20% MetHb on the first day indicating the development of hypoxia in these animals. The peak MetHb concentration increased over the seven days of the experiment for the animals dosed once a day with 40 or 50 g of nitrate. In addition, the steers treated with 50 g divided into two doses also had their peak MetHb concentrations doubled over seven days. Moreover, the rate of incline (% MetHb/h) of MetHb for once a day dosing of 40 and 50 g of nitrate doubled over the seven days.

Leng (2008) hypothesised that nitrate may be recycled in the saliva of ruminants. The recycling of nitrate in saliva may explain why the steers at the higher dose rate of nitrate increased the rate of response to dosing, as measured by the rate of incline to peak MetHb values. The reduction of nitrate to nitrite and then to ammonia is controlled by the bacterial

populations of the rumen and alimentary tract. If nitrate was absorbed into the blood and recycled into the rumen, via saliva or other transport mechanisms, then the production of nitrite from one dose may continue for greater than 24 hours, thereby, maintaining an elevated MetHb concentration in the blood. The demonstration of cause and effect for this hypothesis is beyond the data collected during this experiment; nevertheless, our data support the nitrate recycling hypothesis of (Leng, 2008). These are important findings because the consistent trend evident in the development of MetHb in the steers treated with 40 or 50 g of nitrate once a day would suggest that an adaptation to nitrate does not occur under these experimental conditions.

The increase in carboxyhaemoglobin over time for the once a day treated animals is a notable finding that has not been previously reported. Carbon monoxide competes with oxygen for haem sites on haemoglobin molecules and decreases the ability of haemoglobin to carry and release oxygen to tissues (Hall, 2011). The only endogenous source of carbon monoxide in the mammalian body results from the metabolism of intravascular haemoglobin to biliverdin, which is catalysed by haem oxygenase-1 (Maines, 1988). Haem oxygenase-1 is induced in response to a broad spectrum of stimuli and agents: such as, haem, metal ions, oxidative stress, bacterial toxins, starvation and haemolytic diseases such as babesiosis (Maines, 1988, Taylor et al., 1991, Kadinov et al., 2002). It is likely that oxidative damage to red blood cells, induced by the once a day treatments of nitrate resulted in an expression of hemeoxygenase-1, thereby increasing carboxyhaemoglobin concentrations. As the experiment progressed, the increase in carboxyhaemoglobin as would have compounded the hypoxia caused by the MetHb fraction in the nitrate treated animals.

The oxyhaemoglobin fraction in the present study was affected by nitrate in the diet of steers. The oxyhaemoglobin fraction in the blood of steers decreased each day, in a dose dependant manner, for steers dosed once a day. However, twice a day dosing of nitrate treatments did

not have the same effect. This would imply that multiple meals throughout the day impart a degree of protection against elevated dyshaemoglobins when nitrate is consumed.

Total haemoglobin and haematocrit values showed little biological change over the seven days of the experiment. Large increases in total haemoglobin and haematocrit have been reported in dairy heifers (Jainudeen et al., 1964). Jainudeen et al. (1964) stated that the increases in haematocrit and total haemoglobin were adaptive mechanisms by the animal in order to deal with high levels of nitrate in the diet. Others have found no increases in haematocrit and total haemoglobin in rats administered with acute doses of nitrate salts (Imaizumi et al., 1980).

Dry matter intake by *Bos indicus* steers was not influenced by nitrate dose. In the present study, all supplement treatments were iso-nitrogenous and supplied 15 g N on a daily basis. This finding is consistent with recent results reported by Nolan et al. (2010) and Li et al. (2012) in sheep supplemented with nitrate or urea.

4.5 Conclusion

The dose rate of nitrate consumed and feeding frequency affected the development of nitrate toxicity in *Bos indicus* steers consuming low quality native pasture hay. If nitrate salts are fed as a single dose of 50 g of nitrate to a 320 kg steer then it is likely that a dyshaemoglobinaemia will develop over time until the animal experiences a hypoxic crisis. However, the severity of nitrate toxicoses in cattle can be reduced if the daily nitrate dose is divided over two intake events, or is fed at a lesser rate than 50 g/320 kg LW. The authors recommend that caution should be exercised when intentionally feeding nitrate salts as a urea substitute and/or a methane abatement strategy, due to the inherent variation in intake of dry season supplements and, therefore, the risk of over consumption of the nitrate salt.

CHAPTER 5 THE EFFECT OF NITRATE SUPPLEMENTATION ON ARTERIAL BLOOD GASES, HAEMOGLOBIN FRACTIONS AND HEART RATE IN BOS INDICUS CATTLE AFTER EXERCISE

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Authors: I. Benu, L.A. Fitzpatrick, M.J. Callaghan, N. Tomkins and A.J. Parker

Abstract

The objective of this study was to investigate the effects of nitrate treatment on the arterial blood gas and haemoglobin fractions from *Bos indicus* steers after exercise. *Bos indicus* steers (n = 12; Mean body weight ± SEM, 397 kg ± 10.84 kg) were used in this experiment to investigate the effects of three dose rates of nitrate salts (0, 30 or 50 g of nitrate/day) on arterial blood gases, MetHb concentration, carboxyhaemoglobin concentration, oxyhaemoglobin concentration, total haemoglobin concentration, haematocrit, heart rate, and respiratory rate, after exercise. Doses of supplements were placed directly into the rumen of individual steers once daily, at 0630 h, for 7 days. Increasing the dose rate of nitrate resulted in a decrease in the partial pressure of oxygen (P = 0.004) in blood. Steers treated with 50 g nitrate/day had a decrease in oxyhaemoglobin concentration (P = 0.001), and a concomitant increase in MetHb (P = 0.001) and carboxyhaemoglobin (P = 0.001) compared with steers treated with 0 or 30 g nitrate/day. Steers dosed with 50 g of nitrate had greater heart rates immediately after the exercise regimen than the steers dosed with 30 g of nitrate (P = 0.043) or no nitrate (P = 0.018). There was no difference between treatments for respiratory rate (P = 0.673) or rectal temperature (P = 0.207) after the exercise regimen. Feeding nitrate to *Bos indicus* cattle resulted in a decrease in the oxygen carrying capacity of their blood. It is likely

that doses of nitrate greater than 50 g per day for this class of animal could induce hypoxic trauma if cattle have exercise imposed after consuming a nitrate supplement.

5.1 Introduction

Dietary nitrate has the potential to reduce methane emissions in ruminants by providing an alternative electron sink in the reduction of nitrate to nitrite and, ultimately, decrease the production of ruminal ammonia (Nolan et al., 2010). It has been proposed that nitrate salts could replace urea in rangeland supplements, to provide an ammonia source for microbial growth while decreasing the carbon emissions from ruminant based grazing industries (Leng, 2008). Furthermore, the Australian Government has endorsed the feeding of nitrate to cattle in rangelands if beef producers are to claim carbon credits (Australian Government, 2014). However, excess nitrate in the diet can be toxic to ruminants. Feeding nitrate to cattle results in increased concentrations of nitrite in the blood. In such circumstances blood nitrite concentrations will oxidise haemoglobin to methaemoglobin (MetHb). Increased concentrations of MetHb cause a decrease in the ability of the blood to transport oxygen to active tissues, and hypoxemia ensues. The hypoxemia may result in dyspnoea, muscle tremors, ataxia, incoordination, and weakness in the animal affected by the ingestion of nitrate (Vermunt and Visser, 1987).

In northern Australia, cattle will walk long distances when grazing; however, the animal's ability to walk will be compromised if the animal is hypoxemic. Therefore, we questioned the effect of a nitrate supplement on the arterial blood gases and heart rate of *Bos indicus* steers when exercised. The working hypothesis was that a dose of 50 g of nitrate once a day would compromise the respiratory and cardiac function of cattle compared with consumption of an iso-nitrogenous amount of non-protein-nitrogen without nitrate.

5.2 Materials and Methods

5.2.1 Animals and management

The present research was conducted at the Fletcherview Research Station of James Cook University (20°04.603'S / 146°15.812'E) near Charters Towers, Queensland, Australia. The experimental procedures were reviewed and approved by the James Cook University Animal Ethics Committee (No. A1929). *Bos indicus* steers (n = 12; 2 years of age; mean body weight = 397 kg ± 11 kg), with indwelling rumen cannulas, were used in this study. The animals were kept as one group in the cattle yards, with no access to grazing and they were provided Flinders grass (*Iseilema* spp.) hay and water *ad libitum* throughout the experiment. Samples of Flinders grass hay offered were taken, bulked over the course of the experiment, and sub-sampled for analysis of nutritive value by a private feed analysis laboratory (Table 5.1; Symbio Laboratories, Brisbane, QLD, Australia).

The animals were trained and familiarized with the handling and sampling procedures before the commencement of the experiment. Steers were ranked on body weight and then randomly allocated to one of three nitrate treatments; 0, 30, and 50 g of nitrate daily (Table 5.2). The 50 g of nitrate treatment is the recommended intake of nitrate, by cattle of this class, which is required to reduce methane emissions in accordance with Australian legislation (Australian Government, 2014). Previous research suggested that the 30 g of nitrate was thought to be the safe dose of nitrate for this class of cattle from (Benu et al., 2015). Preliminary chemical analysis of the Flinders grass hay demonstrated the forage comprised (g/kg DM) N: 0.88; P: 0.1; Na: 0.14 and S: 1.0.

Feeding standards indicate that when cattle consuming poor quality forages are supplemented with NPN, the efficiency of microbial protein production (eMCP) will peak at ~130 g MCP/kg total digestible nutrients, or digestible organic matter (DOM; NRC 1996; CSIRO

2007). Thus, all treatments were supplied with 15 g N daily (Table. 5.2) which was the amount calculated to ensure adequate supply of rumen degradable nitrogen. These calculations were reliant upon the preliminary chemical analysis of forage and assumed daily individual forage consumption at 5 kg DM. Further assumptions included 45% OMD (Panjaitan et al., 2015) and rumen degradability of the forage protein was 0.8 (Bowen et al., 2008). The calculation ignored urea recycling to ensure there was an over-supply, rather than undersupply, of RDN. The doses of 0, 30 and 50 g nitrate daily were achieved by blending urea and calcium nitrate decahydrate (Swancorp, Rocklea, QLD, Australia). To correct P, Na and S deficiencies in the forage, 25 g of MDCP (Biofos®) and 25 g sodium sulphate were added to all nitrogen supplements daily. Supplements containing urea and/or nitrate, MDCP, and sodium sulphate were premixed and stored in desiccators until required for daily dosing.

Doses of supplements were placed directly into the rumen of individual steers once daily, at 0630 h, for 7 days. On Day 7 after treatment initiation, the animals were randomly allocated to one of four blocks for the imposition of exercise regimens. Each block of three steers contained an animal from each of the treatments. The animals representing each treatment group were walked in groups of three, by two stockmen on horses. A GPS hiking device (Garmin Etrex 20, Garmin Corp. Olathe, Kansas) was attached to the halter of each of the steers. Horsemen, placed at the front and rear of the group of three steers, attempted to standardise the distance and speed of the animals by directing the steers through a set course. The steers walked for an average period of 24 minutes at a speed of 3.3 km/h. All steers assigned to the various treatment blocks were walked over the same terrain for a mean distance of 2.8 km. The first group of three animals was released at 0830 h. The experiment concluded with the final measurements completed at 1230 h.

Heart rate was measured immediately before and after exercise on Day 7 of treatments. Heart rate was measured by placing a Polar Equine Belt (Polar electro Oy, Kempele, Finland)

around the chest of the animal. Respiration rate was determined by counting the number of flank movements for the period of 15 seconds and multiplying the value by four. Body temperature was recorded using a thermometer inserted into the rectum of the animal for 1 minute. Arterial blood samples for blood gas and co-oximetry analysis were obtained from the caudal auricular artery (Riley and Thompson, 1978), immediately after exercise. A 22 G (0.9 x 25 mm) intra-arterial catheter (Optiva, Johnson and Johnson Int. Belgium) was utilized with a 1 mL blood gas syringe containing lithium heparin to sample arterial blood. Blood gas syringes were capped and placed into iced water for immediate analysis of blood gases. All blood gas assays were performed within 0.5 h of collection. Arterial blood pH, partial pressure of oxygen (pO_2), partial pressure of carbon dioxide (pCO_2), bicarbonate, base excess (BE), and blood co-oximetry were measured using a blood gas analyser (Siemens Rapid Lab 1265, Seimens Health Care, Sydney, NSW).

Table 5.1. Composition[a] of bulked Flinders grass (*Iseilema* spp.) hay throughout the experiment.

Test	Flinders Grass Hay
Protein, g/kg	2. 9
Fat, g/kg	1. 2
Moisture, g/kg	8. 3
Ash, g/kg	8. 6
Crude Fibre, g/kg	39. 1
Dry Matter, g/kg	91. 7
Nitrogen Free Extract DWB, g/kg	48. 1
Acid Detergent Fibre, g/kg	52. 4
Neutral Detergent Fibre, g/kg	72. 1
Calcium, g/kg	3. 20
Phosphorous, g/kg	0. 64

Sodium, g/kg	0. 25
Sulphur, g/kg	0. 91

Source: Symbio Laboratories, Brisbane, Queensland.

[a]All test results except moisture are reported on a dry matter (DM) basis; Moisture is reported on as fed basis.

Table 5.2. Composition of raw materials and formulated analysis of N, NO3, P, Na and S of supplement treatments.

Composition	Treatments g NO_3/day		
	0	30	50
Urea, g/day	32.5	16.5	5.5
Calcium Nitrate decahydrate, g/day	0	48	80
Sodium Sulphate, g/day	25	25	25
MDCP, g/day	25	25	25
N, g/day	15.16	15.16	15.01
NO_3, g/day	0	30.3	50.5
S, g/day	5.6	5.6	5.6
P, g/day	5.3	5.3	5.3
Na, g/day	8.1	8.1	8.1

5.2.2 Data Analysis

Statistical sample size determination was performed using a sample size calculator for the oxyhaemoglobin concentrations in cattle (Austvet Epi Tools; sample size calculation, Sergeant (2016). Least squares means and standard errors are presented. Data were analysed using a one way ANOVA with treatment as the sole source of variation in the model. The ANOVA, was conducted using the IBM SPSS Statistics for Windows, Version 22.0 software package (IBM corp. Armonk, NY). Multiple comparison tests were undertaken using Fisher's LSD test, where the level of significance was set at $P < 0.05$.

5.3 Results

Arterial blood pH did not differ between treatments (P = 0.124, Table 5.3). Similarly, there were no differences between treatments for pCO_2 (P = 0.579), HCO_3 (P = 0.514), and BE (P = 0.516). However, differences between treatments were recorded for pO_2 (P = 0.005). *Post hoc* tests for pO_2 indicated that administration of 50 g of nitrate per day caused a decrease in the pO_2 value in arterial blood, compared with the animals not receiving nitrate (P = 0.004). There was no difference between pO_2 means for steers dosed with 50 g of nitrate or 30 g of nitrate per day (P = 0.098).

A difference could not be detected between treatments for total haemoglobin concentration among treatments (P = 0.059; Table 5.4). Fractional proportions of deoxyhaemoglobin (FHHb) did not differ between treatments either (P = 0.289). However, oxyhaemoglobin concentrations were statistically different between treatments (P = 0.001). *Post hoc* tests indicated oxyhaemoglobin was less in the animals treated with 50 g of nitrate compared with the animals not treated with nitrate (P = 0.001), or those treated with 30 g of nitrate (P = 0.001). There were differences among treatments for MetHb concentrations (P = 0.000). Dosing steers with 50 g of nitrate resulted in a greater concentration of MetHb in the blood than steers dosed with 30 g of nitrate (P = 0.003), or those not treated with nitrate (P = 0.000). Carboxyhaemoglobin also differed between treatments (P = 0.001). Treatment with 50 g of nitrate/day caused a higher concentration of carboxyhaemoglobin in the blood compared with steers dosed with 30 g of nitrate (P = 0.003), or those not treated with nitrate (P = 0.002). There were no differences between the steers dosed with 30 g of nitrate per day and the steers dosed with no nitrate per day (P = 0.879). Haematocrit values did not differ between treatments (P = 0.583).

There was no difference among treatments for heart rate before the imposition of the exercise regimen (P = 0.282; Table 5.5). However, immediately after walking 3 kilometres, the steer's' heart rates were different among treatments (P = 0.016). Steers dosed with 50 g of nitrate had higher heart rates, immediately after the exercise regimen, than the steers dosed with 30 g of nitrate (P = 0.043), or those that did not receive nitrate (P = 0.018). There was no difference between treatments for respiratory rate (P = 0.673) or rectal temperature (P = 0.207) after the imposition of the exercise regimen.

Table 5.3. Least squares means ± SD for arterial blood pH, partial pressure of oxygen (pO_2), partial pressure of carbon dioxide (pCO_2), bicarbonate (HCO_3) and base excess (BE) for *Bos indicus* steers treated with 0, 30 or 50 g of nitrate per day for seven days and walked three kilometres.

Blood variables	Treatment, g NO_3/day			ANOVA
	0	30	50	P values
pH	7.48 ± 0.01	7.50 ± 0.01	7.47 ± 0.00	0.124
pO_2, mm Hg	99.42 ± 5.90[a]	82.90 ± 6.78[ab]	64.60 ± 3.07[b]	0.005
pCO_2, mm Hg	39.45 ± 1.28	38.95 ± 2.05	41.47 ± 1.83	0.579
HCO_3, mmol/L	29.15 ± 1.16	30.67 ± 0.87	29.95 ± 0.56	0.514
BE, mmol/L	5.12 ± 1.25	6.75 ± 0.93	6.05 ± 0.58	0.516

[a,b] values within a row with different superscripts differ, P < 0.05.

n = four steers/group

Table 5.4. Least squares means ± SD for total haemoglobin (tHb), deoxyhaemoglobin (FHHb), Oxyhaemoglobin (FO_2Hb), MetHb (FMetHb), carboxyhaemoglobin (FCO_2Hb) and hematocrit for *Bos indicus* steers treated with 0, 30 or 50 g of nitrate per day for 7 days and walked 3 kilometres.

Co-oximetry variables	Treatment, g NO_3/day			ANOVA
	0	30	50	P values

tHb, g/L	124.50 ± 1.25	117.75 ± 6.26	125.75 ± 6.06	0.059
FHHb, %	1.80 ± 0.55	2.20 ± 1.43	3.33 ± 1.70	0.289
FO_2Hb, %	97.07 ± 0.88[a]	88.22 ± 8.48[a]	61.82 ± 8.76[b]	0.000
FCO_2Hb, %	0.85 ± 0.42[a]	0.95 ± 0.17[a]	1.90 ± 0.22 [b]	0.001
FMetHb, %	0.30 ± 0.05[a]	8.60 ±8.61[b]	32.90 ±9.16[c]	0.000
Hct, %	36.50 ± 1.00	34.75 ± 3.77	37.00 ± 3.74	0.583

[a,b,c] values within a row with different superscripts differ, $P < 0.05$.

n = four steers/group

Table 5.5. Least squares means ± SD for heart rate before and immediately after walking, and respiration rate, and rectal temperature after walking 3 kilometres from *Bos indicus* steers treated with 0, 30 or 50 g of nitrate per day for 7 days.

Variables	Treatment, g NO_3/day			ANOVA P values
	0	30	50	
Heart rate pre, beats/min	76.00 ± 20.05	66.50 ± 7.55	84.75 ± 14.99	0.282
Heart rate post, beats/min	104.75 ± 28.74[a]	116.00 ± 39.88[a]	175.25 ± 10.34[b]	0.016
Respiration rate, breaths/min	30.50 ± 6.70	37.00 ± 7.68	40.50 ± 9.11	0.673
Rectal temperature, °C	39.20 ± 0.14	39.70 ± 0.21	39.53 ± 0.19	0.207

[a,b] values within a row with different superscripts differ, $P < 0.05$.

n = four steers/group

5.4 Discussion

All of the steers appeared to tolerate the exercise regimen; however, the partial pressure of oxygen ($_PO_2$) for the steers given 50 g of nitrate a day was compromised compared with those of steers not treated with nitrite ($_PO_2$ = 64.60 mm Hg; Table 5.3). The $_PO_2$, methaemoglobin and the oxyhaemoglobin values for the steers given 50 g of nitrate indicated a reduction in the oxygen carrying capacity of the blood from these steers. Nitrate is known to result in haem Fe_2^+ to be oxidised to the Fe_3^+ state, which inhibits oxygen binding to the haemoglobin sub-units in the blood causing methaemoglobinemia (Haymond et al., 2005). The response to the hypoxemia in the present study, caused by the 50 g of nitrate treatment, did not involve a greater respiration rate after the imposition of an exercise regimen than when either 0 or 30 g nitrate was administered.

All treatments resulted in similar $_PCO_2$ values in respective mean arterial blood samples – a finding that supported the lack of difference in respiration rate among treatment groups at the completion of the exercise regimen. Carbon dioxide and hydrogen ions control the centre of the brain that responds to oxygen concentration of the arterial blood by regulation of respiration rate when there are decreases to below 60 mmHg. The aortic bodies become the principle sensors involved in regulation of respiration rate when oxygen concentrations decrease to below 60 mmHg (Guyton and Hall, 2011). However, on completion of the exercise regimen, the steers dosed with 50 g of nitrate compensated for the hypoxemia by increasing their heart rate up to 60% greater than the steers not treated with nitrate.

Nitrate and nitrite salts are known to cause pronounced vasodilation in cattle (Vermunt, 1992). The blood vessels that are most sensitive to the dilatory effects of nitrate are those of the head, brain, meninges, and coronary vessels (Valli, 2008). However, it has been suggested that vasodilation of the peripheral vessels is possible when cattle consume nitrates

in their diet (Vermunt, 1992). It is, therefore, likely that vasodilation decreased venous return to the heart in the steers treated with 50 g of nitrate per day, which in conjunction with the reduced oxygen carrying capacity of the blood, resulted in a higher heart rate after exercise in the steers treated with 50 g of nitrate compared with those not treated with nitrate or those treated with 30 g of nitrate. The steers in the present study were exercised for a period of 24 minutes at an average speed of 0.92m/sec. The steers treated with 50 g of nitrate had a mean heart rate of 175 beats/min which is a greater value than recorded in previous studies with cattle and buffalo where exercise regimens were imposed. Kuhlmann et al. (1985) exercised, 199 kg trained, Hereford steers at a speed of 1.0m/sec and there was a heart rate of 140 beats/min after imposition of exercise. Furthermore, draught buffalo weighing 363 kg, pulling a work load of 11% of their body weight and walking at a speed of 0.69m/sec for 3 hours, have been recorded with a heart rate of 100 beats/min (Martin, 1993). Thus, it is thought the steers in the present study were under considerable exercise stress in maintaining oxygenation in tissues that were responding to imposition of the exercise regimen when there was supplementation with 50 g nitrate per steer.

The MetHb concentrations in the cattle in the present study were similar to the values reported in a previous study in which *Bos indicus* steers consuming poor quality forage diets were subjected to 0, 30, 40 and 50 g of nitrate/day in 300 kg steers. Methaemoglobin concentrations increased in a dose-dependent manner in these steers with the peak value occurring between 2 and 5 hours after the treatments were applied (Benu et al., 2015). Therefore, the values recorded for MetHb concentration in the steers in the present study can be regarded as around the peak MetHb concentration (Callaghan et al., 2014). In other species, an increase in MetHb concentration has been recorded with imposition of exercise for a given dose rate of nitrate. In fish, the rate of nitrite entry into red blood cells is greater during forced swimming than the rate of entry into the cells of resting fish; thus, leading to a

greater MetHb formation in fish that are swimming (Brauner et al., 1993). It is unknown if cattle increase the rate of MetHb formation due to exercise. However, if this phenomenon were to apply to ruminants, then an acute toxic dose of nitrate salts may occur at lesser concentrations in exercising animals, compared with what occurs with the feeding of cattle using current dietary recommendations (Vermunt et al., 2010). The extent to which exercise influenced the MetHb concentrations in the cattle in the present study is unknown; however; this provides an interesting research question to consider in the future.

In the present study, the fraction of carboxyhaemoglobin in the blood of the 50 g of nitrate treated steers was approximately double the concentration of the other treatment groups (0 and 30 g nitrate). Benu et al. (2015) also reported an effect of increasing carboxyhaemoglobin concentrations with time for *Bos indicus* steers treated with varying doses of nitrate over 7 days. This suggest a further inhibition of the oxygen carrying capacity of blood cells of cattle treated with nitrate. An important consideration here is that the increase in carboxyhaemoglobin with the largest dose of nitrate in the present study is indicative of oxidative stress, through production of haem oxygenase-1 (Benu et al., 2015).

Arterial blood pH values were similar for all treatment groups in the present study, with there being a slightly alkaline blood pH (of 7.47 - 7.50). It is probable that the daily supplementation of non-protein nitrogen sources, such as urea and nitrate, influenced the arterial blood pH values in the cattle used in the present study. In comparison, Parker et al. (2003) conducted a study using a similar genotype, and cattle of a similar body weight as the *Bos indicus* steers used in the present research, and found an arterial blood pH of 7.44 ± 1.01 when the animals were penned.

5.5 Conclusion

Feeding nitrate to *Bos indicus* cattle resulted in a decrease in the oxygen carrying capacity of their blood. When 400 kg steers consumed 50 g of nitrate per day, and had a mild exercise regimen imposed, there was a significant load placed on the cardio-respiratory system. It is likely that doses of nitrate which exceed 50 g per day for this class of animal would induce a hypoxemic state if exercise was imposed after consuming a nitrate supplement.

CHAPTER 6 BOS INDICUS STEERS FED FLINDERS GRASS (ISEILEMA SPP.) HAY FOR 70 DAYS RESPOND TO NITRATE SUPPLEMENTATION IN A CONSISTENT AND PREDICTABLE PATTERN

This chapter is in preparation for submission to Animal Production Science

Authors: I. Benu, M.J. Callaghan, N. Tomkins, G. Hepworth, L.A. Fitzpatrick and A.J. Parker

Abstract

A 70 day trial was conducted to assess the growth rates and forage intakes of cattle supplemented with an isonitrogenous supplement containing nitrate (50 g/head/day), or no nitrate (0 g/head/day), and offered chaffed Flinders grass (*Iseilema spp*.) hay. Ten three-year-old fistulated *Bos indicus* steers (mean live weight ± sd., 400.7 kg ± 26.2) were randomly allocated into two groups (n = 5). The steers were dosed with their respective treatments, by placing the treatments (nitrate or no nitrate) directly into the rumen of each animal for 70 days. The blood samples were collected at 0, 2, 4 and 6 hours after the animals dosed with the treatments, on days 10, 30, 50 and 70 for MetHb concentration measurement. Mean blood MetHb concentrations were significantly ($P < 0.001$) higher for animals dosed with nitrate compared with the control treatments. A highly significant effect was demonstrated for the peak MetHb fraction ($P < 0.001$) and the carboxyhaemoglobin ($P = 0.008$) in the blood of steers dosed with nitrate compared with the control group. However, nitrate treatment had no significant general effect on the tHb and oxyhaemoglobin. Nitrate supplementation at a rate of 7.1 g NO_3/kg DMI tended to produce a consistent MetHb profile that appeared to return to normal concentrations within 24 h. In addition, there was no reduction in DMI and no difference in live weight of steers fed nitrate compared with the control animals. Therefore, the results suggest there is no production advantage to feeding nitrate, compared to feeding an equivalent amount of urea to steers.

6.1 Introduction

Extensive beef cattle herds contribute to greenhouse gas production through enteric methane emissions (Charmley et al., 2008). The intensity of methane emissions increases with seasonal droughts, when forage digestibility decreases and the forage is largely nitrogen deficient (Johnson and Johnson, 1995, Ulyatt et al., 2002). Providing non-protein-nitrogen supplements, such as urea, reduce body weight loss in the extensive beef herds of northern Australia during seasonal droughts. Nitrate salts can be utilized by the rumen bacteria as an NPN source, and have been demonstrated to reduce enteric methane emissions (Li et al., 2012). Most studies that demonstrate a decrease in methane emissions have fed nitrate salts to ruminant animals over a short period of time (Nolan et al., 2010, Li et al., 2012, Lee et al., 2015a, Lee et al., 2015b). Although a study of Van Zijderveld et al. (2010) conducted over greater time periods of 89 days where cattle fed nitrate salts, but the diets offered to the animal were highly digestible and not reflective of grazing systems in northern Australia where cattle graze low quality forage during the dry season when NPN supplements are usually fed. Furthermore, in most studies involving nitrate supplementation in a diet, a total mixed ration was fed or the daily nitrate dose was dispersed evenly throughout the hay allocated to the animal. This allows for the consumption of small amounts of nitrate at each feeding event (Alaboudi and Jones, 1985, Nolan et al., 2010, Li et al., 2013).

Supplement delivery systems in northern Australia provide for *ad libitum* with minimal human control over the supplement consumption resulting in variable intakes of the supplement by the animal (Eggington et al.1990; Dixon et al. 2003). Furthermore, a single feeding event could result in the prescribed consumption of the daily dose of nitrogen. Consequently, the supplement delivery systems in northern Australia increase the risk of nitrite toxicity in beef cattle if nitrate salts make up the dominant supply of NPN to cattle

(Benu et al. 2017). However, the risk of toxicity can be controlled with a dose of < 7.5 g NO_3/kg DMI over a 7 day period (Benu et al. 2017). The Australian Government recommends a dose rate of 7 g NO_3/kg DMI if beef cattle producers wish to capitalize on the government's carbon trading scheme (Australian Government, 2014). However, there is no information available about long-term studies at this dose rate, and that reflect the grazing conditions and cattle genotypes of northern Australia. Therefore, the hypothesis was that a single bolus of nitrate salts given to *Bos indicus* cattle daily would not change the onset of nitrite toxicity, as measured by haemoglobin fractions in the blood throughout a 70 day treatment period.

6.2 Materials and methods

6.2.1 Animals and management

Ten three-year-old *Bos indicus* steers with indwelling rumen cannulas (an initial body weight 360-456 kg) were used in this study. The animals were allocated to individual pens (4.28m x 1.26m) within a covered cattle housing facility throughout the experiment. The animals were fed hay only for 7 days as an adaptation period. The animals were offered chaffed Flinders grass (*Iseilema* spp) hay at two times (0800 and 1400 h) each day, in approximately equal amounts to ensure *ad libitum* intakes. The amount of hay given to individual animals was calculated from the previous day's actual intake + 20% extra hay (w:w). Data on total dry matter intake were obtained by total hay offered minus daily refusals. Samples of Flinders grass hay offered were taken, bulked over the course of the experiment and sub-sampled for analysis by a private feed analysis laboratory (Table 6.1; Symbio Laboratories, Brisbane, Queensland). Water was available to the animals *ad libitum* in 25 L containers. Hay refusals were recorded once daily at 0800 h. The animals were familiarized with handling and sampling procedures before the start of the experiment. The steers were dosed with their

respective treatments at 0900 h each day, by placing the treatments (nitrate or no nitrate) directly into the rumen of each animal for 70 days. Live weights of the steers were recorded on day 0, and they were then routinely weighed every two weeks until the end of the experiment.

The steers were randomly assigned to one of two treatment groups of five steers, which were given an iso-nitrogenous supplement of 15 g of nitrogen/day. The iso-nitrogenous treatments were either a control group that received 0 g/head/day of nitrate, or a nitrate treated group that received 50 g/head/day of nitrate (Table 6.2).

Blood samples were collected from the jugular vein of each animal using a 1 mL heparinized blood gas syringe, following restraint in a cattle crush. The blood samples were collected before dosing at 0900 h and at two hours intervals post dose introduction (1100, 0100, and 0300 h), on days 10, 30, 50 and 70 of the experiment. Blood samples were placed onto crushed ice and carried to the laboratory for analysis of haemoglobin fractions in the blood of the cattle, using a blood gas analyser (Siemens, Rapid Lab 1265, Sydney, NSW).

6.2.2 Statistical analysis

The data were analysed using ANOVA in the GENSTAT statistical package by considering the effect of dose treatment (nitrate or urea), time of blood collection (0hrs [before dosed], and 2, 4, 6 hours post dosed), day (10, 30, 50 and 70) and their interactions on the fractions of haemoglobin in the steers' blood. Adjusted means were compared using the method of least significant differences (l.s.d.), with significance was set at $P < 0.05$.

Table 6.1. Composition[a] of bulked Flinders grass (Iseilema spp.) hay throughout the experiment.

Test	Flinders Grass Hay
Protein, g/kg	2. 9
Fat, g/kg	1. 2
Moisture, g/kg	8. 3
Ash, g/kg	8. 6
Crude Fibre, g/kg	39. 1
Dry Matter, g/kg	91. 7
Nitrogen Free Extract DWB, g/kg	48. 1
Acid Detergent Fibre, g/kg	52. 4
Neutral Detergent Fibre, g/kg	72. 1
Calcium, g/kg	3. 20
Phosphorous, g/kg	0. 64
Sodium, g/kg	0. 25
Sulphur, g/kg	0. 91

Source: Symbio Laboratories, Brisbane, Queensland.

[a]All test results except moisture are reported on a dry matter (DM) basis; moisture is reported on as fed basis.

Table 6.2. Composition of raw materials and formulated analysis of N, NO_3, P, Na and S in supplement treatments.

Composition	Treatments (g NO_3/day)	
	0	50
Urea (g/day)	32.5	5.5
Calcium Nitrate decahydrate (g/day)	0	80
Sodium Sulphate (g/day)	25	25
MDCP (g/day)	25	25

N (g/day)	15.2	15.0
NO_3 (g/day)	0	50
S (g/day)	5.6	5.6
P (g/day)	5.3	5.3
Na (g/day)	8.1	8.1

6.3 Results

6.3.1 Methaemoglobin

Treating steers with nitrate caused an increase in the mean MetHb concentration in their blood, in comparison with the blood of steers given an iso-nitrogenous supplement without nitrate ($P < 0.001$, Figure 6.1). A significant effect of the time of sampling ($P < 0.001$), and the treatment x time of sampling interaction ($P < 0.001$), was found for the mean MetHb concentrations. Methaemoglobin concentrations were not statistically different between groups at 0 h before giving the no nitrate and nitrate treatments. However, an increase in MetHb concentration occurred between 2 and 4 h after treatment, followed by a decline in MetHb concentrations at 6 h after treatment, on each sampling day, for the nitrate treated steers. We did not detect any differences due to day of sampling for the mean MetHb concentration (10 d = 4.9%, 30 d = 5.04%, 50 d = 4.79%, 70 d = 4.82%; $P = 0.076$).

6.3.2 Peak methaemoglobin

The peak MetHb concentration in blood was greater for the steers given nitrate, compared with the steers given no nitrate ($P< 0.001$, Figure 6.2). However, we did not detect a significant difference for the peak MetHb concentration between any day ($P = 0.293$), or in the interaction of treatment and day ($P = 0.296$)

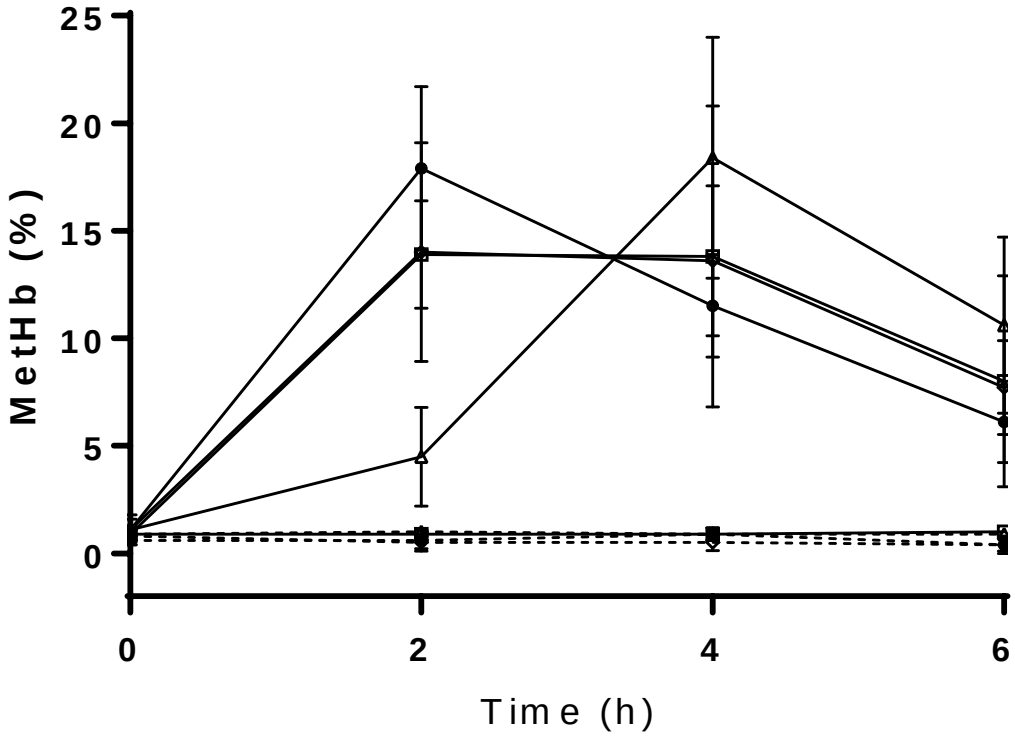

Figure 6.1. Mean methaemoglobin concentration (%) from venous blood of *Bos indicus* steers sampled at 0, 2, 4 and 6 hours after treatment with 50 g of nitrate (—) or no nitrate (-■-) as a non-protein-nitrogen supplement on day 10 (○), day 30 (□), day 50 (Δ) and day 70 (◊).

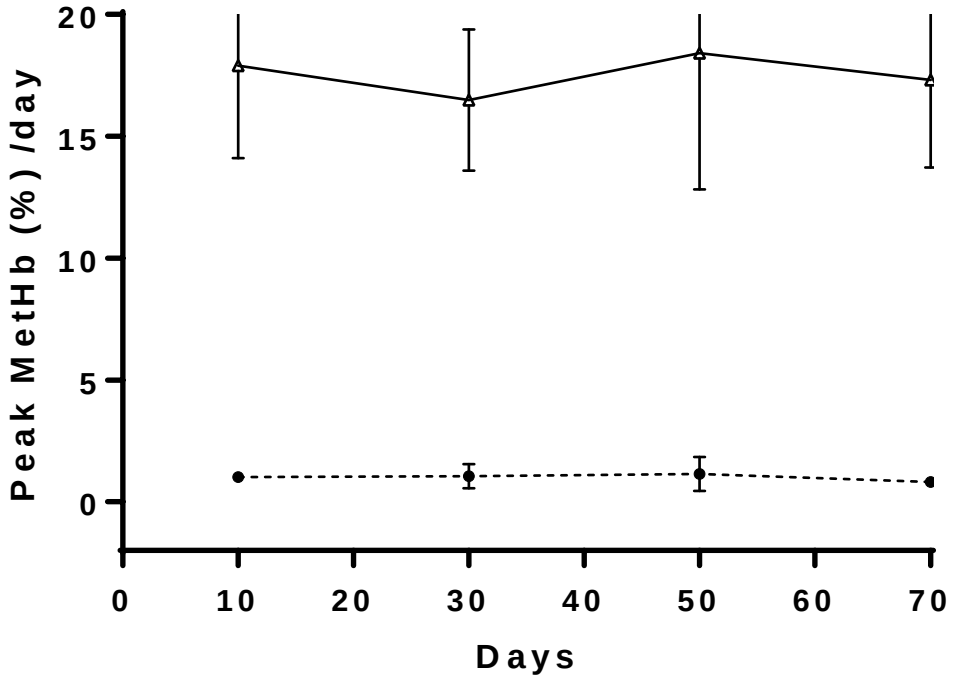

Figure 6.2. Adjusted mean peak MetHb concentration (%) from venous blood of *Bos indicus* steers treated with 50 g of nitrate (─Δ─) or no nitrate (--●--) as a non-protein-nitrogen supplement for a 70 day period (l.s.d. = 4.867).

6.3.3 Carboxyhaemoglobin

Nitrate treatment caused carboxyhaemoglobin concentrations to be greater in the blood of steers, compared with the steers given no nitrate, with back transformed means of 5.53% for nitrate, and 1.45% for the no nitrate groups ($P = 0.008$). A significant effect of day was demonstrated ($P = 0.020$) whereby the mean back transformed carboxyhaemoglobin value for 70 d (1.19%) was less than all other sample days (10 d, 2.63%; 30 d, 4.72%; 50 d, 4.35%). There was a highly significant difference for time of sampling ($P < 0.001$), due to the basal carboxyhaemoglobin concentrations, before treatments were given to the steers at 0 h. There was a significant interaction for dose x time, demonstrating the two and four hour sampling times to be greater for the nitrate treated steers, compared with the steers given no nitrate ($P <$

0.001, Figure 6.3.). There were no differences detected for the interactions of day x dose, or day x dose x time.

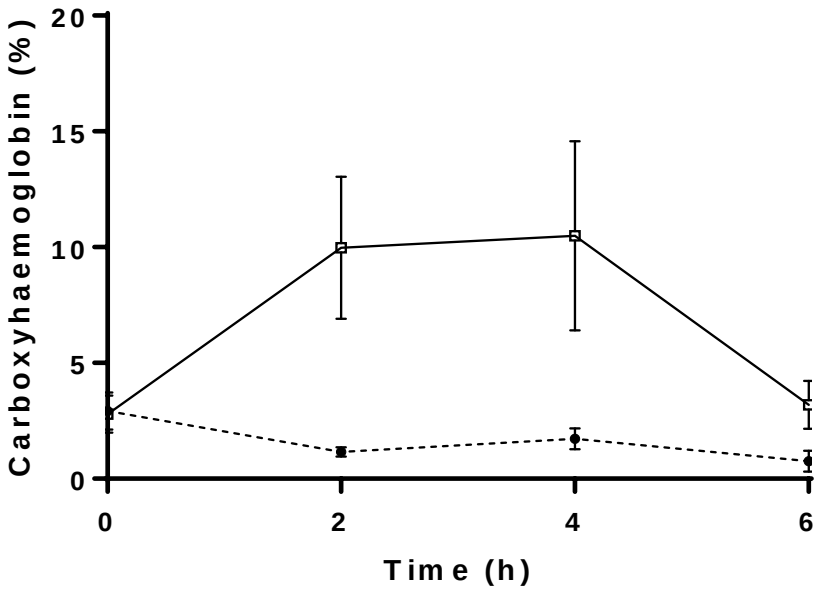

Figure 6.3. Adjusted back transformed mean carboxyhaemoglobin concentration (%) from venous blood of *Bos indicus* steers sampled at 0, 2, 4 and 6 hours after treatment with 50 g of nitrate (—□—) or no nitrate (--●--) as a non-protein-nitrogen supplement for a 70 day period.

6.3.4 Total haemoglobin

Nitrate treatment had no effect on the tHb concentration of treated animals when compared with the steers given no nitrate (P = 0.927). Steers sampled on day 10 had lower concentrations of tHb in their blood, compared with all other sampling days (P < 0.001). The predicted means in the model for days 10, 30, 50, and 70 were 104, 115, 112, 109 g tHb/L (l.s.d. = 3.35), respectively. A time effect was demonstrated, whereby the sampling at 0 h was greater than all other sampling times, with predicted means in the model for times at 0, 2, 4 and 6 h throughout the study being 114, 109, 109, 108 g tHb/L (l.s.d. = 1.64).

6.3.5 Oxyhaemoglobin

Nitrate treatment had no effect on the adjusted mean oxyhaemoglobin concentrations in the blood of steers throughout the study (P = 0.108). However, there was a significant day effect demonstrated (P = 0.003) for oxyhaemoglobin concentrations, which were greater on day 70 compared to all other days. The adjusted means for the sampling on days 10, 30, 50, and 70 were 61, 58, 58 and 68 (l.s.d.= 5.46), respectively. However, this effect was largely due to the elevated concentrations of oxyhaemoglobin on day 70 in the steers given no nitrate. The treatment x day interaction was statistically significant for oxyhaemoglobin concentrations (P = 0.043, Figure 6.4). Steers given no nitrate had greater concentrations of oxyhaemoglobin on days 10 and 70, compared with steers given nitrate on days 10, 30, and 50 but not day 70. The time of blood sampling had a significant effect in this study, with the mean oxyhaemoglobin concentration at 6 h being greater than all other times (0, 2, and 4 h; P = 0.004). Time interacted with nitrate dose, in that steers treated with no nitrate had higher concentrations of oxyhaemoglobin at 6 h after treatments were given, compared with nitrate treated steers at all other sampling times (P = 0.033). Time also interacted with day demonstrating an oxyhaemoglobin concentration greater than at all other sampling times and days, at 6 h after giving treatments on day 70 (P = 0.014). Treating steers with nitrate caused the lowest oxyhaemoglobin concentrations with mean values of 54.2 % and 43.8% for the no nitrate and nitrate treated steers, respectively (P =0.040).

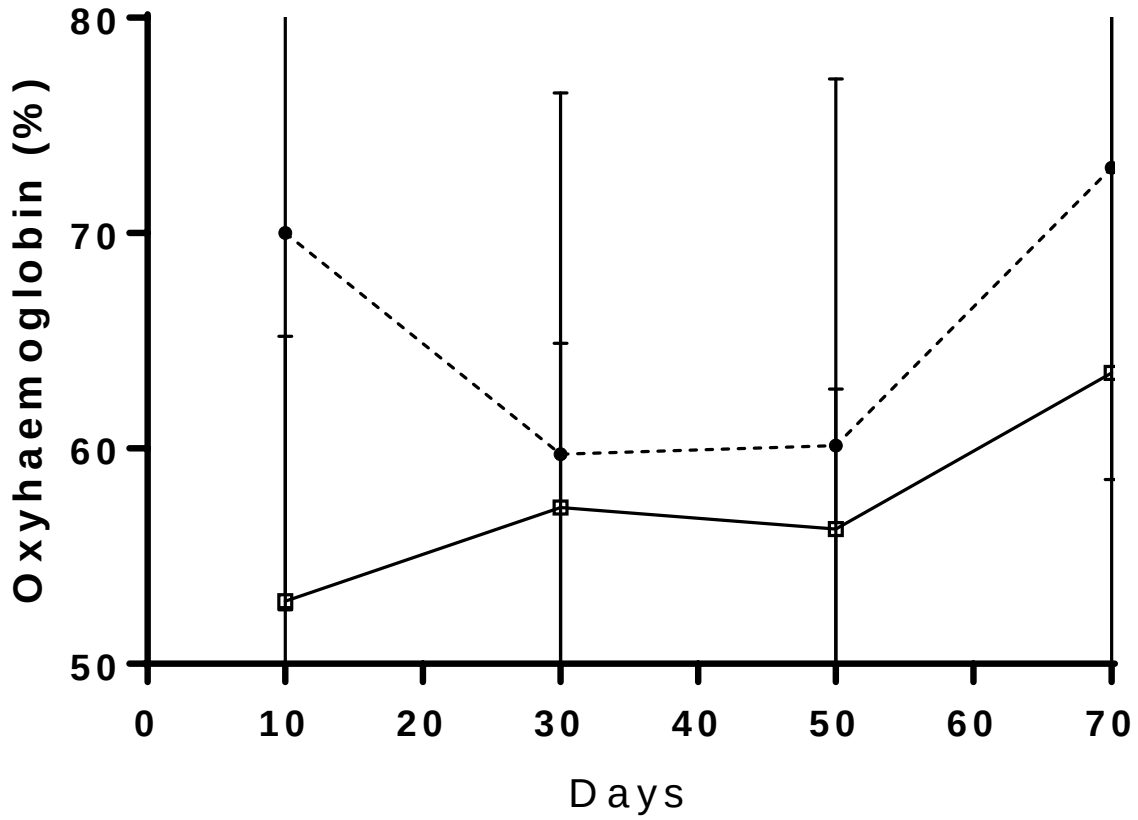

Figure 6.4. Mean oxyhaemoglobin concentration (%) from venous blood of *Bos indicus* steers treated with 50 g of nitrate (—□—) or no nitrate (--●--) as a non-protein-nitrogen supplement for a 70 day period.

6.3.6 Dry matter intake and body weight

Nitrate treatment had no effect on dry matter intake of Flinders grass hay by steers in this experiment. However, an effect of day on dry matter intake ($P < 0.001$; Figure 6.5) was demonstrated on day 70. There was no detectable difference of in the body weight of *Bos indicus* steers due to feeding nitrate (Figure 6.6).

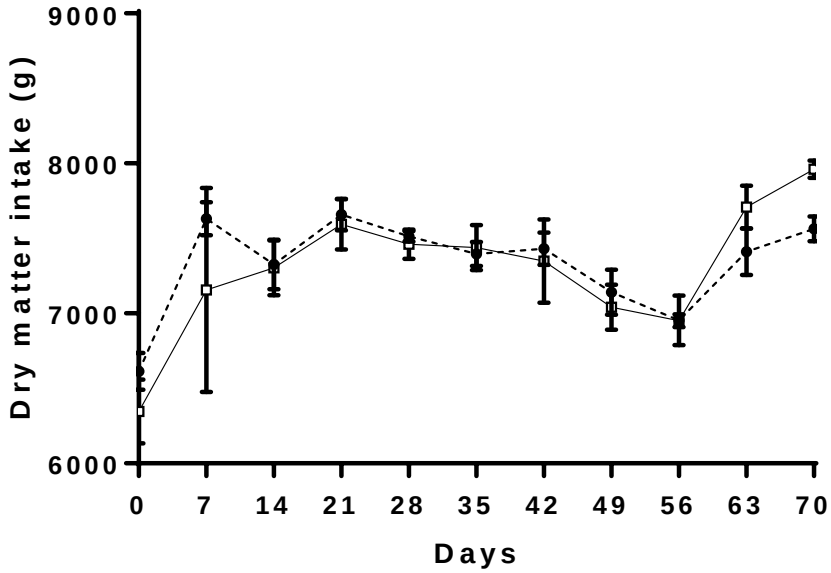

Figure 6.5. Means ± SEM dry matter intake of Flinders grass hay by steers given no nitrate (--●--) or 50 g of nitrate (—□—) for 70 days.

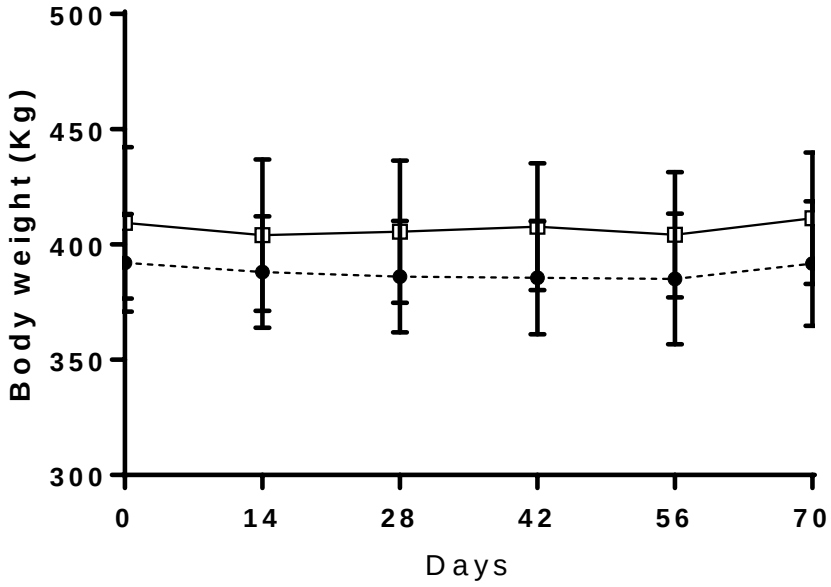

Figure 6.6. Mean ± SEM body weight of steers given no nitrate (--●--) or 50 g of nitrate (—□—) for 70 days.

6.4 Discussion

Bos indicus steers fed a poor-quality forage diet, and supplemented with nitrate as an NPN source, developed nitrite toxicity in a consistent and predictable pattern (Callaghan et al, 2014). There was no difference detected in the development of methaemoglobinemia in the cattle treated with nitrate on any of the days sampled in this study (10, 30, 50 or 70 d). In addition, there was no difference detected between days sampled for the mean peak MetHb concentration of the steers treated with nitrate. *Bos indicus* steers treated with a similar rate of nitrate on a dry matter intake basis in the study of Benu et al. (2015) (7.5 g NO_3/kg DMI), produced MetHb profiles each day for seven days that were similar to the steers in the current study up to day 70 (7.1 g NO_3/kg DMI). Within the dose range of 7.1 to 7.5 g NO_3/kg DMI, there was a clearance of nitrate induced MetHb and carboxyhaemoglobin from the blood of *Bos indicus* steers, which is illustrated by the time zero samples each day being similar in haemoglobin fractions, for both the no nitrate and nitrate treated steers (Benu et al., 2015). When concentrations of nitrate ≥ 7.5g NO_3/kg DMI occurred in the diet of *Bos indicus* cattle, MetHb concentrations in blood (0.3 – 2%) did not return to normal within 24 h. The maintenance of a greater concentration of nitrate in the daily diet (7.5 g NO_3/kg DMI), in the form of a single bolus, resulted in a subsequent peak in MetHb concentrations, which increased each day until a hypoxemic crisis ensued (Benu et al., 2015).

There were no overt clinical signs of nitrite toxicity displayed by the cattle in this study. However, subtle sub-clinical signs of blue colored gums and brown discolored blood at 2 and 4 h samples occurred on each of the days sampled. In addition, the peak MetHb concentration of each animal was less than the concentration considered to be clinically hypoxemic by some authors (20% MetHb; Parkinson et al. 2010). However, no apparent adaptation to nitrite toxicity had occurred over the 70 d experimental period, as measured by the haemoglobin

fractions in the blood. The current method for feeding nitrate, endorsed by the Australian Government, requires a minimum adaptation period of 14 d, in which cattle receive 3.5 g NO_3 / kg DMI, followed by a maximum feeding rate of 7g NO_3 / kg DMI (Australian Government, 2014).

Rumen microbiota in sheep adapt to dietary nitrate by increasing the reduction of nitrate and nitrite, and increasing the numbers of nitrate reducing bacteria in *in vitro* and *in vivo* models (Allison and Reddy, 1984, Alaboudi and Jones, 1985). However, where the bacterial reduction of nitrate has occurred in the rumen environment the MetHb in the animal has not been reported (Allison and Reddy, 1984), or MetHb was sampled at a time that did not coincide with the daily peak MetHb in the animal (Alaboudi and Jones, 1985). In addition, the amount of nitrate consumed by the sheep before the sampling of adaptive measures was not stated (Allison and Reddy, 1984, Alaboudi and Jones, 1985). It is most probable that bacterial adaptations to nitrate did not occurred in the rumen of the cattle in this study. However, if the rumen environment adapted to the nitrate with an increase in the rate of reduction of nitrate and nitrite the protective effects on the animals' haemoglobin fractions in the blood were limited.

The steers in this study were housed in a confined space in a cattle housing facility and their energy expenditure was minimal, thus, there was no change in the body weight of the steers in either group when fed an iso-nitrogenous diet. These data are consistent with others in demonstrating no difference in dry matter intake or body weights of ruminants when fed iso-nitrogenous diets of either nitrate or another NPN source (Nolan et al., 2010, Li et al., 2012, Benu et al., 2015).

6.5 Conclusion

Nitrate supplementation at a rate of 7.1 g NO_3/kg DMI produced a consistent daily MetHb profile that appeared to return to normal concentrations within 24 h. At this rate of nitrate supplementation, cattle did not have a reduction in dry matter intake in comparison with steers fed an iso-nitrogenous supplement without nitrate each day. The live weight of steers fed nitrate was not different to the steers fed an equivalent amount of urea. Therefore, the results of the present study suggest there is no production advantage to feeding nitrate, compared to feeding an equivalent amount of urea to steers.

CHAPTER 7 GENERAL DISCUSSION

Beef cattle in Australia contribute a considerable amount to greenhouse gas emissions, due to their enteric methane gas production. Approximately 60% of Australia's national beef cattle are based on pasture rangelands and have been targeted by the government to reduce methane production (Callaghan et al., 2014). The use of nitrate feeding as a NPN supplement, to replace urea's function, during dry seasons is well accepted (Nolan et al., 2010), as well as being a means to reduce methane production from cattle and sheep (Leng, 2008). Moreover, nitrate feeding to ruminants has been demonstrated to reduce enteric methane emissions (Li et al., 2012). However, most studies involving feeding nitrate salts to ruminants were based on a high quality diet, such as concentrates, and forages, or even nitrate, which were mixed with water and sprayed on the top of hays, and ultimately produced a low risk of nitrate toxicity. In addition, highly digestible diets provide a degree of protection against nitrate toxicity (Burrows et al., 1987) and do not reflect tropical forages and supplements, due to labour cost constraints alone. Nevertheless, there are few data available for *Bos indicus* cattle, which are fed nitrate and graze low quality forage in northern Australia. This thesis described some studies relating to nitrate feeding in *Bos indicus* cattle when consuming low quality rangeland hay in northern Australia. These included a validation of co-oximeter for MetHb concentration measurement in the blood of ruminants with regards to nitrate feeding, a dose rate and frequency of nitrate feeding to cattle, exercise tolerance of cattle when fed nitrate supplements, and long term nitrate feeding effects on MetHb in the blood and growth rate of cattle.

Measurement of MetHb concentration in ruminants is based on the method of Evelyn and Malloy (1938) as a gold standard. Despite variances on this method, there are a number of problems. Firstly, it is timely consuming; the method of Evelyn and Malloy (1938) takes a

long time and the MetHb has to be analysed quickly, or at least the red blood cells (RBC's) have to be lysed quickly. Secondly, the method of Evelyn and Malloy (1938) requires the use of cyanide to convert MetHb to cyanMetHb, which is a dangerous and poisonous substance. In addition, operators have to be licenced and account for its use. This increases the administration costs on the operation and imposes a health risk on the operator and environment. Thirdly, human error in handling samples, such as pipetting, may cause inaccuracy in the results.

The co-oximetry method, on the other hand, offers a rapid and accurate process for measuring MetHb concentrations in the blood; it is immediately calibrated, it measures multiple-wavelengths, it does not require reagents, and it has internal standard deviations. However, the co-oximeter method has not been validated in ruminants.

A highly significant correlation between the Evelyn and Malloy (1938) and co-oximetry methods has been demonstrated in Chapter three. The analysis of Bland-Altman and Lin's concordance coefficients showed a substantial agreement in the results generated using both methods, using samples from sheep and goats. However, there was a greater bias in the cattle samples, causing a poor agreement between the two different methods. This was, perhaps, due to human error in pipetting within the Evelyn and Malloy (1938) method, since the cattle samples were the first taken to evaluate the similarity between the results produced from both methods. However, the data of sheep and goats were significantly improved after the identification of pipetting issue. This study validated that the co-oximeter method measured the same concentrations of MetHb in the blood of cattle, sheep and goats compared with the Evelyn and Malloy (1938) method. Therefore, all the subsequent experiments utilised the co-oximeter method to measure MetHb in the blood of cattle.

Chapter four demonstrated that increasing dose rate of nitrate feeding increased the development of MetHb in the blood of cattle fed low quality Flinders grass (*Iseilema* spp) hay. For once a day nitrate feeding, dose rate of 30, 40 or 50 g/day caused a greater increase in the MetHb concentration in the blood of steers than the equivalent dose divided into two equal portions and administered in the morning and afternoon. These findings agree with a study reported by Tomkins et al. (2016) where a consistent increase in MetHb concentrations in blood of *Bos indicus* cattle fed Flinders grass (*Iseilema* spp) hay and supplemented with nitrate salts. The consistent trend evident in the development of MetHb in blood of steers, perhaps suggests that there was no adaptation of nitrate feeding by rumen microbes under these experimental conditions. The ability of the rumen microbial population to detoxify nitrate, or the nitrite derive from it, is believed to be energy-dependent and therefore progressively reduced under conditions of poor feed quality (Burrows et al, 1987; Valli, 2008). Ruminants fed highly digestible diets and supplemented with nitrate salts are often reported to have low MetHb concentrations in their blood (Alaboudi and Jones, 1985, van Zijderveld et al., 2010). Another important consideration of studies reporting low concentrations of MetHb in the blood of ruminants fed nitrate compounds is that the authors do not quantify the amount of nitrate consumed before the blood sample was taken (Alaboudi and Jones, 1985, van Zijderveld et al., 2010; Li et al, 2012). Therefore, the timing of blood sampling may or may not align with the peak MetHb concentration for the day. The peak of MetHb concentration is important because it determines the amount of time the animal is placed in a hypoxic state. Furthermore, the steers receiving 40 and 50 g of nitrate once a day demonstrated a peak of over 20% MetHb on the first day. This peak of MetHb continued to increase over seven days experiment, indicating development of hypoxia in these animals.

It has been hypothesised that the mechanism of nitrate transportation into the rumen may be recycled in the saliva of ruminants (Leng, 2008). The recycling of nitrate may explain why

the steers at the higher dose rate of nitrate increased the response to dosing as measured by the rate of incline to peak MetHb values. The reduction of nitrate to nitrite and then to ammonia is controlled by the bacterial population of the rumen and alimentary tract.

The carboxyhaemoglobin concentration in the blood of the steers treated with nitrate once a day was also increased over time during the course of the experiment. It is probable that oxidative damage to red blood cells by the once a day nitrate treatments resulted in an expression of hemeoxigenase-1 thereby increasing carboxyhaemoglobin concentration. The increase in carboxyhaemoglobin as the experiment progressed would have compounded the hypoxia caused by the MetHb fractions in the nitrate-treated animals.

Increasing of MetHb and carboxyhaemoglobin concentrations in the blood of *Bos indicus* steers resulted in a decreased of oxyhaemoglobin each day in a dose-dependent manner for steers dosed once a day during the experiment. However, twice a day dosing of nitrate feeding did not affect the oxyhaemoglobin concentration. This would imply that multiple meals throughout the day impart a degree of protection against elevated dyshaemoglobins when nitrate is consumed. However, other haemoglobin fractions, such as total haemoglobin, deoxyhaemoglobin, and haematocrit were not affected by increasing nitrate dose feeding on cattle. Therefore, care should be taken when intentionally feeding nitrate salts as a urea substitute and or a methane abatement strategy due to the inherent variation in intake of dry season supplements and thus the risk of over consumption of the nitrate salt.

During the study of dose rate and feeding frequency of nitrate (Chapter four), the animals were stalled or penned individually, under low physiological stress and, therefore, we questioned what would happen if exercise was imposed on the animal when feeding nitrate. Nitrate feeding causing hypoxemia, via increased concentrations of MetHb and carboxyhaemoglobin in the blood of the animal. The effects of nitrate on the kinetics of

arterial blood gases and heart rate can be investigated while the animals are exercised. The greatest impact to the animals fed nitrate and were exercised as demonstrated in Chapter five was the increased in the MetHb and carboxyhaemoglobin concentrations and a decreased in pO_2 particularly for those steers treated with 50 g nitrate per day, indicated a reduction in the oxygen carrying capacity of the blood from these steers. Nitrate is known to oxidise haemoglobin in the blood, producing MetHb, which reduces the blood's ability to transport oxygen around the tissues in the body (Haymond et al., 2005). The response to the hypoxemia in the present study caused by 50 g of nitrate treatment did not involve a greater respiration rate after imposition of an exercise regime than that when nitrate was not administered or when the 30 g nitrate treatment was imposed. Furthermore, there were a decrease in deoxyhaemoglobin concentrations and oxygen carrying capacity in the blood, but an increased in heart rate after exercise. This indicates that the animal had to compensate for the hypoxemia. The pCO_2, however, did not differ between the treatments. This, therefore, suggests that the animals' bodies had to work harder to maintain homeostasis.

Effects of dose rate of nitrate feeding have been demonstrated to increase MetHb concentrations in the blood of cattle, a finding which is supported by the results of Chapter four. Acclimation is believed to occur overtime with nitrate feeding (Allison and Reddy, 1984, Alaboudi and Jones, 1985), with an implied suggestion that MetHb will decrease in the animal over time. Therefore, Chapter six was designed to assess the safety of long term feeding of nitrate on cattle.

A long-term study of nitrate feeding to *Bos indicus* cattle consuming low quality hay showed an increase in MetHb and carboxyhaemoglobin concentrations in the blood compared to the other group fed no nitrate (Chapter six). Nitrate supplementation at a rate of 7.1 g NO_3/kg DMI produced a consistent MetHb blood profile that appeared to clear out of the blood within 24 hours. The consistent development of MetHb and carboxyhaemoglobin

concentrations in the blood over 70 days indicated that the cattle's rumen microorganisms did not adapt to the nitrate feeding. This finding agrees with the results of previous studies, in which cattle were consuming low quality hay and were supplemented with calcium nitrate (Benu et al., 2015, Tomkins et al., 2016). Other studies have reported a reduction of nitrate and nitrite that occurred in a rumen of ruminant adapted to dietary nitrate either in *in vitro* or *in vivo* models (Allison and Reddy, 1984, Alaboudi and Jones, 1985). However, the MetHb concentration in the animal has not been reported (Allison and Reddy, 1984), or MetHb was sampled at a time that did not coincide with the daily peak MetHb in the animal (Alaboudi and Jones, 1985). Therefore, it is most probable that bacterial adaptations to nitrate did not occurred in the rumen of the cattle in this study.

In conclusion, data from the studies included in this thesis add to the knowledge of nitrate feeding supplementation in tropical areas, where cattle are mainly grazed in low quality rangelands. Nitrate feeding increases MetHb concentrations in the blood of cattle consuming low quality Flinders grass hay. Once a day feeding of 50 g nitrate/day/head is more likely cause toxicity, whereas the same amount of nitrate divided and fed twice a day is a safer option for the animal. The results of this research highlight that caution should be exercised when feeding nitrate as a non-protein-nitrogen to cattle consuming low quality rangelands hay in northern Australia.

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APPENDICES

CHAPTER 3. ANOVA TABLES

Table A3.1. Unpaired t test of log_{10} MetHb of the Evelyn and Malloy (1938) and co-oximetry methods for cattle blood samples

Table Analyzed	t test cattle
Column B	log 10 COOXY
vs.	vs.
Column A	log 10 EM
Unpaired t test	
P value	0.5007
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.6829 df=26
How big is the difference?	
Mean ± SEM of column A	1.048 ± 0.1288, n=14
Mean ± SEM of column B	0.9118 ± 0.1526, n=14
Difference between means	-0.1364 ± 0.1997
95% confidence interval	-0.5469 to 0.2741
R squared	0.01762
F test to compare variances	
F,DFn, Dfd	1.403, 13, 13
P value	0.5505
P value summary	ns
Significantly different? (P < 0.05)	No

Table A3.2. Unpaired t test of log_{10} MetHb of the Evelyn and Malloy (1938) and co-oximetry methods for sheep blood samples

Table Analyzed	t test sheep
Column B	log 10 COOXY
vs.	vs.
Column A	log 10 EM
Unpaired t test	
P value	0.8688
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.1662 df=42
How big is the difference?	
Mean ± SEM of column A	1.115 ± 0.1669, n=22
Mean ± SEM of column B	1.077 ± 0.1545, n=22
Difference between means	-0.03779 ± 0.2274
95% confidence interval	-0.4967 to 0.4212
R squared	0.0006570
F test to compare variances	
F,DFn, Dfd	1.166, 21, 21
P value	0.7285
P value summary	ns
Significantly different? (P < 0.05)	No

Table A3.3. Unpaired t test of log_{10} MetHb of the Evelyn and Malloy (1938) and co-oximetry methods for goat blood samples

Table Analyzed	t test goat
Column B	log 10 COOXY
vs.	vs.
Column A	log 10 EM
Unpaired t test	
P value	0.8887
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.1409 df=40
How big is the difference?	
Mean ± SEM of column A	1.212 ± 0.1476, n=21
Mean ± SEM of column B	1.183 ± 0.1405, n=21
Difference between means	-0.02871 ± 0.2038
95% confidence interval	-0.4405 to 0.3831
R squared	0.0004961
F test to compare variances	
F,DFn, Dfd	1.104, 20, 20
P value	0.8268
P value summary	ns
Significantly different? (P < 0.05)	No

Table A3.4. Pearson correlation of log_{10} MetHb values produced from cattle blood samples using the Evelyn and Malloy (1938) and co-oximetry methods

Pearson r	
r	0.9116
95% confidence interval	0.738 to 0.972
R squared	0.8311
P value	
P (two-tailed)	<0.0001
P value summary	****
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	14

Table A3.5. Pearson correlation of log_{10} MetHb values produced from sheep blood samples using the Evelyn and Malloy (1938) and co-oximetry methods

Pearson r	
r	0.9925
95% confidence interval	0.9816 to 0.9969
R squared	0.985
P value	
P (two-tailed)	<0.0001
P value summary	****
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	22

Table A3.6. Pearson correlation of log_{10} MetHb values produced from goat blood samples using the Evelyn and Malloy (1938) and co-oximetry methods

Pearson r	
r	0.9906
95% confidence interval	0.9764 to 0.9962
R squared	0.9812
P value	
P (two-tailed)	<0.0001
P value summary	****
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	21

Table A3.7. Bland and Altman analysis of log_{10} MetHb values produced from cattle blood samples for the Evelyn and Malloy (1938) and co-oximetry methods with bias and 95% limits of agreement

Bias	-0.1364
SD of bias	0.2468
95% Limits of Agreement	
From	-0.62
To	0.3473

Table A3.8. Bland and Altman analysis of $\log_{10}$ MetHb values produced from sheep blood samples for the Evelyn and Malloy (1938) and co-oximetry methods with bias and 95% limits of agreement

Bias	-0.03779
SD of bias	0.1115
95% Limits of Agreement	
From	-0.2564
To	0.1808

Table A3.9. Bland and Altman analysis of $\log_{10}$ MetHb values produced from goat blood samples for the Evelyn & Malloy (1938) and co-oximetry methods with bias and 95% limits of agreement

Bias	-0.02871
SD of bias	0.1011
95% Limits of Agreement	
From	-0.2268
To	0.1694

Table A3.10. Lin's concordance correlation coefficient (Rc) for the $\log_{10}$ MetHb values produced from cattle blood samples for the Evelyn and Malloy (1938) and co-oximetry methods

```
Run MATRIX procedure:


Lin's Concordance Coefficient

X & Y Statistics

             var1     var2

Mean        1.060     .949

Variance     .242     .255

Nr. of valid pairs

       14

Association statistics

     Cov.        R

    .2146    .8636

Lin's Concordance Coefficient & related statistics

       Rc       Cb Mn.Shift

     .870     .974     .214

95%CI for Rc

    Lower    Upper

     .679     .951

Lower one-sided 95% CL for Rc

     .720

Fisher transformation:

        Z    SE(Z)

    1.224     .279
```

Table A3.11. Lin's concordance correlation coefficient (Rc) for the $\log_{10}$ MetHb values produced from sheep blood samples for the Evelyn and Malloy (1938) and co-oximetry methods

```
Run MATRIX procedure:


Lin's Concordance Coefficient

X & Y Statistics

             var1     var2

Mean        1.115    1.077

Variance     .642     .550

Nr. of valid pairs

       22

Association statistics

     Cov.        R

    .5898    .9925

Lin's Concordance Coefficient & related statistics

       Rc       Cb Mn.Shift

     .988     .996     .048

95%CI for Rc

    Lower    Upper

     .975     .994

Lower one-sided 95% CL for Rc

     .978

Fisher transformation:

        Z    SE(Z)

    2.569     .191
```

Table A3.12. Lin's concordance correlation coefficient (Rc) for the $\log_{10}$ MetHb values produced from goat blood samples for the Evelyn and Malloy (1938) and co-oximetry methods

```
Run MATRIX procedure:


Lin's Concordance Coefficient

X & Y Statistics

             var1     var2

Mean        1.212    1.183

Variance     .503     .456

Nr. of valid pairs

       21

Association statistics

     Cov.        R

    .4745    .9906

Lin's Concordance Coefficient & related statistics

       Rc       Cb Mn.Shift

     .988     .998     .040

95%CI for Rc

    Lower    Upper

     .973     .995

Lower one-sided 95% CL for Rc

     .977

Fisher transformation:

        Z    SE(Z)

    2.574     .216
```

CHAPTER 4. REML VARIANCE TABLES

Table A4.1. REML variance of interaction between nitrate dose, frequency and time for log_{10} MetHb in the blood of *Bos indicus* steers treated with nitrate once a day

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose 0.014	19.06	3	6.35	8.9
Time <0.001	458.10	11	41.65	818.9
Dose.Time <0.001	272.66	33	8.26	818.9
day <0.001	127.49	6	21.25	47.2
day.Dose <0.001	91.45	18	5.08	47.3

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose.Time <0.001	280.25	33	8.49	818.9
day.Dose <0.001	91.45	18	5.08	47.3

Table A4.2. REML variance of interaction between nitrate dose, frequency and time for log MetHb in the blood of *Bos indicus* steers treated with nitrate twice daily

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose <0.001	89.38	3	29.79	7.0
Time <0.001	594.84	11	54.08	755.6
Dose.Time <0.001	260.47	33	7.89	755.7
day <0.001	29.99	6	5.00	42.2
day.Dose 0.118	28.01	18	1.56	42.3

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose.Time <0.001	262.12	33	7.94	755.7
day.Dose 0.118	28.01	18	1.56	42.3

Table A4.3. REML variance of interaction between nitrate dose, frequency and time for Oxihaemoglobin in the blood of *Bos indicus* steers treated with nitrate once daily

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose 0.959	0.30	3	0.10	4.8
Time <0.001	40.37	11	3.67	792.9
Dose.Time 0.035	49.73	33	1.51	793.1
day <0.001	56.54	6	9.42	47.0
day.Dose 0.283	22.00	18	1.22	47.2

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose.Time 0.037	49.36	33	1.50	793.1
day.Dose 0.283	22.00	18	1.22	47.2

Table A4.4. REML variance of interaction between nitrate dose, frequency and time for Oxihaemoglobin in the blood of *Bos indicus* steers treated with nitrate twice daily

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose 0.941	0.38	3	0.13	7.0
Time <0.001	72.13	11	6.56	753.9
Dose.Time 0.224	39.03	33	1.18	754.1
day 0.127	10.69	6	1.78	41.1
day.Dose 0.609	15.74	18	0.87	41.3

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose.Time 0.218	39.21	33	1.19	754.1
day.Dose	15.74	18	0.87	41.3

Table A4.5. REML variance of interaction between nitrate dose, frequency and time for total haemoglobin in the blood of *Bos indicus* steers treated with nitrate once daily

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose 0.090	8.90	3	2.97	9.0
Time <0.001	178.16	11	16.20	806.4
Dose.Time <0.001	147.50	33	4.47	806.7
Day 0.083	3.09	1	3.09	65.5
Dose.Day <0.001	19.53	3	6.51	65.6

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose.Time <0.001	150.73	33	4.57	806.7
Dose.Day <0.001	19.53	3	6.51	65.6

Table A4.6. REML variance of interaction between nitrate dose, frequency and time for total haemoglobin in the blood of *Bos indicus* steers treated with nitrate twice daily

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose 0.862	0.79	3	0.24	3.3
Time <0.001	91.24	11	8.29	748.6
Dose.Time 0.767	26.75	33	0.81	748.8
day <0.001	38.38	6	6.40	38.4
day.Dose 0.903	10.17	18	0.56	38.5

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose.Time 0.789	26.23	33	0.79	748.8
day.Dose 0.903	10.17	18	0.56	38.5

Table A4.7. REML variance of interaction between nitrate dose, frequency and time for deoxyhaemoglobin in the blood of *Bos indicus* steers treated with nitrate once daily

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose 0.537	2.41	3	0.78	7.7
Time <0.001	53.74	11	4.89	815.5
Dose.Time 0.009	55.82	33	1.69	815.6
day <0.001	32.93	6	5.49	47.4
day.Dose 0.304	21.48	18	1.19	47.5

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose.Time 0.009	55.85	33	1.69	815.6
day.Dose 0.304	21.48	18	1.19	47.5

Table A4.8. REML variance of interaction between nitrate dose, frequency and time for deoxyhaemoglobin in the blood of *Bos indicus* steers treated with nitrate twice daily

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose 0.938	0.40	3	0.13	7.0
Time <0.001	62.81	11	5.71	758.1
Dose.Time 0.429	33.86	33	1.03	758.4
Day 0.023	5.33	1	5.33	58.6
Dose.Day 0.674	1.54	3	0.51	58.6

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose.Time 0.404	34.39	33	1.04	758.4
Dose.Day 0.674	1.54	3	0.51	58.6

Table A4.9. REML variance of interaction between nitrate dose, frequency and time for log carboxyhaemoglobin in the blood of *Bos indicus* steers treated with nitrate once daily

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose 0.891	0.61	3	0.20	8.8
Time <0.001	33.38	11	3.03	817.5
Dose.Time 0.103	43.91	33	1.33	817.7
Day 0.013	6.51	1	6.51	67.0
Dose.Day 0.354	3.31	3	1.10	67.1

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose.Time 0.098	44.18	33	1.34	817.7
Dose.Day 0.354	3.31	3	1.10	67.1

Table A4.10. REML variance of interaction between nitrate dose, frequency and time for log carboxyhaemoglobin in the blood of *Bos indicus* steers treated with nitrate twice daily

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose 0.162	6.96	3	2.32	7.0
Time <0.001	58.03	11	5.28	753.0
Dose.Time <0.001	99.04	33	3.00	753.2
day 0.150	10.08	6	1.68	41.2
day.Dose 0.500	17.62	18	0.98	41.4

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose.Time <0.001	100.34	33	3.04	753.2
day.Dose 0.500	17.62	18	0.98	41.4

Table A4.11. REML variance of interaction between nitrate dose, frequency and time for haematocrit in the blood of *Bos indicus* steers treated with nitrate once daily

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose 0.346	3.78	3	1.26	9.0
Time <0.001	130.92	11	11.90	790.1
Dose.Time <0.001	81.81	33	2.48	790.3
Day 0.004	8.78	1	8.78	64.9
Dose.Day 0.016	11.06	3	3.69	64.9

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose.Time <0.001	84.12	33	2.55	790.3
Dose.Day 0.016	11.06	3	3.69	64.9

Table A4.12. REML variance of interaction between nitrate dose, frequency and time for haematocrit in the blood of *Bos indicus* steers treated with nitrate twice daily

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose 0.879	0.66	3	0.22	7.0
Time <0.001	89.65	11	8.15	748.3
Dose.Time 0.754	27.07	33	0.82	748.4
day <0.001	37.03	6	6.17	38.1
day.Dose 0.914	9.87	18	0.55	38.2

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f. F pr
Dose.Time 0.771	26.65	33	0.81	748.4
day.Dose 0.914	9.87	18	0.55	38.2

CHAPTER 5. ANOVA TABLES

Table A5.1. One Way ANOVA of arterial blood pH, partial pressure of oxygen (pO_2), partial pressure of carbon dioxide (pCO_2), bicarbonate (HCO_3) and base excess (BE) for *Bos indicus* steers treated with 0, 30 or 50 g of nitrate per day for seven days and walked three kilometres

		Sum of Squares	df	Mean Square	F	Sig.
pH	Between Groups	.002	2	.001	2.655	.124
	Within Groups	.004	9	.000		
	Total	.006	11			
pCO2	Between Groups	14.302	2	7.151	.580	.579
	Within Groups	110.908	9	12.323		
	Total	125.209	11			
pO2	Between Groups	2427.662	2	1213.831	10.075	.005
	Within Groups	1084.368	9	120.485		
	Total	3512.029	11			
HCO3	Between Groups	4.655	2	2.328	.717	.514
	Within Groups	29.227	9	3.247		
	Total	33.883	11			
BE	Between Groups	5.315	2	2.658	.713	.516
	Within Groups	33.528	9	3.725		
	Total	38.843	11			

Table A5.2. One Way ANOVA of total haemoglobin (tHb), deoxyhaemoglobin (FHHb), Oxyhaemoglobin (FO_2Hb), MetHb (FMetHb), carboxyhaemoglobin (FCO_2Hb) and hematocrit for *Bos indicus* steers treated with 0, 30 or 50 g of nitrate per day for 7 days and walked 3 kilometres

		Sum of Squares	df	Mean Square	F	Sig.
Hct	Between Groups	11.167	2	5.583	.573	.583
	Within Groups	87.750	9	9.750		
	Total	98.917	11			
tHb	Between Groups	148.167	2	74.083	.717	.059
	Within Groups	930.500	9	103.389		
	Total	1078.667	11			
sO2	Between Groups	24.185	2	12.093	3.944	.514
	Within Groups	27.595	9	3.066		
	Total	51.780	11			
FO2Hb	Between Groups	2690.460	2	1345.230	26.999	.000
	Within Groups	448.423	9	49.825		
	Total	3138.883	11			
FCOHb	Between Groups	2.687	2	1.343	15.908	.001
	Within Groups	.760	9	.084		
	Total	3.447	11			
FMetHb	Between Groups	2305.445	2	1152.723	21.885	.000
	Within Groups	474.045	9	52.672		
	Total	2779.490	11			
FHHb	Between Groups	5.002	2	2.501	1.429	.289
	Within Groups	15.748	9	1.750		
	Total	20.749	11			

Table 5. 3. One Way ANOVA of heart rate before and immediately after walking, and respiration rate, and rectal temperature after walking 3 kilometres from *Bos indicus* steers treated with 0, 30 or 50 g of nitrate per day for 7 days

		Sum of Squares	df	Mean Square	F	Sig.
HR pre	Between Groups	666.500	2	333.250	1.462	.282
	Within Groups	2051.750	9	227.972		
	Total	2718.250	11			
HR post	Between Groups	11476.500	2	5738.250	6.821	.016
	Within Groups	7571.500	9	841.278		
	Total	19048.000	11			
RR	Between Groups	206.000	2	103.000	.413	.673
	Within Groups	2242.000	9	249.111		
	Total	2448.000	11			
RT	Between Groups	.515	2	.257	1.888	.207
	Within Groups	1.227	9	.136		
	Total	1.742	11			

CHAPTER 6. ANOVA TABLES

Table A6.1. ANOVA of interaction between nitrate dose, day and time for MetHb in the blood of *Bos indicus* steers.

Variate: MetHb

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
ID stratum					
Dose	1	2712.609	2712.609	132.25	<.001
Residual	8	164.087	20.511	1.62	
ID.Day stratum					
Day	3	1.434	0.478	0.04	0.990
Day.Dose	3	4.430	1.477	0.12	0.949
Residual	24	303.180	12.632	2.21	
ID.Day.*Units* stratum					
Time	3	1055.923	351.974	61.58	<.001
Day.Time	9	326.931	36.326	6.36	<.001
Dose.Time	3	1057.178	352.393	61.66	<.001
Day.Dose.Time	9	339.747	37.750	6.60	<.001
Residual	96	548.677	5.715		
Total	159	6514.195			

Table A6.2. ANOVA of interaction between nitrate dose, day and time for tHb in the blood of *Bos indicus* steers.

Variate: tHb

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
ID stratum					
Dose	1	10.00	10.00	0.01	0.927
Residual	8	9001.85	1125.23	21.37	
ID.Day stratum					
Day	3	2589.75	863.25	16.40	<.001
Day.Dose	3	155.35	51.78	0.98	0.417
Residual	24	1263.65	52.65	3.88	
ID.Day.*Units* stratum					
Time	3	1068.85	356.28	26.23	<.001
Day.Time	9	815.30	90.59	6.67	<.001
Dose.Time	3	286.95	95.65	7.04	<.001
Day.Dose.Time	9	99.30	11.03	0.81	0.606
Residual	96	1304.10	13.58		
Total	159	16595.10			

Table A6.3. ANOVA of interaction between nitrate dose, day and time for MetHb_peak in the blood of *Bos indicus* steers.

Variate: MetHb_peak

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
ID stratum					
Dose	1	2729.10	2729.10	122.68	<.001
Residual	8	177.96	22.24	1.94	
ID.Day stratum					
Day	3	5.86	1.95	0.17	0.915
Day.Dose	3	4.79	1.60	0.14	0.935
Residual	24	274.88	11.45		
Total	39	3192.60			

Table A6.4. ANOVA of interaction between nitrate dose, day and time for FCOHb in the blood of *Bos indicus* steers.

Variate: FCOHb

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
ID stratum					
Dose	1	51.3022	51.3022	22.42	0.001
Residual	8	18.3030	2.2879	3.45	
ID.Day stratum					
Day	3	7.9203	2.6401	3.98	0.020
Day.Dose	3	1.4023	0.4674	0.71	0.558
Residual	24	15.9050	0.6627	0.87	
ID.Day.*Units* stratum					
Time	3	16.2662	5.4221	7.14	<.001
Day.Time	9	11.9672	1.3297	1.75	0.088
Dose.Time	3	20.1723	6.7241	8.85	<.001
Day.Dose.Time	9	10.6412	1.1824	1.56	0.140
Residual	96	72.9480	0.7599		
Total	159	226.8278			

Table A6.5. ANOVA of interaction between nitrate dose, day and time for log FCOHb in the blood of *Bos indicus* steers.

Variate: logFCOHb

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
ID stratum					
Dose	1	13.4771	13.4771	12.02	0.008
Residual	8	8.9672	1.1209	3.58	
ID.Day stratum					
Day	3	9.0576	3.0192	9.66	<.001
Day.Dose	3	1.1096	0.3699	1.18	0.337
Residual	24	7.5049	0.3127	1.09	
ID.Day.*Units* stratum					
Time	3	4.2731	1.4244	4.94	0.003
Day.Time	9	5.6735	0.6304	2.19	0.029
Dose.Time	3	5.2817	1.7606	6.11	<.001
Day.Dose.Time	9	2.6177	0.2909	1.01	0.438
Residual	96	27.6583	0.2881		
Total	159	85.6208			

Table A6.6. ANOVA of interaction between nitrate dose, day and time for FHHb in the blood of *Bos indicus* steers.

Variate: FHHb

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
ID stratum					
Dose	1	45.1	45.1	0.07	0.800
Residual	8	5231.0	653.9	5.18	
ID.Day stratum					
Day	3	2382.7	794.2	6.29	0.003
Day.Dose	3	1152.0	384.0	3.04	0.048
Residual	24	3032.1	126.3	0.87	
ID.Day.*Units* stratum					
Time	3	4073.5	1357.8	9.31	<.001
Day.Time	9	3378.8	375.4	2.57	0.011
Dose.Time	3	261.3	87.1	0.60	0.618
Day.Dose.Time	9	1360.5	151.2	1.04	0.417
Residual	96	13997.4	145.8		
Total	159	34914.3			

Table A6.7. ANOVA of interaction between nitrate dose, day and time for FO2Hb in the blood of *Bos indicus* steers.

Variate: FO2Hb

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
ID stratum					
Dose	1	2724.2	2724.2	3.28	0.108
Residual	8	6652.3	831.5	5.94	
ID.Day stratum					
Day	3	2634.1	878.0	6.27	0.003
Day.Dose	3	1323.3	441.1	3.15	0.043
Residual	24	3360.8	140.0	0.92	
ID.Day.*Units* stratum					
Time	3	2162.0	720.7	4.73	0.004
Day.Time	9	3404.0	378.2	2.48	0.014
Dose.Time	3	1389.8	463.3	3.04	0.033
Day.Dose.Time	9	1979.3	219.9	1.44	0.180
Residual	96	14618.6	152.3		
Total	159	40248.4			

Table A6.8. ANOVA of treatment effect on body weight

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	8.000	2108.058	.000
Treat	1	8.000	1.204	.305
Weeks	5	45.000	4.122	.004

a. Dependent Variable: Body weight.

Table A6.9. ANOVA of treatment effect on dry matter intake

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	8.000	3668.884	.000
Days	68	612.000	4.094	.000
Treat	1	8.000	.004	.954

a. Dependent Variable: Dry matter intake.