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Practical and sustainable considerations for the mitigation of methane emissions in the northern Australian beef herd using nitrate supplements

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Executive summary

The efficacy of methane reduction, animal productivity and risk of nitrite toxicity were examined when *Bos indicus* cattle consuming low quality tropical forages were fed nitrate to correct a deficiency of rumen degradable nitrogen (RDN). The main findings of this project were:

- Dry matter intake and liveweight gain responses for steers were similar when urea or nitrate salts are fed at isonitrogenous rates.
- There was no significant reduction on daily methane production (g CH_4/d) for individual steers weighing 400 kg when dosed with nitrate up to a rate of 50 g daily (7.9 g nitrate/kg DM), but reductions in methane yield (g CH_4/kg DMI) can be achieved.
- Nitrate supplementation was consistently associated with increased blood methaemoglobin concentrations and suggests an elevated risk of nitrite toxicity.
- Concentrations of blood methaemoglobin reported in this project are higher than other published studies despite using low dietary nitrate concentrations. This project has confirmed that feeding frequency effects have an important role in the development of methaemoglobinaemia.
- A significant respiratory challenge was evident when nitrate supplemented cattle were subjected to exercise.
- The use of nitrate blocks in a grazing trial resulted in lower consumption by cattle compared with urea lick blocks. Cattle consumed insufficient N from nitrate to remedy the underlying deficiency of RDN. This resulted in a lower liveweight response, a reduction in BCS and no impact on daily methane production. Blood methaemoglobin concentrations increased in a dose respondent manner.

Caution should be exercised when feeding nitrate salts as a urea substitute in self-fed supplements to extensively managed beef cattle herds during the dry season. Nitrate consumption is unlikely to reach a level that significantly decreases methane production, or, if this threshold is achieved, there is significant risk of nitrite toxicity. The existing methodology for reducing greenhouse gas emissions by feeding nitrate to beef cattle was approved prior to the findings of this project being released. It is suggested that the methodology and underlying technical assumptions be reviewed using the findings from this project, which contains data specific to the forages, supplements and feeding practices relevant to the northern Australia beef cattle industry.

Future research on the use of nitrate in the context of northern Australian beef cattle industry should focus on mechanisms to minimise the risk of nitrite toxicity, thereby allowing greater levels of nitrate to be safely fed and thereby achieve a reduction in methane production.

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1. Background

Enteric methane from ruminants accounts for 11% of GHG emissions in Australia and beef cattle are the largest emitting sector (Commonwealth of Australia 2014). Australian beef production systems are largely pasture based with approximately 60% of the national beef herd located in the northern rangelands. Cattle in these regions graze C4 tropical pastures and consequently have lower annual liveweight gain and reproductive performance compared with temperate pasture based systems, resulting in higher methane emission intensity (Charmley *et al.* 2008). Efforts to reduce the carbon footprint of cattle production systems in Australia should place considerable emphasis on methane emissions from the northern herd.

Incorporation of dietary additives into existing supplement delivery mechanisms may provide abatement opportunities for the northern beef industry because supplementation of cattle during the dry season, between April and October, is common practice (Bortolussi *et al*. 2005). During this period, nitrogen (N) is the primary limiting nutrient in the diet of grazing cattle in the northern rangelands (Winks 1984). The addition of non-protein nitrogen (NPN) sources, such as urea to low quality forage diets, typical of those consumed over the northern dry season, increases forage intake and liveweight gain (Hennessy and Williamson 1990; Hennessy *et al*. 2000).

Leng (2008) proposed that urea could be replaced with nitrate salts to provide an ammonia source for microbial growth when ruminants consume low quality forages. Nitrate may also provide a mechanism to decrease enteric methanogenesis by providing an energetically favourable alternative electron sink through hydrogen in the reduction of nitrate to nitrite and ultimately the production of ruminal ammonia (Ungerfeld and Kohn 2006). The replacement of dietary urea with nitrate has been validated experimentally in beef cattle (Hulshof *et al*. 2012; Velazco *et al*. 2014), confirming reductions in both daily methane production (g CH_4/d) and methane yield (g CH_4/kg DMI). In these experiments, cattle consumed total mixed rations containing moderate (400 g/kg DM) to high (> 750 g/kg DM) levels of concentrates, which allow for multiple meals of a small amount of nitrate within a day for individual animals.

This is in contrast to grazing systems during the northern Australia dry season where cattle consume a low quality forage diet, are supplemented with NPN in the form of free choice loose mineral mixes or solidified lick blocks and generally have no access to additional concentrates. These 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*a*). 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 a 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). In the context of production systems used in northern Australia, there may be an increased the risk of methaemoglobinaemia if nitrate salts replace urea as the NPN source.

Beef cattle in northern Australia are reared on an extensive basis. Paddock sizes are often very large and cattle must walk long distances during mustering and to seek both pasture and water. When blood methaemoglobin is elevated, the oxygen carrying capacity of the blood is impaired (Parkinson *et al*. 2010). This may reduce exercise tolerance and cattle may be less inclined to walk away from watering points to graze. This may further exacerbate the problem of uneven grazing distribution in rangeland environments (Hunt *et al*. 2007). Nitrates have also been associated with a reduction of dry matter intakes of beef cattle when replacing urea in the diet (Hulshof *et al*. 2012; Hegarty *et al.* 2013). Any reduction in dry matter intake would be considered contrary to a production orientated outcome when supplementing NPN to cattle consuming low quality forage.

A series of experiments were designed to address these knowledge gaps to determine the efficacy of methane reduction, animal productivity, exercise tolerance and risk of nitrite toxicity were examined when *Bos indicus* cattle consuming low quality tropical forages were fed nitrate, as an alternative to urea, to correct a deficiency of rumen degradable nitrogen (RDN). These experiments were progressed from pen studies, where nitrate dosage was pre-determined, through to field testing to measure the effects of nitrate supplements when self-administered under conditions typical of northern Australian dry season.

2. Methodology

Experiment 1: Methane emissions, dry matter intake, rumen fermentation and methaemoglobin concentrations of *Bos indicus* steers fed Flinders grass (*Iseilemia spp*.) hay supplemented with either urea or nitrate

Animals and management

The experimental protocol complied with the Australian Code of Practice for the care and use of Animals for Scientific Purposes (NHMRC 2004) and was approved by the local Animal Experimentation and Ethics Committee (A10/2012). Eight two-year-old fistulated *Bos indicus* steers with an initial live weight of 412 ± 19.3kg (Mean ± SD) were used in this study. The animals were familiarized with handling and sampling procedures before the start of the experiment, including periods of housing in open circuit respiration chambers. Throughout the experiment, the animals were allocated to individual pens within a covered animal house facility at Lansdown Research Station, Woodstock, Australia. The animals were fed chaffed Flinders grass (*Iseilema spp.*) hay once daily at 0800 h. To ensure hay intake was *ad libitum*, the amount of hay offerred to each animal was calculated from the previous days actual intake + 20% additional hay (w:w). Samples of Flinders grass hay were bulked over the course of the experiment and sub-sampled for proximate analysis (Table 1.1). Water was available to the animals *ad libitum*. Hay refusals were recorded once daily at 0800 h.

Experimental Design

Since methane measurement was limited to four open circuit respiratory chambers, the eight steers were divided into two groups of four steers each. The experimental design

consisted of two 4 x 4 Latin squares, one for each group. The design comprised four supplement treatments being allocated to the four measurement periods for each steer. The two groups were staggered 7 d apart, with each group going through four periods so that each steer received each treatment, resulting in 8 replicates per treatment.

The supplement treatments were: Control (nil nitrogen supplement), Urea (urea supplement) and two levels of calcium nitrate supplement CaN1 (equivalent to 30 g nitrate/d) and CaN2 (equivalent to 50 g nitrate/d). Treatments of urea, CaN1 and CaN2 were formulated to be isonitrogenous (Table 1.2). Current Australian feeding standards indicate that when cattle consuming low quality forages are supplemented with non-protein nitrogen (NPN), the efficiency of microbial protein production (eMCP) will peak at ~130 g MCP/kg digestible organic matter (DOM; CSIRO 2007). The Urea, CaN1 and CaN2 treatments were formulated to supply 15 g N daily, which was the amount required to supply adequate rumen degradable nitrogen (RDN). Formulations were based on preliminary chemical analysis of forage (920 g/kg DM, 880 g/kg OM, 50 g/kg CP) and assumed a DMI of 6.0 kg/d. Assumptions also included 45% OMD (Panjaitan *et al*. 2014) and 0.8 for rumen degradability of forage protein (Bowen *et al*. 2008). Urea recycling was ignored to ensure an over-supply, rather than undersupply of RDN. In order to correct P, Na and S deficiency in the forage, 25 g of MDCP (Biofos®) and 25 g sodium sulphate were added to all treatments daily (Table 1.2). All supplements were mixed with a molasses carrier (250 g) to encourage intake and presented to each animal prior to the morning feed. Each experimental period was 28 d. The first 6 d comprised an adaptation to pens where hay only was fed. Supplements were gradually introduced between 7 and 10 d, followed by full dosage between 11 and 28 d. During period 1, the steers consumed supplements within 30 min, with the exception of two steers (from group 2) which rejected all supplements. For the remaining periods, supplements were offered each morning for up to 30 min and any supplement not consumed in this time was dissolved in warm water (up to 500 mL) and dosed directly into the rumen. An extra period was added to the experiment to repeat the treatments rejected by the two steers (period 1) in order to complete the experimental design.

Steers were confined in open circuit respiration chambers on d 27 and 28 of each period for individual 48 h methane measurements. At the completion of each period, steers were removed from the animal house and fed hay in a group pen over 12 d without supplementation. These 12 days served as a washout period prior to the commencement of the next period. One steer succumbed to an infection during period 2 and was withdrawn from the experiment.

Measurement and Sampling

Hay offered was bulked over each period, sub-sampled and stored for chemical analysis. Dry matter of the hay was determined by drying in a vacuum oven at 60°C for 48 h. Daily dry matter intake was determined for a 7-d period immediately prior to entering open circuit respiration chambers (d 20 to 26) and during the 48 h period of confinement in chambers.

Individual methane production was determined over 48 h using four open circuit respiration chambers. Each chamber had an internal volume of 23.04 m^3 and was fitted with an automated water trough and feed bin containing the daily ration. Chambers were maintained at 2.0 °C below external ambient air temperature, approximately -10 Pa, and relative humidity varied between 61 and 90%. Air was drawn through a 250

mm diam duct into each chamber at a rate of approx. 3000 L/min. Exact flow rates, corrected to standard conditions for temperature and pressure (STP) for each chamber were used in calculations for methane production (Takahashi *et al.* 1999; Williams *et al.* 2007). Flow rate through each chamber was measured using thermal flow sensors (SS20.500 SCHMIDT® Flow Sensor). Air samples from each chamber was drawn from a point in the exhaust duct at 4.5 L/min using a micro diaphragm pump located between a multiport gas switching unit (SW & WS Burrage, Ashford Kent UK) and membrane drier (Perma Pure LLC). Air samples from each chamber initially passed through particulate filters (AF30-02 SMC Pneumatics Aust. Pty Ltd) and a four pot fridge drier prior to the multiport gas switching unit which was programmed to cycle through each chamber and two outside air ports. Clean, dry air samples then passed through independent rotameters before compositional analysis for CO_2, O_2 and CH_4 (Servomex 4100, Servomex Group Ltd. Crowborough UK). Data for flow rate, temperature, chamber pressure, and CH_4 concentrations in the inlet and exhaust air was managed in a SQL data base to calculate methane production expressed on a g/d and g/kg DMI basis.

Pre-treatment blood and rumen fluid samples were collected immediately before the introduction of supplements for each period. Samples were also obtained at 3 and 6 h after dosing, for the 7 d period immediately prior to entering open circuit respiration chambers. Blood was collected using indwelling venous jugular catheters fitted to steers using a modified technique adapted from Parker *et al.* (2009). The blood was drawn from tubing using 3-mL heparinised pre-set syringes (Becton Dickinson), placed in crushed ice and analysed immediately for methaemoglobin on a blood gas analyser (Siemens, Rapid Lab 1265, Sydney, NSW). After each blood sample was obtained 10 mL heparinised saline (15000 IU heparin) was flushed through the line to prevent clotting. Rumen fluid samples were drawn directly from three sites using a 50 mL syringe fitted with a rumen probe sleeved with 150 μm nylon. Samples were collected twice daily; 3 and 6 h post dose over 7 d. The rumen fluid was immediately sub sampled; 4mL into duplicate 5 mL tubes containing 1.0 mL 20% metaphosphoric acid for VFA analysis, and 4 mL into duplicate 5mL tubes containing 0.4 mL 25% metaphosphoric acid for determining ruminal ammonia-N concentrations. All samples were frozen until analysis. Volatile fatty acid concentrations in rumen fluid were measured using the method described by Cottyn and Boucque (1968) and Playne (1985) using a gas chromatograph (Varian CP-3800 GC) fitted with a polar capillary column (Varian CP-WAX 52CB). Rumen ammonium-N (NH_3-N) concentration was determined by steam distillation (Buchi, Switzerland) using saturated sodium tetraborate and boric acid, and titration (Titralab TIM 840, Radiometer Analytical, France) against 0.01M HCl. Resultant NH_3-N concentration was expressed as mg/L.
An intensive period of rumen fluid collection was conducted during period 3 at 0, 1, 2, 3, 4, 6, 9, 12 and 24 h post dose to determine the effects of nitrate supplementation on rumen pH, ammonia-N concentration and volatile fatty acid concentration over 24 h.

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 treatment (Control, Urea, CaN1 and CaN2) on mean dry matter intake, methane production, blood methaemoglobin, rumen ammonia and VFA concentrations. Time after dosing (3 or 6 h) and day of treatment period (1-7) were added to the model as fixed effects for

examining blood methaemoglobin, rumen ammonia and VFA concentrations. Random effects in the model included the individual animal, time period and interactions. Where pre-treatment values of the response variables were available, these were included as a covariate in the model if significant at $P < 0.1$. Plots of residuals *vs* fitted values were examined for each analysis. If the plot indicated a departure from the assumption of the homogeneity of variance, a log transformation was applied. Data from the two steers in period 1 (group 2) were excluded from the analysis because they did not consume their treatments then, and replacement data from the additional period was included. Adjusted means for all variables were estimated from the mixed models for comparison between treatments. Adjusted means were compared using the method of least significant differences (LSD), with significance at $P < 0.05$.

Table 1.1. Composition of bulked Flinders grass (*Iseilema spp.*) hay throughout the experiment (g/kg DM).

Test	Flinders Grass Hay
Protein	49
Dry Matter	942
Organic Matter	894
Ash	106
Fat	14
Acid Detergent Fibre	492
Neutral Detergent Fibre	680
Calcium (Ca)	5.27
Phosphorous (P)	1.03
Sodium (Na)	0.14
Sulphur (S) (g/kg)	1.79

Source: Symbio Alliance, Eight Mile Plains, Queensland

Table 1.2. Composition of control, urea and nitrate based treatments offered to *Bos indicus* steers fed chaffed Flinders grass hay *ad libitum*

Composition	Control	Urea	CaN1	CaN2
Urea (g/day)	0	32.5	16.5	5.5
Calcium nitrate decahydrate[1] (g/day)	0	0	48	80
Sodium sulphate (g/day)	25	25	25	25
MDCP(g/day)	25	25	25	25
N (g/day)	0	15.0	15.2	15.0
NO_3 (g/day)	0	0	30.3	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

[1] Swancorp, Rocklea, Queensland

Experiment 2: 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

Animals and management

All 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 live weight (317.8 ± 28.5kg (Mean ± SD) were used in this study. Throughout the experiment, the animals were allocated to individual pens (4.28m x 1.26m) within a covered cattle house facility. The animals were given chaffed Flinders grass (*Iseilema spp.*) hay at three times (0800, 1200, 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 days 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 2.1; 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.

Preliminary chemical analysis of the Flinders grass hay demonstrated the forage comprised (g/kg DM) N: 0.88; P: 1.0; Na: 0.14 and S: 1.0. Feeding standards indicate that when cattle consuming low quality forages are supplemented with non-protein nitrogen (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 2.2) which was the calculated amount 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*. 2014) 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 30, 40 and 50 grams nitrate daily were achieved by blending urea and calcium nitrate decahydrate (Swancorp, Rocklea, QLD, Australia). In order to correct P, Na and S deficiency 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.

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, 30, 40 and 50 grams 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 of 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. Each experimental period consisted of a 6 day preliminary feeding period where steers were fed hay only, then followed by a 7 day treatment period. Previous work at CSIRO Lansdown research station has demonstrated that 10 days is sufficient to return the animal to a normal concentration of methaemoglobin (Tomkins *pers. comm*. 2013).

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 h and continued for a period of 7 days. Blood sampling was timed to coincide with the peak methaemoglobin (%) three hours after dosing. The 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, methaemoglobin, and carboxyhaemoglobin in blood on a blood gas analyser (Siemens, Rapid Lab 1265, Sydney, NSW). 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 two, one steer in the 40 grams of nitrate dosed once a day treatment was found to have a methaemoglobin concentration greater than 75% and was treated with a methylene blue solution and removed from the study in accordance with animal ethics protocols.

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 grams 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 included as well for variables which were not aggregated over time. Separate analyses for once and twice a day feeding frequency 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 methaemoglobin and carboxyhaemoglobin data was log transformed before analysis. Plots of residuals vs fitted values for the other variables were consistent with the assumption of constant variance and therefore they were not transformed. The peak methaemoglobin values were defined by the following rule: When frequency = 1 or 2, select the greatest reading between 0600 and 2400 h inclusive. The rate of incline for methaemoglobin in the model was defined as the peak reading minus the reading four hours prior to peak. The reported means and slopes for rate of incline were divided by four to present an hourly rate.

Table 2.1. Composition[a] of bulked Flinders grass (*Iseilema spp.*) hay throughout the experiment.

Test	Flinders Grass Hay
Protein (%)	2.9
Fat (%)	1.2
Moisture (%)	8.3
Ash (%)	8.6
Crude Fibre (%)	39.1
Dry Matter (%)	91.7
Nitrogen Free Extract DWB (%)	48.1
Acid Detergent Fibre (%)	52.4
Neutral Detergent Fibre (%)	72.1
Organic Matter (%)	91.3
Calcium (Ca) (g/kg)	3.20
Phosphorous (P) (g/kg)	0.64
Sodium (Na) (g/kg)	0.25
Sulphur (S) (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.

Table 2.2. 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	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

Experiment 3: The effect of nitrate supplementation on arterial blood gases, heart rate, respiratory rate and rectal Temperature of *Bos indicus* cattle after exercise

Animals and management

The experiment was carried out at the Fletcher view research station of James Cook University in Charters Towers, Queensland. All the experimental procedures were reviewed and approved by the James Cook University Animal Ethics Committee # A1929. Twelve two-year-old fistuled *Bos indicus* steers with a mean (± SEM) live weight of 397 kg ± 10.84 kg were used in this study. The animals were kept as one group and were given 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 by a private feed analysis laboratory (Table 2.1; Symbio Laboratories, Brisbane, Queensland). The animals were trained and familiarized with handling and sampling procedures before the commencement of the experiment. On day 7, animals representing each treatment group were walked in groups of three, by two stock personal on horses. A GPS hiking device (Garmin Etrex 20, Garmin Corp. USA) was attached to the halter of each of the steers. The steers walked for an average period of 24 minutes at a speed of 3.3 km/h with a maximum speed of 16.5 km/h. All blocked groups were walked over the same terrain for a mean distance of 2.8 km. On the day of the exercise regimen all steers were dosed with their respective treatments at 0630 h. The first group of three animals were released at approximately 0830 h. The experiment concluded with the final measurements completed at 1230 h.

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 in a 15 second interval. Body temperature was recorded using a thermometer inserted into the rectum of the animal for one minute. Arterial blood samples for blood gas and co-oximetry analysis were obtained from the caudal auricular artery (Riley and Thompson 1978). 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 an ice-water slurry 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 and blood co-oximetry were measured on a blood gas analyser (Siemens Rapid Lab 1265, Seimens Health Care, Sydney, NSW).

Experimental design

The experiment was a complete randomized block design. The steers were randomly allocated to one of three nitrate treatments: 0, 30, and 50 grams of nitrate daily (Table 3.1). The nitrate treatment was dosed into the rumen of individual steers once daily at 0630 h for seven days. The animals were then randomly blocked into four different groups for the exercise experiment.

Data Analysis

Least squares means and standard errors are presented. Data were analysed by one way ANOVA with treatment as the sole source of variation in the model. The ANOVA was conducted using the SPSS statistics version 22 software package. Multiple comparison tests were undertaken using Tukeys honestly significant difference test, where the level of significance was set at $P < 0.05$.

Table 3.1. Composition of raw materials and formulated analysis of N, NO_3, 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

Experiment 4: Effects of urea or nitrate supplements on haemoglobin fractions from *Bos indicus* steers fed Flinders grass (*Iseilemia spp*.) hay for 70 days

Animals and management

Ten three-year-old fistulated *Bos indicus* steers with 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 Mitchel 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 of total dry matter intake was 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 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. The steers were dosed with their respective treatments at 0900 h each day by placing the treatments directly into the rumen of each animal for 70 days. Live weights of the steers were recorded on day 0 and 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 that were given an iso-nitrogenous supplement of 15 grams 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 4.2).

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 and at two hours intervals post dose (11 am, 1 pm, and 3 pm) on days 10, 30, 50 and 70 of the experiment. Blood samples were place 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).

Statistical analysis

A repeated measures mixed linear model was performed to analyze the effects of treatment (nitrate or no nitrate), time of blood collection (0hrs, and 2, 4, 6 hours post dosing) and day (10, 30, 50 and 70) and their interactions on the fractions of haemoglobin in the steer's blood. Live weight was analyzed for the effects of treatment and day only and dry matter intake was analyzed for the effects of treatment and day (days 0 to 70)(IBM SPSS version 20).

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

Test	Flinders Grass Hay
Protein (%)	2.9
Fat (%)	1.2
Moisture (%)	8.3
Ash (%)	8.6
Crude Fibre (%)	39.1
Dry Matter (%)	91.7
Nitrogen Free Extract DWB (%)	48.1
Acid Detergent Fibre (%)	52.4
Neutral Detergent Fibre (%)	72.1
Organic Matter (%)	91.3
Calcium (Ca) (g/kg)	3.20
Phosphorous (P) (g/kg)	0.64
Sodium (Na) (g/kg)	0.25
Sulphur (S) (g/kg)	0.91

Source: Symbio Laboratories, Brisbane, Queensland

[a]All tests results except moisture are reported on a dry matter basis. Moisture is reported on an as fed basis.

Table 4.2. 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

Experiment 5: Field experiments for *Bos indicus* cows grazing tropical pastures consuming nitrate lick blocks

Location and pastures

Herd scale supplementation experiments were conducted in successive dry seasons at Fletcherview Research Station (20°53'S 146°11'E), situated 26 km northwest of Charters Towers in the seasonally dry tropics of northern Australia. Mean annual precipitation is 615 mm with a highly seasonal distribution. Most rainfall (70%) falls in the summer months between December and March with an extended dry season occurring during the intervening months. Cattle were grazed in a single 467-ha paddock for the duration of both experiments. The paddock contained two dams, located at the northern and southern end of the paddock. However during the experiments, cattle were allowed access to the dam at the southern end of the paddock only. The soils in this paddock have previously been described by Roth *et al.* (2003) as a mixture of fertile basaltic rocky red (euchrozems) associated with basaltic black soils (heavy grey-brown cracking clays) along with basaltic sediments and some areas where the underlying gradodiorite and sedimentary soils are exposed. The dominant pasture species was forest bluegrass (*Bothriochloa bladhii*), comprising approximately 40% of the pasture biomass. Introduced pasture species accounted for a further 40% of the pasture biomass, split between sabi grass (*Urochloa mosambicensis*) and buffel grass (*Cenchrus ciliaris*). Minor species included black spear grass (*Heteropogon contortus*), wire grass (*Aristida spp.*) and the introduced legume seca stylo (*Stylosanthes scabra*), which was well established and interspersed across the paddock. The pastures were interspersed with narrow leaf ironbark (*Eucalyptus crebra*), red bloodwood (*Corymbia gummifera*) and grey box (*Eucalyptus microcarpa)*.

Experimental design

The experiment utilised a two-way remote automatic drafting unit (Precision Pastoral Pty. Ltd., Alice Springs, Australia) to allow cattle to access differing self-fed supplement regimes whilst being grazed in a common paddock. This was achieved by placing the drafting unit at the dam which was the only watering point available in the paddock. A boundary fence was constructed around the dam and access to water was only possible via one-way spear traps. In both years, prior to the commencement of the experimental period, cattle were gradually familiarised with both spear traps and the drafting unit. Cattle were required to walk firstly next to, then through the drafting unit to access the spear gates exiting the dam. A drafting training schedule was conducted over 14 days including alternate draft gate movements, powered by an oxygen gas cylinder. Two additional yards were constructed leading off the drafting unit. Cattle could then be directed into either yard containing supplement treatments by an automated gate movement programmed from individual animals RFID ear tag. Entry to these yards was solely via the remote automatic drafting unit and exits were via one-way spear gates. During year 1 (Y1) of the experiment, cattle accessed the drafting unit and consequently supplement yards after first accessing the dam. This design allowed some animals to camp at the water source, away from the location of the supplements. Therefore in year 2 (Y2) the yard design was reconfigured. A water trough was made available in each supplement yard and the dam was completely fenced off to cattle, allowing access to both water and supplement in the same location.

Cattle and Treatments

During Y1 the experiment was conducted between 19th November and 20th December 2013 using non-pregnant, *Bos indicus* cows (*n* = 67), aged between four and twelve years of age. The cows were allocated to one of two nutritional treatments following stratified randomisation on the basis of (i) liveweight (LW) (ii) BCS and (iii) age of cow. The mean LW and BCS (1-5 scale; 1 = poor, 5 = fat) of the cows at commencement of Y1 were 343 kg (s.d. = 43) and 2.5 (s.d. = 0.39), respectively. The allocated RFID lists were immediately loaded into the remote automatic drafting unit such that nutritional treatments could be imposed whilst grazing cattle in the same paddock. The treatments were either remote automatic drafting into a yard containing no supplement (Control; CON) or drafting into a yard allowing free-choice access to molasses-based lick blocks containing nitrate (MNB). The MNB blocks were manufactured according to a cold poured patented block process held by Ridley AgriProducts Pty. Ltd. Blocks contained by weight (g/kg as fed), 350 calcium nitrate decahydrate, 240 molasses, 120 dicalcium phosphate, 80 salt, 50 water, 10 vegetable oil and 5 trace mineral mix including Cu, Co, I, Zn and Se. The remaining ingredients were involved primarily with hardening the blocks. Within the supplement yard, lick blocks (*n* = 3) were spaced at 20 m intervals in an attempt to prevent dominant cows restricting access by other cows to the blocks. The threshold for replacing lick blocks was set at consumption of at least three-quarters of the initial block weight.

At the conclusion of Y1, the cows were relocated to Swans Lagoon, Ayr, due to widespread drought in the Charters Towers region. These cows (excluding some culled for age) and a group of replacement heifers were subjected to an artificial insemination (AI) program over the 15th and 16th February 2014. Bulls were joined with the cows and heifers on 4th March 2014. During the period at Swans Lagoon, cattle were

provided unrestricted access to a mixture of equal parts MDCP and salt. The herd was returned to the experimental paddock at Fletcherview during mid-March. On the 1st May 2014, the cattle were mustered, weighed, bulls were removed and pregnancy diagnosis made via manual palpation. Cattle were then allocated to treatments, returned to the paddock and Y2 experiment commenced. The cows (*n* = 52) and heifers (*n* = 24) were allocated to treatments following stratified randomisation on the basis of (i) pregnancy status (ii) parity (primparous *v.* multiparous) and (iii) LW. There were 6 non-pregnant cows and 10 non-pregnant heifers in the herd. The mean LW of cows and heifers at commencement of Y2 were 502 (s.d. = 38) and 413 kg (s.d. = 24), respectively. The allocated RFID lists were loaded into the drafting unit prior to cattle being returned to the paddock. Treatments for Y2 were either remote automatic drafting into a yard containing commercial dry season urea lick blocks, Rumevite 30% Urea + P (30U), or the same nitrate blocks (MNB) as used in the Y1 experiment. Samples of blocks taken over both Y1 and Y2 were bulked over the course of the experiment and sub-sampled for chemical analysis (Table 5.1). Confirmation of initial pregnancy diagnosis was made during early June 2014. The Y2 experiment concluded on 5th November 2014, approximately three weeks before calving was due to commence. Cows and calves were monitored closely in the paddock during the calving period. The newborn calves were tagged, date of birth and the dam ID was recorded.

Table 5.1. Composition of molasses nitrate (MNB; Y1 and Y2) and molten urea (30U; Y2) blocks throughout the experiment (g/kg DM).

Test	MNB	30U
Dry Matter	817	980
Nitrogen (N)	73	155
Calcium (Ca)	110	85
Phosphorus (P)	28	42

Source: Symbio Alliance, Eight Mile Plains, Queensland

Measurements and sampling

Year 1

An estimate of herbage mass was made using a visual assessment against photo standards at the commencement of the experiment. Lick blocks were weighed into the supplement yard when treatments commenced, then twice weekly for the duration of the trial. At day 0 (treatments commenced) and then at d 14, 21 and 28, cows were mustered to yards at 1500 h. Immediately upon arrival at the yards, cows were weighed, BCS estimated and an individual faecal sample manually collected per rectum. Faecal samples were obtained successfully on 92% of occasions. The samples were placed on ice and then frozen prior to diet analysis using faecal near infra-red reflectance spectroscopy (F.NIRS). Blood samples were obtained at the same intervals by tail venipuncture into 10-mL potassium EDTA vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Samples were immediately chilled in iced water and centrifuged (3000G × 15 minutes) at 4°C to separate plasma, and then frozen at -20°C. Prior to centrifuging the blood samples, a sub-sample was drawn from each vacutainer

into 3-mL heparinised pre-set syringes (Becton Dickinson) and analysed immediately by an automated blood gas analyser (Siemens, Rapid Lab 1265, Sydney, NSW).

Measurements of methane flux at the herd scale were determined using open path lasers over 23 days. In brief, a remotely controlled open-path Laser (GasFinder/Scanner, Boreal Laser Inc., AB, Canada) was mounted on a digital scanning motor (PTU D300, FLIR Motion Control Systems, Burlingame, CA,USA), located approximately 30 m between two source areas to detect enhanced methane concentrations relative to the source along three paths. The scanning motor was programmed to cycle the laser between three paths representing two possible upwind and a downwind concentration measurement with prevailing easterly winds In addition, two other paths were scanned continuously using an independent laser mounted on a tripod which was moved according to the measurement protocol. Average path length was 88 m. The termination of each laser path consisted of an array of 6 corner cube reflectors housed in an enclosure with a polycarbonate window. The line-averaged CH_4 mixing ratio (ppmv) was recorded approximately every second for 60 s for each path at a height of 1.6 m. The scanner automatically moved the laser and aligned it with the next retro reflector and sampling was repeated for up to 6 h on a daily basis. Laser data was processed to generate 10 min average concentrations for each path. Measurements with return light levels outside of the manufacturer recommended limits were excluded from the average as well as periods when the laser was transitioning to a new path. A weather station was also installed on the site and fitted with a 3-dimensional sonic anemometer (CSAT-3, Campbell Scientific, Logan UT) to characterize daily wind statistics. Animal emissions were calculated by a backward Lagrangian Stochastic (bLS) procedure (bLS, Flesch *et al*, 2004) using WindTrax software (Thunderbeach Scientific).

For daily emission flux estimates, animals in each group were confined to their respective supplement yard after auto drafting (approx 0.13 ha). This yard was treated as a uniform source area to meet modelling criteria. Emissions from the source area could then then be regarded as the herd emission rate and mean animal emission rate (g/hd/d) calculated on the cumulative number of animals confined in that source area for an equivalent 24 h period. Animals were allowed to exit the source area each afternoon and return to grazing. The routine was repeated the following day when cattle accessed water associated with each source.

A filtering criterion was applied to merged 10 min average data sets to remove low accuracy observations. Flesch *et al.* (2013) suggest estimates become unreliable when the mixing capacity of the atmosphere is compromised (very stable or very unstable conditions, or low u*) or if the emitting source is not truly captured by the laser path (poor wind direction and/or wind speed for site configuration). Data were excluded from the data set if any of the following conditions were met; (i) friction velocity (u*) less than 0.15 (ii) absolute value of Monin Obukhov length (L) <5.

Year 2

Measurements and sampling techniques applied during Y1 were repeated in Y2. Cattle were mustered on a monthly basis to determine LW, BCS, diet quality estimates from faecal NIRS and blood methaemoglobin concentrations as previously described. The exception was blood methaemoglobin measurements of 30U cattle during November 2014 only. At this time point, the blood gas analyser malfunctioned immediately after

MNB samples had been measured. Therefore for 30U samples only, blood gases were determined within 24 h according to the method of Evelyn and Malloy (1938). Faecal samples were obtained successfully on 90% of occasions. Pastures species composition and herbage mass were assessed on a monthly basis using the BOTANAL technique (Tothill *et al.*, 1992).

Measurements of methane flux at the herd scale were determined using open path lasers over two occasions for the Y2 dry season period; from 11th June to 16th July 2014 (mid dry season), and from 1st September to 10th October 2014. The process of measurement was in accordance with Y1. The major difference in Y2 was that the source area for daily measurement was concentrated further (approx 0.07 ha) by dividing each supplement yard with an electrified tape.

Measurements of grazing distribution were determined using GPS collars over two occasions; 21 days in July and up to 14 days in October 2014 following direct measurements of methane emissions. The collars were fitted and retrieved to coincide with scheduled measurements of LW and BCS. In the first deployment 59 animals across both groups were fitted with either CSIRO U-blox5 GPS units, CSIRO U-blox4 GPS units or UNE AniTrak devices. In the second deployment 57 collars were deployed across both groups. Allocation to collars was based on age, pregnancy status of the animal and number of collar types to ensure no bias could be associated with GPS device.

Laboratory analysis and calculations

In Y1, all samples were processed for faecal NIRS analysis. However in Y2 analysis was confined to animals which were pregnant, had a sample for every measurement interval and had a collar allocated at both GPS collar measurement periods. Faecal samples were oven dried (65°C), and ground through a 1 mm screen in a FOSS Tecator Model 1093 Cyclotec mill (Foss Tecator AB, Hoganas, Sweden). Samples were scanned (400 – 2500 nm range) using a monochromator fitted with a spinning cup module (Foss 6500, NIRSystems, Inc., Silver Spring, MD, USA). Chemometric analysis used ISI software (Infrasoft International, Port Matilda, PA, USA). The Coates (2004) and Coates and Dixon (2008*a*) faecal NIRS calibration equations were used to estimate the crude protein (CP) and dry matter digestibility (DMD) of the diet, plus the non-grass component and faecal N content of the faeces. The non-grass component of the diet was calculated from faecal non-grass composition.

Mean daily intake of blocks (as fed), kg/cow.d was calculated from measurements of lick block disappearance. Conceptus-free LW (CFLW) was calculated in pregnant animals by subtracting the estimated conceptus weight from actual LW at each measurement interval. Estimates of conceptus weight were made using values described by O'Rourke (1991) based on the foetal age at each measurement, which was verified by calving date. There were five animals in Y2 (2 MNB and 3 30U), that were pregnant but did not rear a calf. It was not possible to define the time of calf loss because of the extensive nature of the experiment however the only possible explanation is that the calf was aborted or parturition occurred but succumbed to neo-natal mortality. Therefore these animals were excluded from CFLW calculations.

CSIRO U-blox5, U-blox4 GPS units, and UNE AniTrak collars collected GPS positional data at a rate of 4 Hz (ECEF co-ordinates), 3 Hz (GDA94, Northings and Eastings.

MGA Zone 55), and 5 min (latitude, longitude, WGS84), respectively. Collars had up to 4GB of flash memory. AniTrak collars were programmed to optimise tracks so that redundant way points were deleted prior to download. All resultant geo-positional data was converted to latitude and longitude (WGS84) at 5 min intervals using Python (vers.2.7). Spatial analyst tools in ArcMap 9.1 (ESRI, Redlands, California, USA) were used to overlay spatial data from each GPS device and independent GIS layers indicating paddock boundary and infrastructure (water points and source areas). Temporal relationships were derived from 24 h datasets. Euclidean distances travelled by collared animals were based on consecutive positional data and summed to determine total distance travelled over 24 h. Maximum distance from water/supplement point was determined for each animal as an estimate of grazing range.

Statistical analysis

The data were analysed by fitting linear mixed models, using the REML algorithm of the GenStat statistical package. Fixed effects used in the model were treatment (Y1, CON or MNB; Y2, 30U or MNB), time, and the interaction of treatments and time. In Y2, age (heifer or multiparous cow) and pregnancy status were also included in final models if significant at $P < 0.05$. Individual animals were added as random effects for Y1 and Y2. Where pre-treatment values of the response variables were available, these were included as a covariate in the model if significant at $P < 0.1$. Plots of residuals *vs* fitted values were examined for each analysis. All plots were consistent with the assumption of homogeneity of variance, with the exception of blood methaemoglobin, where a log transformation was applied. Random effects in the model included the individual animal, time period and interactions. Adjusted means for all variables were estimated from the mixed models for comparison between treatments. Adjusted means were compared using the method of least significant differences (LSD), with significance at $P < 0.05$.

3. Results

Experiment 1: Methane emissions, dry matter intake, rumen fermentation and methaemoglobin concentrations of *Bos indicus* steers fed Flinders grass (*Iseilemia spp*.) hay supplemented with either urea or nitrate

Intake

Mean daily dry matter intakes (DMI) were not significantly different between Urea, CaN1 and CaN2 during the 7 d period prior to methane measurements (Table 1.3), but were greater ($P < 0.05$) than control steers during the same period. The DMI of steers during the 48 h period of confinement in open circuit respiration chambers was consistent with treatment means measured during the preceding 7 d in individual pens. Consequently there were no significant differences in DMI between Urea, CaN1 and CaN2 steers when confined for individual methane measurements. Control steers had lower DMI ($P < 0.01$) compared to all other treatments during confinement in respiration chambers.

Methane

Mean daily methane production (g/d) for steers measured in open circuit respiration chambers for 48 h did not differ significantly between treatments (Table 1.3). Methane yield (g/kg DMI) from the CaN2 treatment tended to be lower ($P < 0.07$) than either the control or urea treatments. There were no significant differences in methane yield between control, urea or CaN1 treatments.

Methaemoglobin (MetHb)

Blood methaemoglobin concentrations measured prior to the introduction of supplements were not significantly different between treatments, 0.59 ± 0.28% (mean ± SEM). During the 7 d treatment period before entering respiratory chambers, mean blood methaemoglobin concentrations of CaN2, were greater than control, urea or CaN1 treatments (Table 1.4). There were no significant differences in blood methaemoglobin concentrations between control, urea or CaN1 treatments during the same period. There were significant time effects after dosing ($P < 0.001$) and a significant interaction between treatment and time after dosing ($P < 0.001$). Although overall concentrations of methaemoglobin measured 3 h post dosing were greater than at 6 h, this effect was largely confined to the CaN2 treatment. At 3 h after dosing, blood methaemoglobin concentrations of CaN1 were greater than urea treatment, but not different to the control. Mean daily blood methaemoglobin levels at 3 h post dose are shown in Fig. 1.1. At both 3 and 6 h after dosing, CaN2 had greater methaemoglobin concentrations than control, urea or CaN1.

An effect of measurement day was demonstrated ($P < 0.01$), with mean daily blood methaemoglobin concentrations. The first day of the 7 d period, prior to methane measurements, was significantly lower than all other days (Fig. 1.2). There was also a significant interaction between treatment and measurement day ($P < 0.05$). Mean daily blood methaemoglobin concentrations associated with CaN2 were elevated compared to all other treatments. In addition, mean daily blood methaemoglobin concentrations of CaN1 were greater than control or urea treatments on d 6. Concentrations of blood methaemoglobin for control, Urea and CaN1 remained constant over the measurement period. In contrast CaN2 rose at day 2, declined at day 3 and remained unchanged thereafter.

Rumen fermentation

Mean ruminal pH values determined during the intensive sampling periods are summarised in Table 1.5. There was no significant treatment effect on rumen pH, however there was a significant time effect ($P < 0.05$) over 24 h with a decrease in rumen pH between 4 and 12 h post feeding for all treatment groups.

There was no significant treatment effect on total VFA concentration. Overall mean total VFA concentration was 74.0 ± 1.53 mM. A significant effect for both time of sampling (3 h *v*. 6 h post dose) within days and between days (1 to 7) was observed with total VFA concentrations decreasing over time.

With the exception of propionate there was a significant treatment effect on VFA molar proportions. Molar proportion of acetate was found to be higher for CaN1 and CaN2 compared with the control. Similarly the A:P was also significantly higher for CaN1 and

CaN2 compared with the control and within days (3 h *v*. 6 h post dose) resulting in a significant (*P* <0.001) treatment x time effect. The inclusion of calcium nitrate as an alternative NPN source significantly reduced the molar proportions of butyrate (*P* <0.001), iso-butyrate (*P* <0.05) and iso-valerate (*P* <0.001) compared to the control. The molar proportions of these VFAs were also significantly affected by time of sampling (3 h *v*. 6 h post dose) with butyrate tending to increase and iso-butyrate and iso-valerate decreasing between sampling events.

Ruminal ammonia-N increased significantly (*P* <0.001) with the inclusion of a NPN source (Table 1.5). There was a highly significant treatment by time interaction, but no significant treatment by day interaction. Three hours post dose mean ruminal ammonia-N concentrations were 145.6, 141.9 and 146.9 mg/L for the urea, CaN1 and CaN2 treatments, respectively. These concentrations decreased to 81.4, 72.9 and 70.8 mg/L, respectively by 6 h post dose compared to 65.8 (3 h) and 44.3 mg/L (6 h) for the control.

During the intensive sampling periods mean ammonia-N concentrations decreased over time (1 h to 24 h post dose) by 43, 85, 87 and 81 % for the control, urea, CaN1 and CaN2 treatments, respectively.

Table 1.3. Mean dry matter intake (DMI) and methane production for *Bos indicus* steers fed a Flinders grass hay diet with and without urea, or calcium nitrate at two levels[1]

	Treatments					
	Control	Urea	CaN1	CaN2	l.s.d	*P*-value
DMI, kg/d						
Pen, 7 d	5.7[a]	6.3[b]	6.5[b]	6.3[b]	0.49	0.024
Chambers, 2 d	5.7[a]	6.3[b]	6.8[b]	6.5[b]	0.57	0.008
Methane						
g/day	113.8	110.8	115.0	102.2	15.5	0.300
g/kg DMI	19.9[a]	19.3[a]	18.8[ab]	16.9[b]	2.35	0.065

[1] Mean values shown are pooled means for control (0.0 g urea, 0.0 g calcium nitrate per day), Urea (32.5 g urea, 0.0 g calcium nitrate per day), CaN1 (16.5 g urea, 48 g calcium nitrate per day) and CaN2 (5.5 g urea, 80 g calcium nitrate per day) treatment groups throughout the experimental period

Within rows, different alphabetical superscripts indicate significant differences.

Table 1.4. Adjusted mean venous blood methaemoglobin concentrations (%); 7 d mean, 3 and 6 h concentrations, for *Bos indicus* steers fed Flinders grass hay with and without urea, or calcium nitrate at two doses[1]

	Treatments					
	Control	Urea	CaN1	CaN2	l.s.d	*P*-value
Mean, 7 d						
Transformed[2]	0.005[a]	-0.209[a]	0.041[a]	1.453[b]	0.324	<0.001
Back-transformed	0.91	0.71	0.94	4.17		
Mean, 3h						
Transformed[2]	-0.073[ab]	-0.230[a]	0.211[b]	1.897[c]	0.383	<0.001
Back-transformed	0.83	0.69	1.13	6.57		
Mean, 6h						
Transformed[2]	0.083[a]	-0.188[a]	-0.129[a]	1.007[b]	0.395	<0.001
Back-transformed	0.99	0.73	0.78	2.64		

[1] Mean values shown are pooled means for control (0.0 g urea, 0.0 g calcium nitrate per day), Urea (32.5 g urea, 0.0 g calcium nitrate per day), CaN1 (16.5 g urea, 48 g calcium nitrate per day) and CaN2 (5.5 g urea, 80 g calcium nitrate per day) treatment groups throughout the experimental period

[2] Transformation; $y = \ln(x+0.1)$
Within rows, different alphabetical superscripts indicate significant differences.

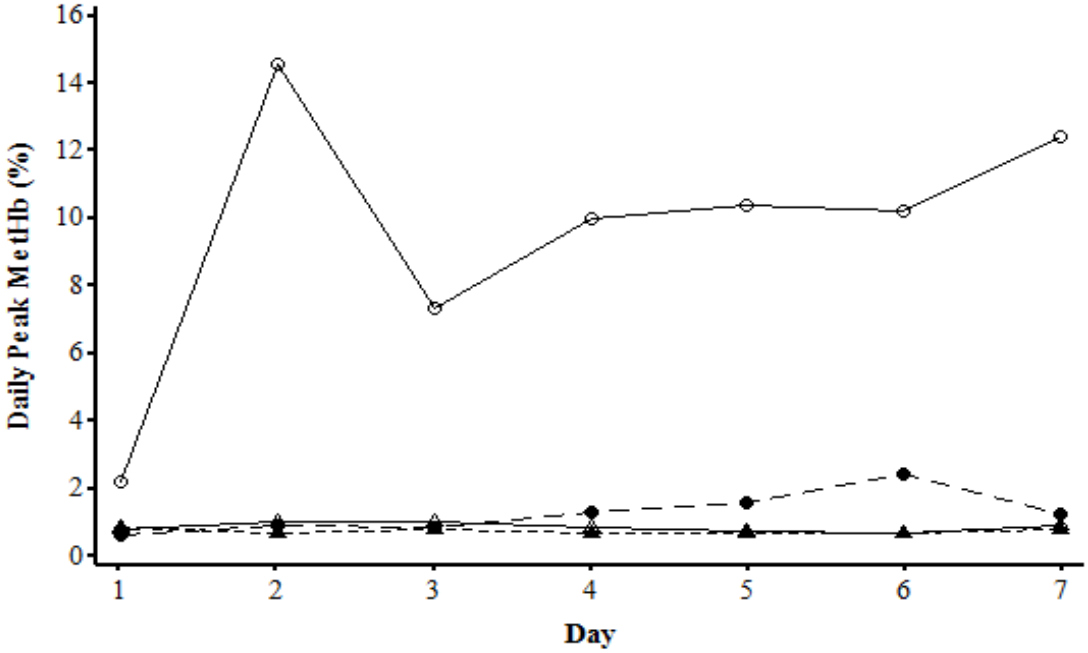

Figure 1.1 Back transformed adjusted means for peak (3 h) blood methaemoglobin (%) of *Bos indicus* steers consuming Flinders grass hay, after receiving either 0 (Control; △) or 15 g N supplement containing Urea (▲), 30 g (CaN1; ●) or 50 g (CaN2; ○) nitrate daily.

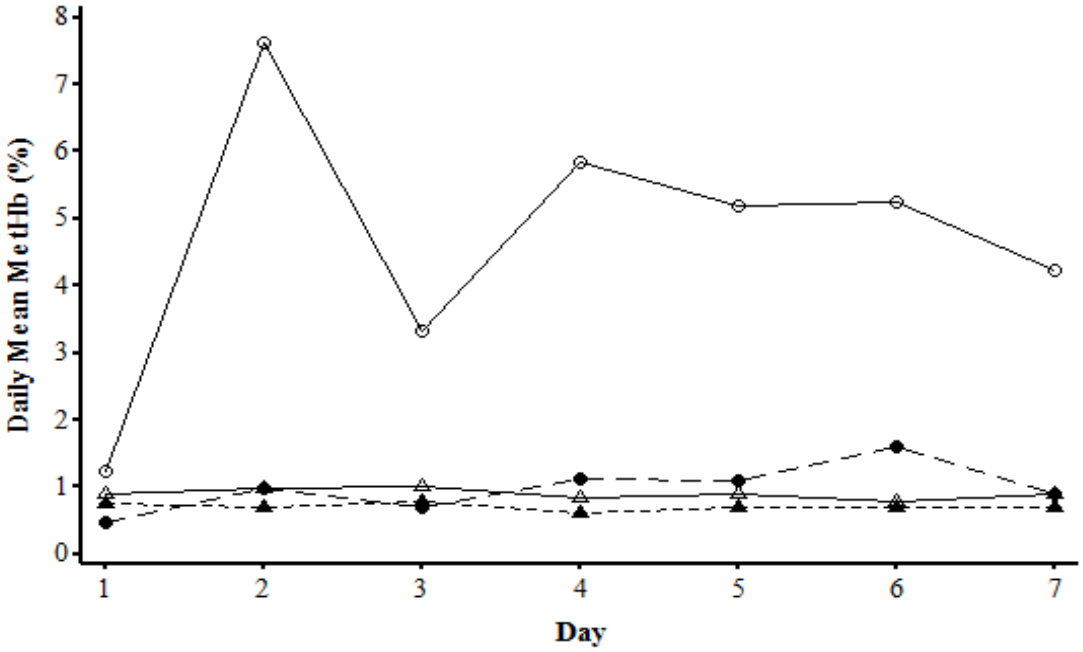

Figure 1.2. Back transformed adjusted mean blood methaemoglobin (%) of *Bos indicus* steers consuming Flinders grass hay, sampled at 3 and 6 h after receiving either 0 (Control; △), 15 g N supplement containing Urea (▲) or 30 g (CaN1; ●) or 50 g (CaN2; ○) nitrate daily .

Table 1.5. Mean ruminal fermentation parameters; ruminal pH, total VFA concentration, VFA molar proportions, and rumen ammonia-N concentrations for steers fed a Flinders grass hay diet *ad libitum* with and without urea, or calcium nitrate at two doses[1]

	Treatments					P-value[2]		
	Control	Urea	CaN1	CaN2	SEM	Treatment	day	time
n	*34*	*38*	*41*	*32*				
Ruminal pH[3]	6.7	6.8	6.9	6.7	0.09	ns	nd	<0.05
Total VFA, mM	73.7	73.8	74.8	73.6	2.41	ns	<0.001	<0.05
VFA proportions, % Total								
Acetate	74.8^{a}	75.9^{ab}	76.7^{b}	76.9^{b}	0.55	<0.05	ns	ns
Propionate	14.3	13.8	13.7	13.7	0.39	ns	ns	<0.001
Butyrate	9.8^{a}	9.8^{a}	8.7^{b}	8.7^{b}	0.23	<0.001	ns	<0.001
Iso-butyrate	0.44^{a}	0.31^{ab}	0.28^{b}	0.22^{b}	0.05	<0.05	ns	<0.001
Valerate	0.31	0.35	0.34	0.32	0.02	0.05	ns	<0.05
Iso-valerate	0.32^{a}	0.19^{b}	0.18^{b}	0.16^{b}	0.03	<0.001	ns	<0.001
A:P[4]	5.33^{a}	5.43^{a}	5.60^{b}	5.64^{b}	0.13	<0.05	ns	<0.001
NH_3-N, mg/L	55.05^{a}	113.5^{b}	107.4^{b}	108.9^{b}	9.81	<0.001	ns	<0.001

[1] Mean values shown are pooled means for control (0.0 g urea, 0.0 g calcium nitrate per day), Urea (32.5 g urea, 0.0 g calcium nitrate per day), CaN1 (16.5 g urea, 48 g calcium nitrate per day) and CaN2 (5.5 g urea, 80 g calcium nitrate per day) treatment groups throughout the experimental period; n indicates number of observations for VFA parameters only; [2] Main effects for treatment (TRT), day (between days for up to 7d), or time (within day at 3h or 6h post dose), ns non-significant, nd not determined; [3] ruminal pH measured at 1, 2, 3, 4, 6, 9, 12 and 24 h post feeding only; [4]Acetate : Propionate. Within rows, different superscripts indicate significant differences for treatment effect only.

Experiment 2: 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

Total Methaemoglobin

The total mean methaemoglobin concentration (%) in the blood of steers increased when the nitrate concentration in the diet increased and was given as a singular bolus ($P = 0.014$; Figure 2.1 & 2.3) or given as two equal portions throughout the day ($P < 0.001$; Figure 2.2 & 2.3). The response was also time dependent with increasing methaemoglobin values over the seven days of the experiment when the steers were dosed once ($P < 0.001$; Figure 2.1 & 2.3) and twice ($P < 0.001$; Figure 2.2 & 2.3) 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 grams of nitrate per day showed a greater increase in mean methaemoglobin values than for the 0 and 30 grams of nitrate per day (Figure 2.3). However, the interaction of dose rate x day was not significant for twice a day feeding of the nitrate treatments.

Methaemoglobin Peaks

The daily peak methaemoglobin values increased when the nitrate concentrations in the diet increased and was given as a single bolus ($P = 0.000$; Figure 2.1 & 2.4) or was divided equally into two portions and given twice daily ($P = 0.002$; Figure 2.2 & 2.4). The daily peak methaemoglobin concentrations demonstrated time dependent effects when nitrate was administered once ($P < 0.001$; Figure 1 & 4) and twice ($P < 0.001$; Figure 2.2 & 2.4) a day. A dose rate x day interaction was highly significant ($P < 0.001$, Ave SED = 12.5%; Figure 2.4) for daily peak methaemoglobin concentrations in the steers given nitrate once a day. Although daily peak methaemoglobin values were non-existent for the zero nitrate treated animals and the dose rate of 30 grams of nitrate produced relatively stable adjusted mean daily peak values of between 7 and 11 % methaemoglobin from day 1 to day 7. The dose rates of 40 and 50 grams of nitrate per day demonstrated adjusted mean daily peak methaemoglobin concentrations that increased over time from 21.2 and 28.5% on day 1 to 53.3 to 59.4% on day 7 respectively.

A dose rate x day interaction for daily methaemoglobin peak values was also demonstrated when nitrate treatments were dosed twice a day ($P = 0.018$, Ave SED 3.34; Figure 4). As expected the adjusted means for the daily peak methaemoglobin concentrations in the zero nitrate treated steers was not remarkable. However, the adjusted means for the daily peak methaemoglobin values from the steers treated with 30 grams 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 methaemoglobin values from the steers treated with 50 grams of nitrate increased progressively each day from 9.2% on day 1 to 19.4% on day 7.

Methaemoglobin rate of incline

The adjusted means for the rate of incline in methaemoglobin per hour for the four hours prior to the daily peak methaemoglobin value increased with dose rate ($P = 0.014$) and day ($P < 0.001$) for the steers dosed once a day. Furthermore a dose rate x day interaction was evident for once a day dosing of treatments ($P = 0.029$, Ave SED 2.5%). Specifically, when steers were administered with 40 or 50 grams of nitrate the adjusted means for the rate of incline for methaemoglobin concentrations demonstrated an increase from 4.3 and 6.1%/h on day 1 to 10.1 and 13.1%/h on day 7 respectively. The dose rate of 30 grams of nitrate per day resulted in adjusted means that ranged from 1.4% on day 1 to 2.2% on day 7 and the adjusted means for the rate of incline for the zero nitrate treatment 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 methaemoglobin concentration in the blood of steers with increasing dose rate ($P = 0.001$). The adjusted means for the hourly rate of incline for methaemoglobin for the 0, 30, 40 and 50 grams of nitrate treatments were 0.16, 1.2, 1.7 and 2.8% per hour (Ave. SED = 0.32%) for the four hours immediately before the peak daily methaemoglobin

concentration. There was no effect of day or dose rate x day interaction when the nitrate treatments were dosed twice a day.

Oxyhaemoglobin

The oxyhaemoglobin concentration in blood decreased when the nitrate concentration of the diet increased and was given as a single dose (Figure 2.5). This response was both dose dependent ($P = 0.040$) and time dependent ($P < 0.001$). In addition a dose rate x day interaction was demonstrated for oxyhaemoglobin ($P = 0.006$, Ave SED = 5.5%). Oxyhaemoglobin remained consistent for the 0 nitrate treated animals, and decreased by 7.3% between day 1 and day 7 for steers dosed with 30 grams of nitrate per day. Furthermore, oxyhaemoglobin decreased by 18% between day 1 and day 7 in the animals dosed with 40 and 50 grams of nitrate per day (Figure 2.5). However, with twice daily dosing there was no effect of dose rate, although there was a time effect ($P < 0.001$).

Total Haemoglobin

Neither dose rate nor day had a significant effect overall on total haemoglobin (tHb) concentration in steers dosed once a day. However, a day x dose rate interaction was highly significant for steers dosed once a day ($P < 0.001$). The steers treated with 50 grams of nitrate had the lowest tHb concentration on day 4 compared to the other treatments. The mean total haemoglobin 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/litre.day for 0 grams, -1.64g tHb/litre.day for 30 grams, -1.44g tHb/litre.day for 40 grams and 1.099g tHb/litre.day for 50 grams.

Dose rate had no significant effect on total haemoglobin 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 86g tHb/L (sed = 2g 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.

Deoxyhaemoglobin

Overall deoxyhaemoglobin increased over the seven 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% (sed = 2%) for the steers dosed once a day. However, there was no significant effect of dose rate or interaction between dose rate and day for either singular or twice a day dosing.

Carboxyhaemoglobin

Carboxyhaemoglobin was log transformed to better satisfy the assumption of constant variance. A day effect 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 day effect 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. A dose rate x day interaction was not significant for either the once or twice a day treatments.

Haematocrit

Dose rate had no significant effect on haematocrit values when treatments were dosed once or twice per day. A day effect was observed for haematocrit values for once (P = 0.004) and twice (P < 0.001) a day dosing. The steers dosed once a day demonstrated a decrease in haematocrit values until day 4. A 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 (Ave. SED = 0.47).

Dry Matter Intake

The dry matter intake of Flinders grass hay was not affected by dose rate or day for both frequencies of nitrate intake. The mean dry matter intake of the hay for the 0, 30, 40 and 50 grams of nitrate treatments was 4.2 kg, 4.4 kg, 4.1kg and 4.6 kg respectively (Ave SED 0.52 kg).

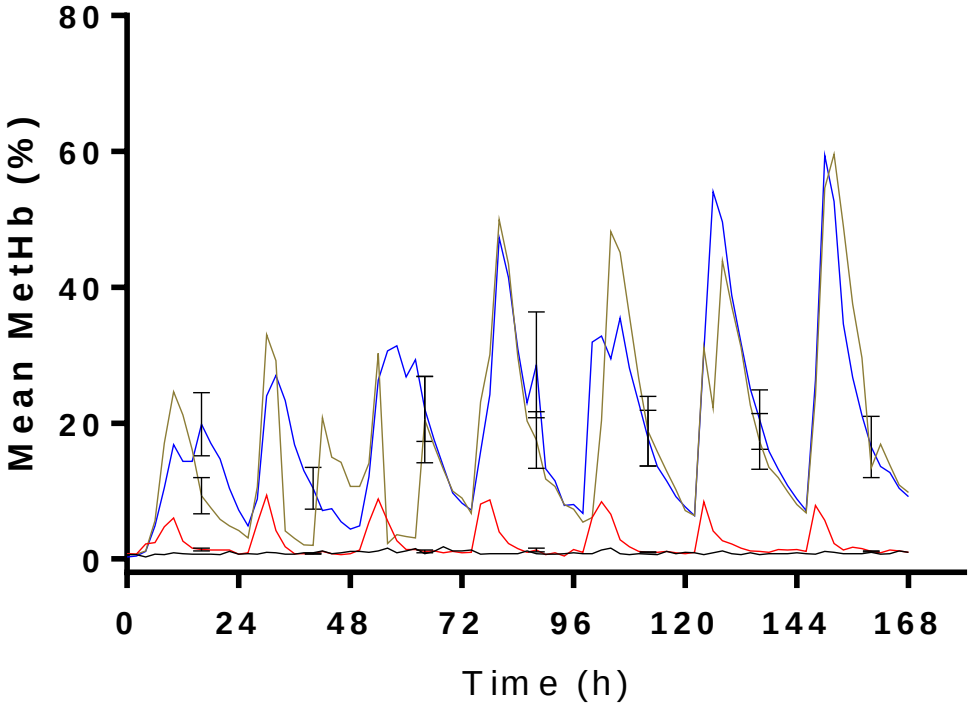

Figure 2.1. Actual mean ± SEM methaemoglobin concentration (%) from venous blood of *Bos indicus* steers treated with 0 (—), 30 (—), 40 (—)or 50 (—) grams of nitrate as a non-protein nitrogen supplement once a day in a single dose at 0700 hours for seven days.

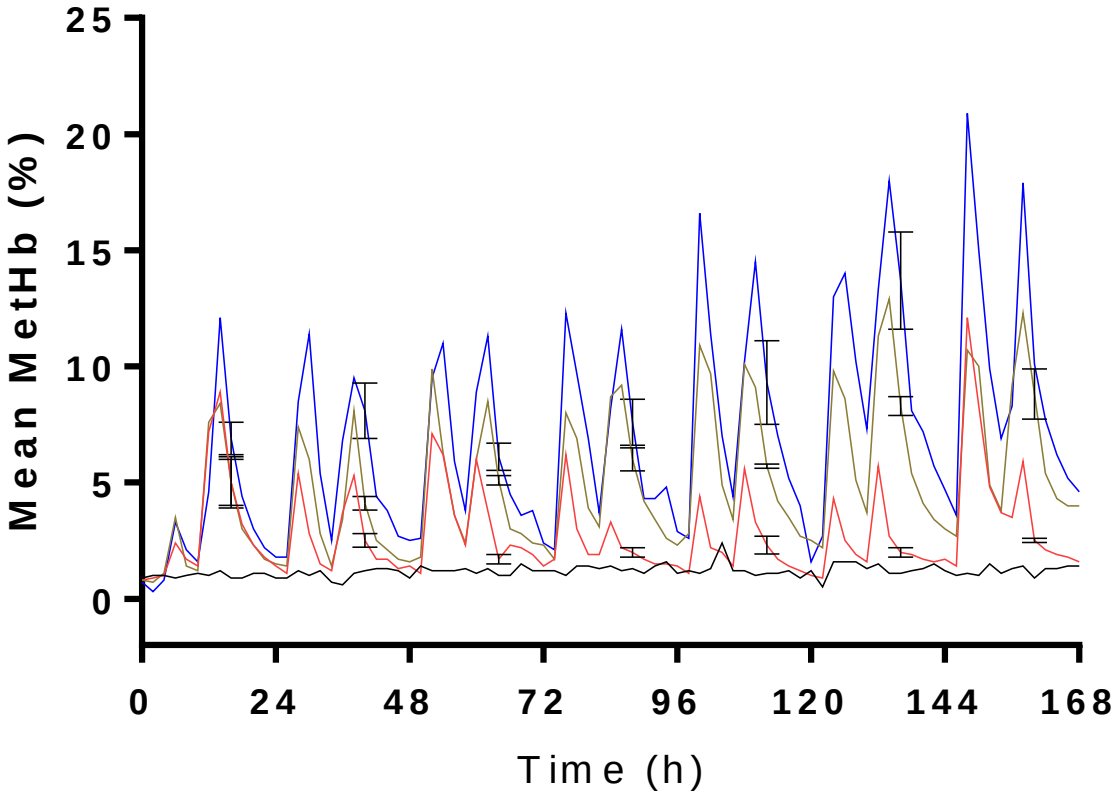

Figure 2.2. Actual mean ± SEM methaemoglobin concentration (%) from venous blood of *Bos indicus* steers treated with 0 (—), 30 (—), 40 (—)or 50 (—) grams of nitrate as a non-protein nitrogen supplement divided into two equal portions and given at 0700 and 1700 hours for seven days.

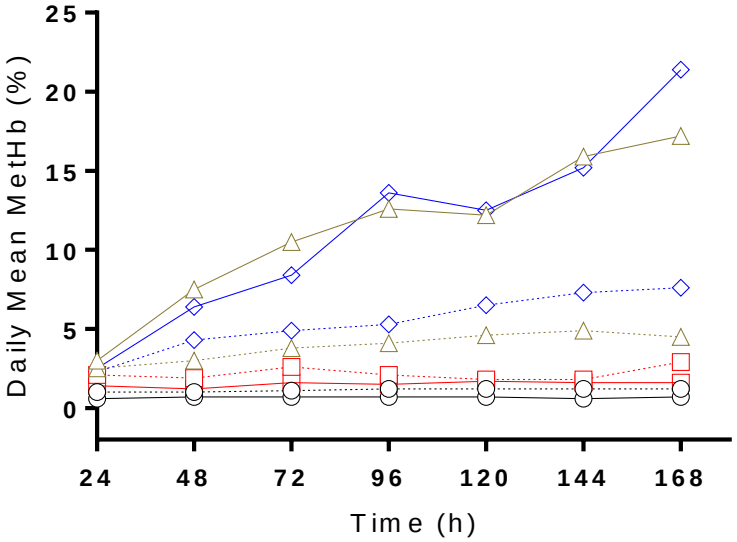

Figure 2.3. Back transformed adjusted means for methaemoglobin (%) in venous blood of *Bos indicus* steers dosed with 0 (—○—), 30 (—□—), 40 (—Δ—)or 50 (—◊—) grams of nitrate once a day at 0700 hours or dosed with 0 (--○--), 30 (--□--), 40 (--Δ--)or 50 (--◊--) grams of nitrate divided into two equal portions and administered at 0700 hours and 1700 hours for seven days

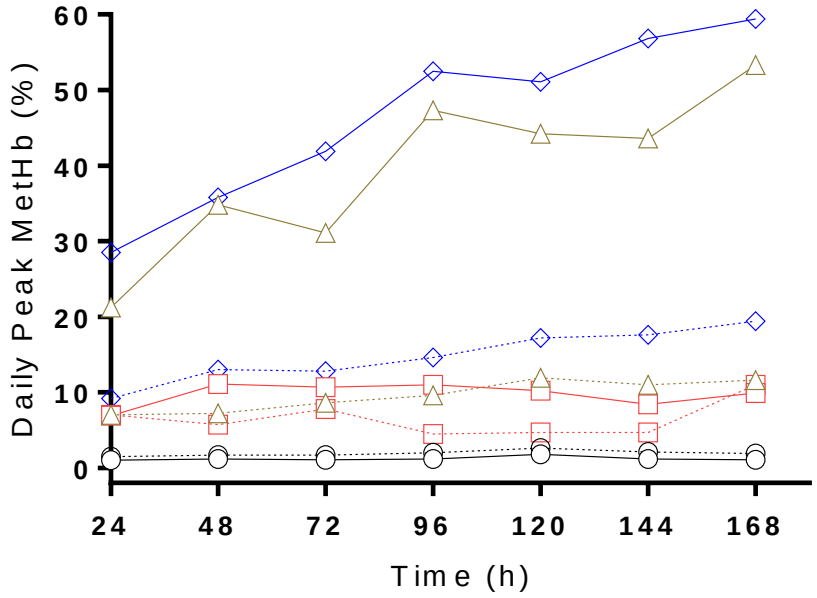

Figure 2.4. Adjusted means for daily peak methaemoglobin (%) in venous blood of *Bos indicus* steers dosed with 0 (—○—), 30 (—□—), 40 (—Δ—)or 50 (—◊—) grams of nitrate once a day at 0700 hours (Ave SED = 12.5) or dosed 0 (--○--), 30 (--□--), 40 (--Δ--)or 50 (--◊--) grams of nitrate divided into two equal portions and administered at 0700 hours and 1700 hours for seven days (Ave SED = 3.34)

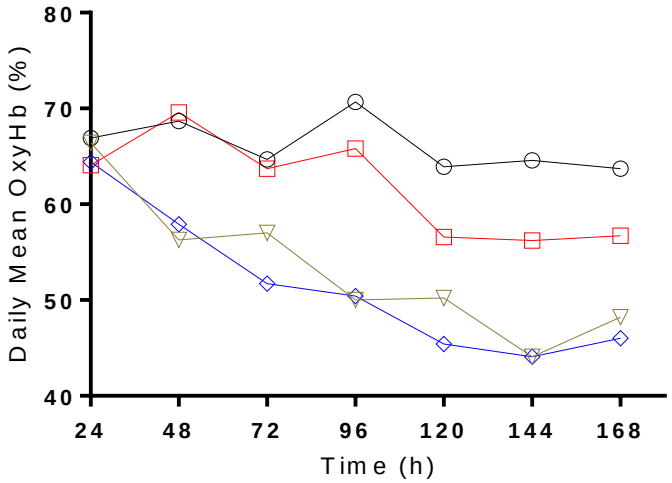

Figure 2.5. Adjusted means for daily oxyhaemoglobin (%) in venous blood of *Bos indicus* steers treated with 0 (—○—), 30 (—□—) 40 (—△—)or 50 (—◊—) grams of nitrate as a non-protein nitrogen supplement once a day in a single dose at 0700 hours for seven days (Ave SED = 5.5).

Experiment 3: The effect of nitrate supplementation on arterial blood gases, heart rate, respiratory rate and rectal Temperature of *Bos indicus* cattle after exercise

Arterial blood gas parameters are presented in Table 3.2. Arterial blood pH did not differ between treatments (*P* = 0.124). 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 demonstrated that 50 grams of nitrate per day caused a lower pO_2 value in arterial blood than the animals receiving no nitrate as a treatment (*P* = 0.004). In addition a trend was evident for 50 grams of nitrate to produce lesser values for pO_2 than animals dosed with 30 grams of nitrate per day (*P* = 0.098)

Total haemoglobin was not different between treatments (*P* = 0.059; Table 3.3). Fractional proportions of deoxyhaemoglobin (FHHb) were also not different between treatments (*P* = 0.289). Oxyhaemoglobin demonstrated a difference between treatments (*P* = 0.001). Post hoc tests revealed that oxyhaemoglobin was reduced in the animals treated with 50 grams of nitrate compared with the animals given no nitrate (*P* = 0.001) or 30 grams of nitrate (*P* = 0.001). There were differences between treatments for methaemoglobin concentrations (*P* = 0.000). Dosing steers with 50 grams of nitrate caused a greater concentration of methaemoglobin in the blood than steers dosed with 30 grams of nitrate (*P* = 0.019) or no nitrate (*P* = 0.002). Carboxyhaemoglobin also demonstrated differences between treatments (*P* = 0.001). Dosing steers with 50 grams of nitrate caused a greater concentration of carboxyhaemoglobin in the blood than steers dosed with 30 grams of nitrate (*P* = 0.003) or no nitrate (*P* = 0.002). Haematocrit values did not differ between treatments (*P* = 0.583).

There was no difference between treatments for heart rate before the exercise regimen (P = 0.282; Table 3.4). However, the steer's heart rate immediately after walking three kilometres was different between treatments (P < 0.016). Steers dosed with 50 grams of nitrate had greater heart rates immediately after the exercise regimen than the steers dosed with 30 grams of nitrate (P = 0.043) or urea (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.

Table 3.2. Least square means ± SEM 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 grams of nitrate per day for seven days and walked three kilometres.

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

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

Table 3.3. Least square means ± SEM for total haemoglobin (tHb), deoxyhaemoglobin (FHHb), Oxyhaemoglobin (FO_2Hb), methaemoglobin (FMetHb), carboxyhaemoglobin (FCO_2Hb) and haematocrit for *Bos indicus* steers treated with 0, 30 or 50 grams of nitrate per day for seven days and walked three kilometres.

Co-oximetry variables	Treatment (g NO_3/day)			ANOVA P values
	0	30	50	
tHb (g/L)	124.50 ± 1.25 [a]	117.75 ± 6.26 [a]	125.75 ± 6.06 [a]	0.059
FHHb (%)	1.80 ± 0.27 [a]	1.20 ± 0.71 [a]	3.32 ± 0.85 [a]	0.289
FO_2Hb (%)	97.07 ± 0.44 [a]	88.22 ± 4.23 [a]	61.82 ± 4.38 [b]	0.001
FCO_2Hb (%)	0.85 ± 0.21 [a]	0.95 ± 0.86 [a]	1.90 ± 0.10 [b]	0.001
Hct (%)	36.50 ± 0.50 [a]	34.75 ± 1.88 [a]	37.00 ± 1.87 [a]	0.583
FMetHb (%)†	0.28[a]	5.01[b]	31.62[c]	0.000

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

† Back transformed FMetHb values

Table 3.4. Least square means ± SEM for heart rate before (pre) and immediately after (post) walking, and respiration rate, and rectal temperature after walking three kilometres from *Bos indicus* steers treated with 0, 30 or 50 grams of nitrate per day for seven days

Variables	Treatment			ANOVA P values
	0	30	50	
Heart rate pre (beats/min)	76.00 ± 10.02 [a]	66.50 ± 3.77 [a]	84.75 ± 7.49 [a]	0.282
Heart rate post (beats/min)	104.75 ± 14.37[a]	116.00 ± 19.94[a]	175.25 ± 5.17[b]	0.016
Respiration rate (breaths/min)	30.50 ± 6.70[a]	37.00 ± 7.68[a]	40.50 ± 9.11[a]	0.673
Rectal temperature (°C)	39.20 ± 0.14[a]	39.70 ± 0.21[a]	39.53 ± 0.19[a]	0.207

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

Experiment 4: Effects of urea or nitrate supplements on haemoglobin fractions from *Bos indicus* steers fed Flinders grass (*Iseilemia spp*.) hay for 70 days

Methaemoglobin

A significant effect of treatment ($P < 0.001$) and time of sampling ($P < 0.001$) was demonstrated for methaemoglobin concentrations in the steers (Fig. 4.1). However, there was no effect for day of blood sampling on MetHb concentration. Nitrate and control treated steers had the same MetHb concentrations at time 0 on each day of sampling. However, at all other times sampled post dosing the nitrate treated animals recorded greater concentrations of MetHb than the control steers.

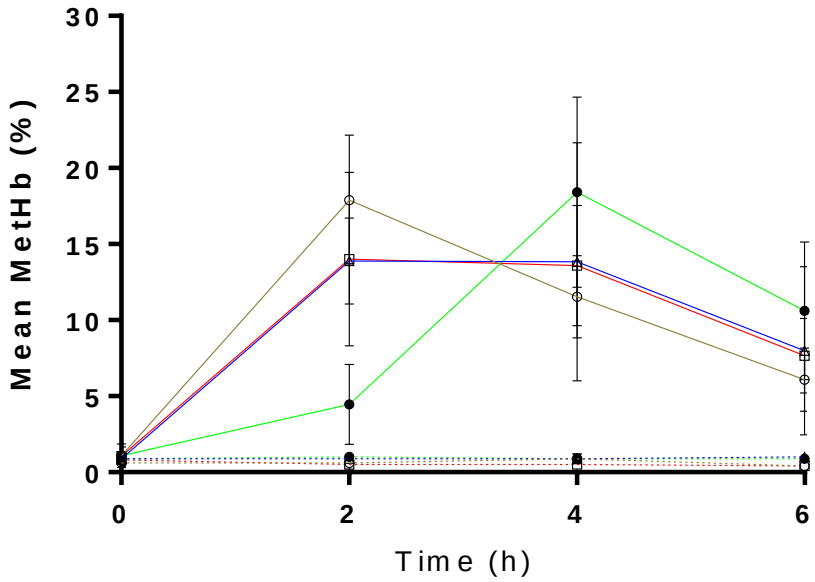

Figure 4.1. Mean ± SEM Methaemoglobin (%) in venous blood from *Bos indicus* steers at two hour intervals for six hours after dosing with 50 g nitrate (—) or no nitrate (--) on days 10 (—○—)(--○--), 30 (— Δ—) (--Δ--), 50 (—●—) (--●--) and 70 (—□—) (--□--).

Total haemoglobin

There were highly significant effects of day ($P < 0.001$) and time ($P < 0.001$) of blood sampling on tHb (Figure 4.2). However there was no treatment difference on tHb concentration of steers in this experiment (Figure 4.3).

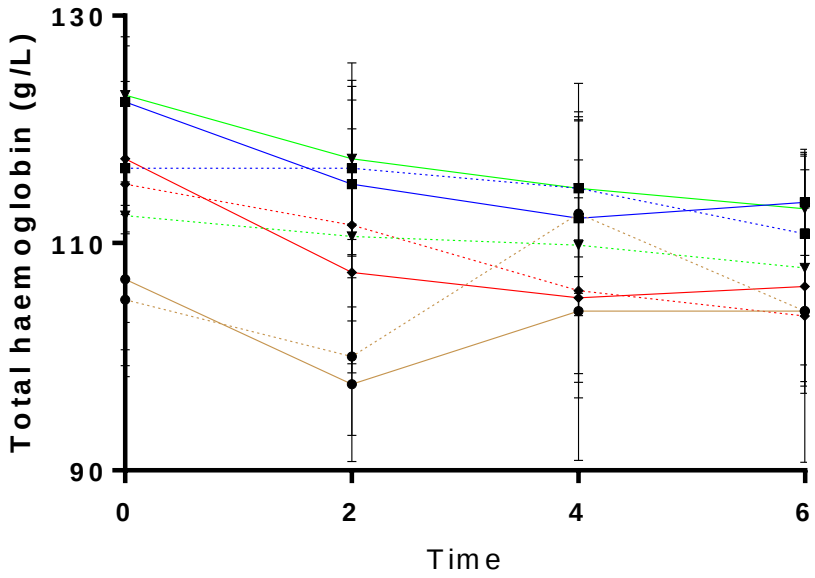

Figure 4.2. Mean total haemoglobin (g/L) in venous blood of *Bos indicus* steers dosed with 50 g NO_3 (—) and no nitrate (--) on d 10 (—○—)(--○--), d 30 (— Δ—) (--Δ--), d 50 (—●—) (--●--) and d 70 (—□—) (--□--).

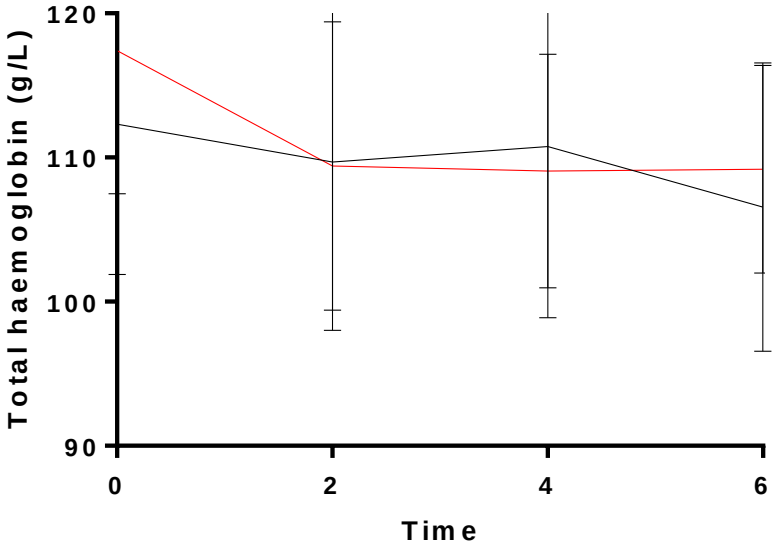

Figure 4.3. Mean total haemoglobin (g/L) in venous blood of *Bos indicus* steers dosed with 50 g NO_3 (—) and no nitrate (—)

Oxyhaemoglobin

There were no treatment or time effects for oxyhaemoglobin concentration (Fig. 4.4, 4.5).

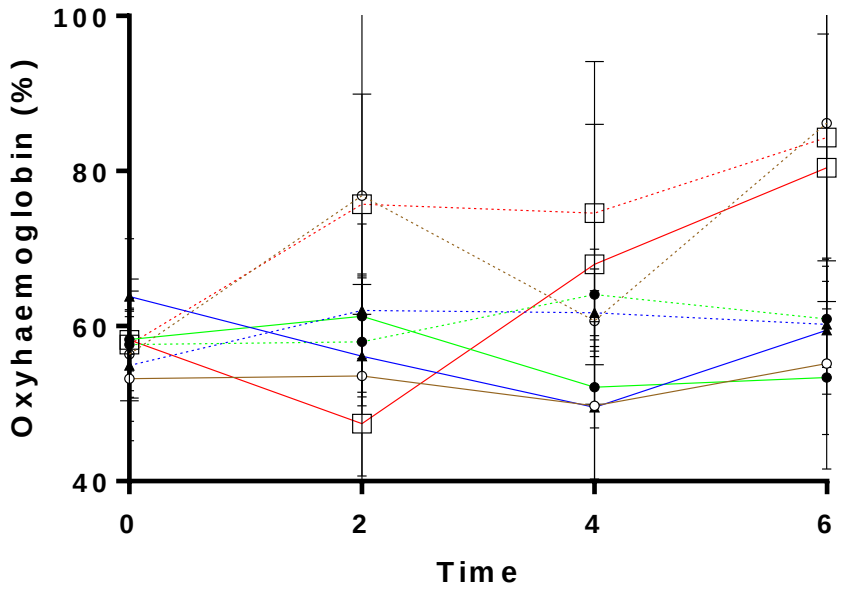

Figure 4.4. Mean ± SEM Oxyhaemoglobin (%) in venous blood of *Bos indicus* steers dosed with 50 g NO_3 (—) and no nitrate (--) on d 10 (—○—)(--○--), d 30 (— Δ—) (--Δ--), d 50 (—●—) (--●--) and d 70 (—□—) (--□--).

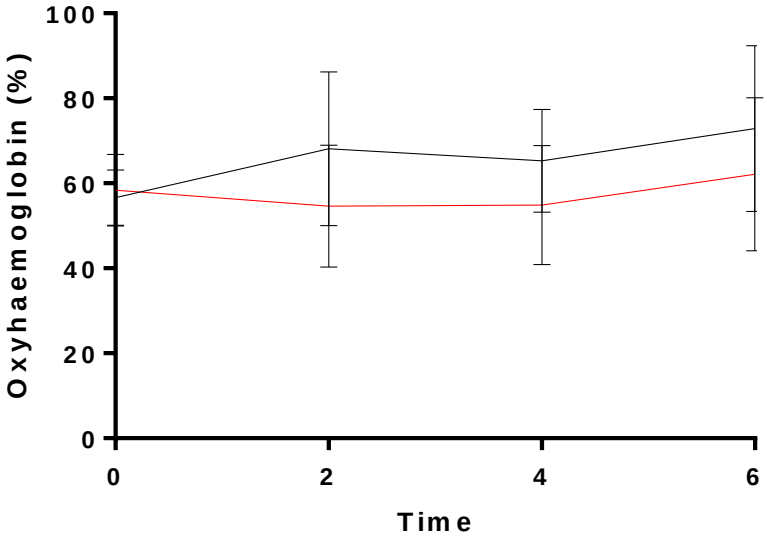

Figure 4.5. Mean ± SEM oxyhaemoglobin (%) in venous blood of *Bos indicus* steers dosed with 50 g NO_3 (—) and no nitrate (—)

Carboxyhaemoglobin

A highly significant effect of treatment on carboxyhaemoglobin was demonstrated. Steers fed nitrate had greater concentrations of carboxyhaemoglobin in their blood (P = 0.001; Figure 4.6) compared to steers treated with no nitrate. Time of blood sampling demonstrated significant effects on carboxyhaemoglobin concentrations (P = 0.001). However, there was no day effect demonstrated for carboxyhaemoglobin concentration.

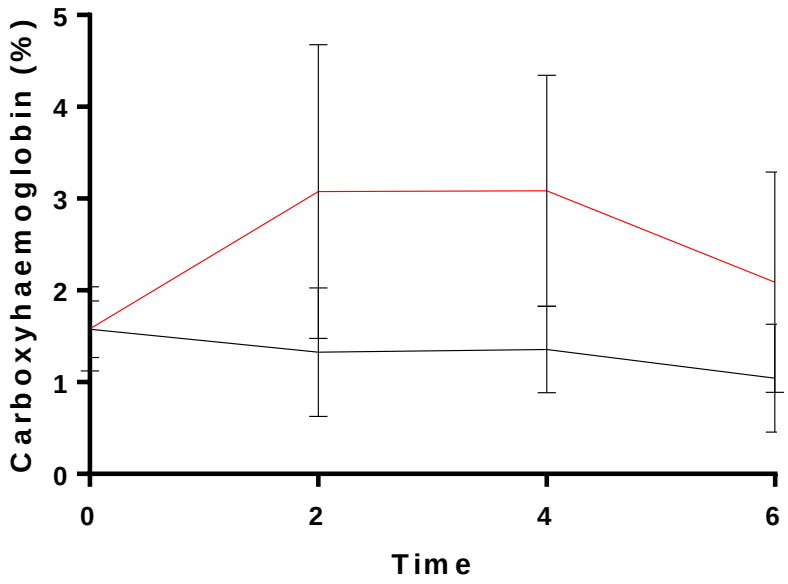

Figure 4.6. Means total carboxyhaeglobin (%) in venous blood of *Bos indicus* steers dosed with 50 g NO_3 (─) and no nitrate (─)

Dry matter intake

There were no treatment effects on dry matter intake of Flinders grass hay by steers in this experiment. However, a highly significant effect of day on dry matter intake (P < 0.001; Figure 4.7) was recorded.

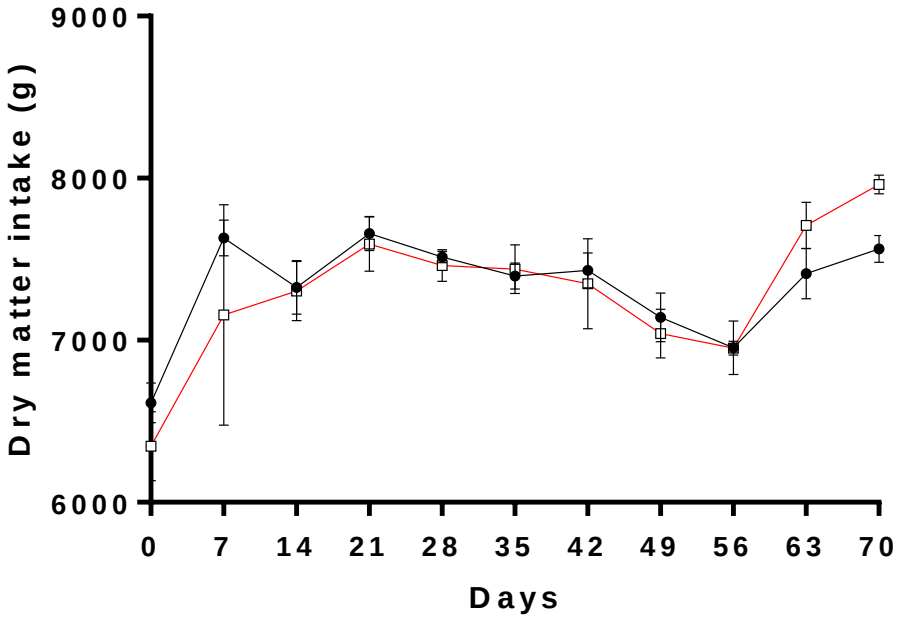

Figure 4.7. Means dry matter intake of Flinders grass hay by steers given no nitrate (—●—) and 50 g NO_3 (—□—) for 70 days

Body weight

There was no treatment effect on body weight of *Bos indicus* steers (Figure 4.8).

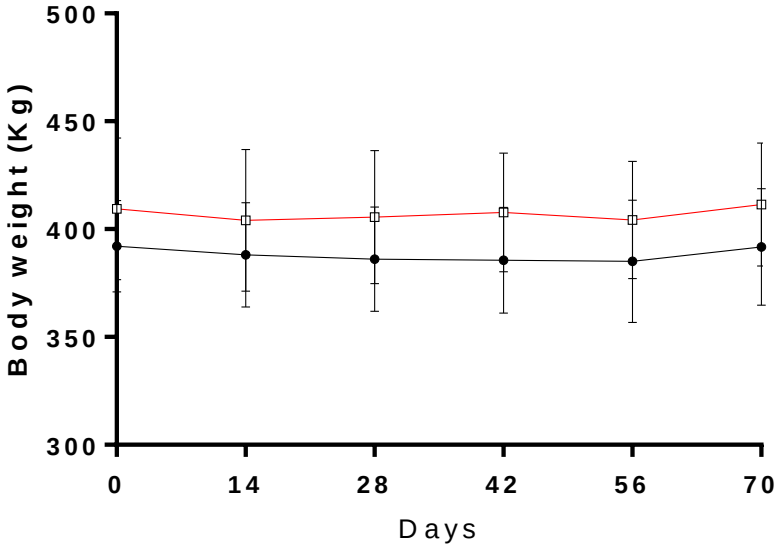

Figure 4.8. Mean ± SEM body weight of steers given no nitrate (—●—) and 50 g NO_3 (—□—) for 70 days

Experiment 5: Field experiments for *Bos indicus* cows grazing tropical pastures consuming nitrate lick blocks

Pastures

The herbage mass at the commencement of the experiments were 1200 and 2093 kg DM/ha for Y1 and Y2 respectively. In Y2, pasture biomass declined linearly across as the dry season progressed and had reached 807 kg DM/ha during November 2014.

Rainfall

The rainfall during 2013 was 51% of the long term average of the site (Table 5.2). The year was characterised by a failed 2012/13 wet season, with only January rainfall comparable with long term average totals. The low pasture availability at the commencement of Y1 reflected the lack of rainfall during the pasture growing season. A series of rainfall events during late November 2013, coinciding with week 2 of the treatment period, resulted in a visible green shoot on the pasture. This visual change to the pasture was short-lived under sustained high daily maximum temperatures typical of the late dry season. The pastures quickly senesced and dry weather prevailed until the conclusion of Y1. During Y2, rainfall totals were similar to the long term average of the site. The combination of near average 2013/14 wet season rainfall and pasture spelling whilst cattle were relocated to Swans Lagoon, led to an improvement in the herbage mass at the commencement of Y2. However the greater number and heavier liveweight of cattle carried through Y2 resulted in low pasture availability at the completion of the trial.

Table 5.2. Monthly rainfall (mm) preceding (January to October 2013), during Y1 (November – December 2013), during Y2 (May – November 2014) and the long term average at the site**.**

Month	2013	2014	40-year average
January	163	186	141
February	21	157	120
March	10	43	71
April	28	20	38
May	18	0	28
June	0	37	17
July	13	0	14
August	0	6	12
September	0	0	9
October	0	0	27
November	63	-	53
December	0	-	87
Total	316		615

Supplement intake

Voluntary intake of MNB during Y1 averaged 78 g/cow.d as fed. Thus the N intake from MNB averaged 7 g N/cow.d. Supplement intake across the 4-week trial period was similar at 91, 67, 68 and 85 g/cow.d at week 1, 2, 3 and 4 respectively.

Block intakes of both 30U and MNB blocks during Y2 increased across the experimental period (Fig. 5.1). Average intake of 30U blocks during the dry season was 93 g/animal.d, which equated to 15 g N/animal.d. The corresponding values for MNB supplement intakes were 72 g/animal.d and 6.5 g N/animal.d. When expressed in terms of mean nitrate intake, MNB blocks delivered 16 g nitrate/animal.d. Intakes increased sharply during the late dry season, between September and November, peaking in the final week of the experimental period at 219 and 193 g/animal.d for 30U and MNB treatments respectively.

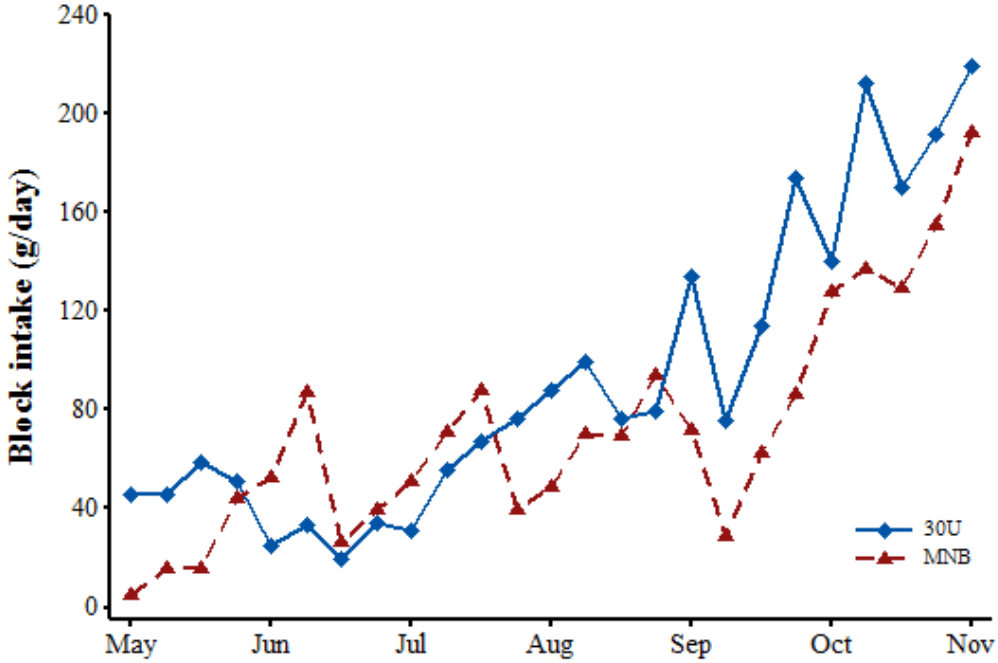

Fig 5.1. Average weekly voluntary intake of molten urea (30U) or molasses nitrate (MNB) blocks by *Bos indicus* heifers and cows grazing tropical pastures during the 2014 dry season at Fletcherview, Charters Towers.

Liveweight (LWT), Conceptus-free liveweight (CFLW), CFLW change

There were no differences in LW between MNB and CON animals prior to the commencement of the experiment in Y1 (332.0 ± 6.4 kg *v*. 329.5 ± 5.8 kg). There were effects of time (P<0.001), but not treatment (Table 5.3) on LW. Animal LW increased over the 4-week trial period to peak at 351.3 ± 4.5 kg. At the conclusion of Y1, CON cows were heavier ($P < 0.05$; 355.5 ± 5.9 kg) than MNB cows (347.0 ± 7.0 kg).

Mean LWT, CFLW and CFLW change throughout Y2 dry season are summarised in Table 5.4. There were no statistical differences between treatment groups for either LW or CFLW prior to commencement of the experiment (Fig 5.2 and 5.3). There were also no statistical differences for LW, CFLW and CFLW change between heifers and cows.

The LW of pregnant cows was greater than dry cows throughout the treatment period, with the mean difference being 9 kg ($P < 0.05$). The pattern of changes over time was similar with both LWT and CFLW peaking in August before declining linearly until the conclusion of the experiment in November. At the September yarding, 30U cattle had greater LWT (P<0.05) than those supplemented with nitrate (502.3 *vs.* 495.4*)*. There was an overall effect of treatment on CFLW change ($P < 0.05$). Cattle supplemented with 30U had a CFLW change advantage of approximately 50 grams/day compared to MNB cattle.

Table 5.3. Mean liveweight (LWT), body condition score (BCS) and blood methaemoglobin concentrations of non-pregnant *Bos indicus* cows grazing tropical pastures during the late 2013 dry season allocated to either nil supplement (CON) or molasses nitrate (MNB) blocks at Fletcherview, Charters Towers.

	Treatment			P-value[1]		
	CON	MNB	SEM	Treatment	Time	Treatment × Time
LWT (kg)	350.7	347.0	2.6	ns	< 0.001	< 0.05
BCS	2.57	2.55	0.02	ns	<0.001	ns
Methaemoglobin[2] (%)	0.30[a]	0.46[b]	0.09	< 0.001	< 0.001	< 0.001

[1] Main effects for treatment or time (monthly intervals), ns non-significant; [2] Methaemoglobin concentrations presented are back-transformed values. Within rows, different superscripts indicate significant differences for treatment effect only.

Table 5.4. Mean liveweight (LWT), conceptus-free liveweight (CFLW), CFLW change, body condition score (BCS) and blood methaemoglobin concentrations of *Bos indicus* heifers and cows grazing tropical pastures during the 2014 dry season with unrestricted access to either molten urea (30U) or molasses nitrate (MNB) blocks at Fletcherview, Charters Towers.

	Treatment			P-value[1]		
	30U	MNB	SEM	Treatment	Time	Treatment × Time
LWT (kg)	498.2	494.7	2.4	ns	< 0.001	< 0.05
CFLW (kg)	486.8	483.1	2.5	ns	< 0.001	ns
CFLW change (kg/d)	0.019[a]	-0.035[b]	0.03	< 0.05	< 0.001	ns
BCS	4.27[a]	4.17[b]	0.02	< 0.05	<0.001	<0.01
Methaemoglobin[2] (%)	0.36[a]	0.61[b]	0.04	< 0.001	< 0.001	< 0.001

[1] Main effects for treatment or time (monthly intervals), ns non-significant; [2] Methaemoglobin concentrations presented are back-transformed values. Within rows, different superscripts indicate significant differences for treatment effect only.

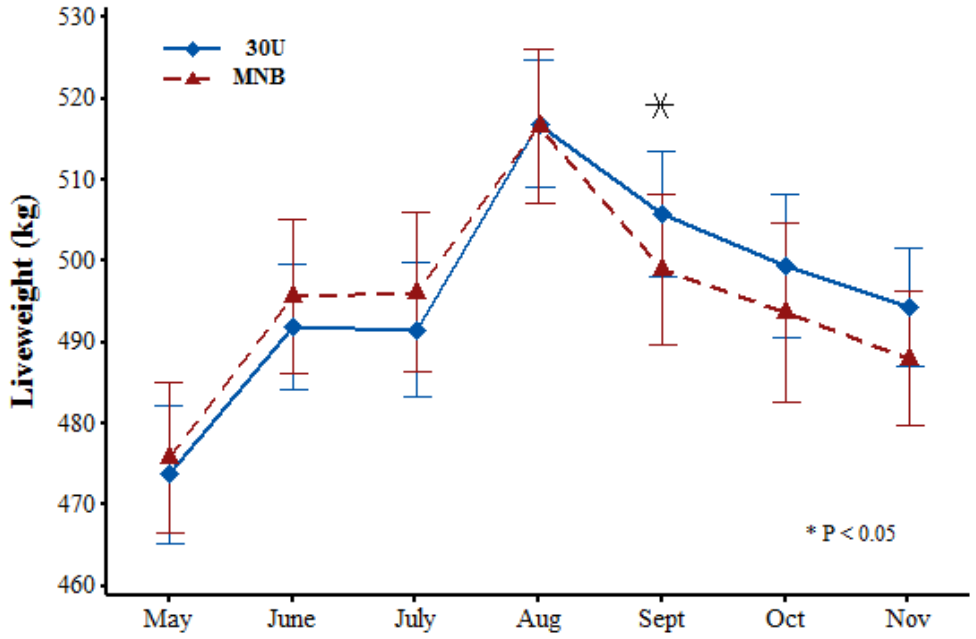

Fig 5.2. Mean liveweight (LWT) of *Bos indicus* heifers and cows grazing tropical pastures during the 2014 dry season with unrestricted access to either molten urea (30U) or molasses nitrate (MNB) blocks at Fletcherview, Charters Towers.

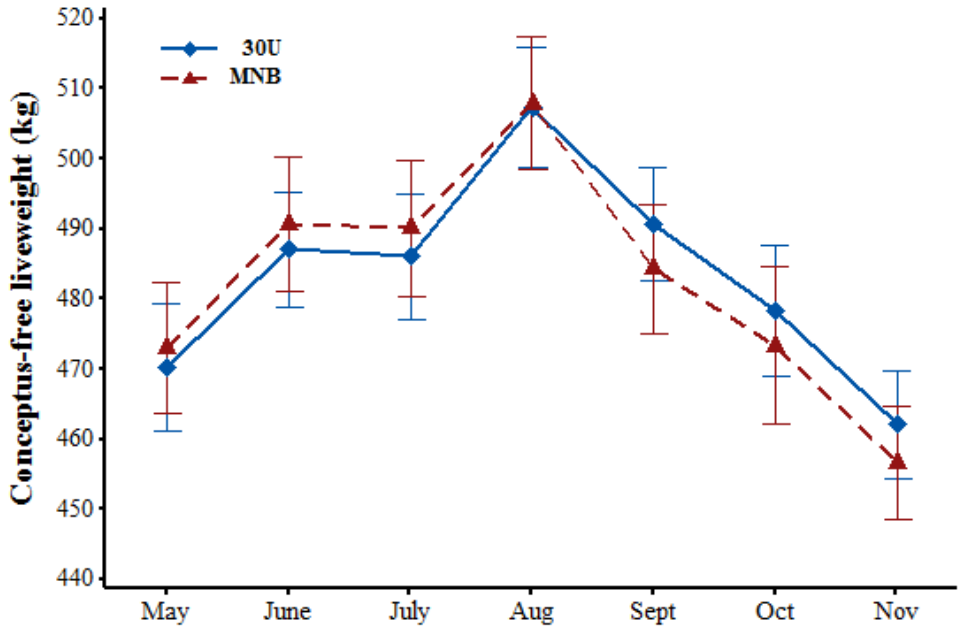

Fig 5.3. Mean conceptus-free liveweight (CFLW) of *Bos indicus* heifers and cows grazing tropical pastures during the 2014 dry season with unrestricted access to either molten urea (30U) or molasses nitrate (MNB) blocks at Fletcherview, Charters Towers.

Body Condition Score

During Y1, BCS was not different between CON or MNB treatments (Table 5.3). Although there were effects of time (P<0.001) on BCS the changes were biologically insignificant.

There were no statistical differences in BCS between treatments prior to the commencement of the trial in Y2, nor were there effects of either age or pregnancy status during the experimental period. Cattle supplemented with 30U were associated with greater overall BCS (P < 0.05; Table 5.4). The BCS of cattle held between June and September, with no differences between treatment groups during this period. However the BCS of cattle decreased in both October and November (*P* < 0.001). At these measurements, 30U cattle retained greater BCS compared to the MNB treatment group (Fig 5.4; *P* < 0.01).

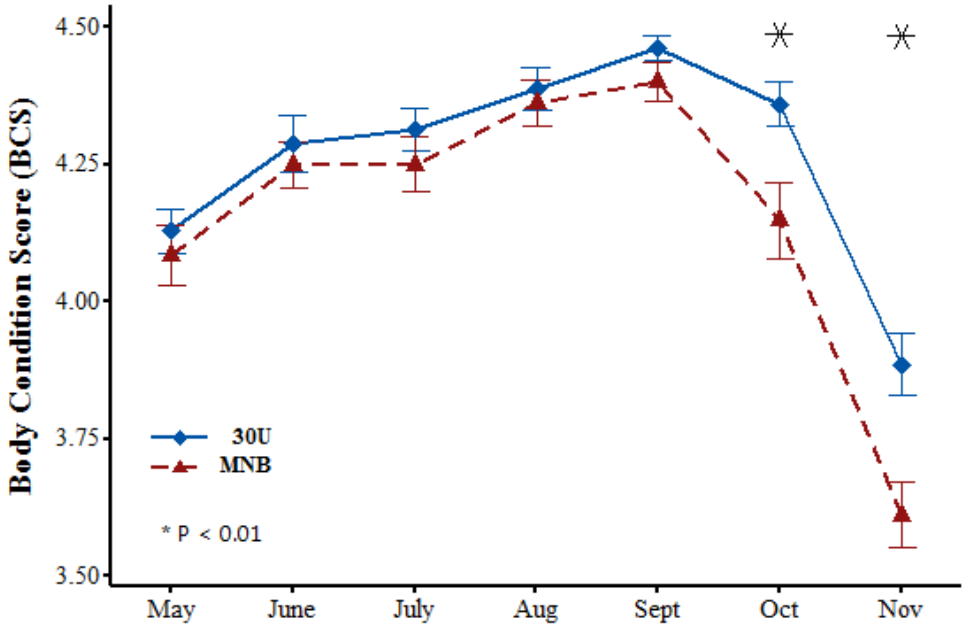

Fig 5.4. Mean body condition score (BCS) of *Bos indicus* heifers and cows grazing tropical pastures during the 2014 dry season with unrestricted access to either molten urea (30U) or molasses nitrate (MNB) blocks at Fletcherview, Charters Towers.

Blood Methaemoglobin

During Y1 there were treatment, time and treatment-by-time interaction effects (*P* < 0.001; Table 5.3) on blood MetHb concentrations. The blood MetHb concentrations of CON animal did not change over the experimental period. Mean blood MetHb concentrations of MNB cows were greater than control cows. The concentration of blood methaemoglobin in MNB animals was greater than CON animals in week 3 and 4, at 0.43% and 0.97%, respectively. The maximum value in week 3 was 13.9% and 6.0% in week 4.

Mean blood methaemoglobin concentrations of MNB cattle were greater (P < 0.001) than 30U cattle over the experimental period (Table 5.4). The mean blood methaemoglobin concentration of 30U cattle remained consistent at between June and

October at 0.41 ± 0.01% between June and October (Fig. 5.5). However blood methaemoglobin concentrations in 30U cattle declined to 0.19% ± 0.03% during November (P < 0.001). Although blood methaemoglobin concentrations of treatment groups did not differ between June and September, MNB cattle had greater concentrations that the 30U treatment during both October and November (P < 0.001). This coincided with an increasing range of upper methaemoglobin concentrations in MNB cattle, with maximum values of 7.5% and 5.7% recorded in October and November, respectively.

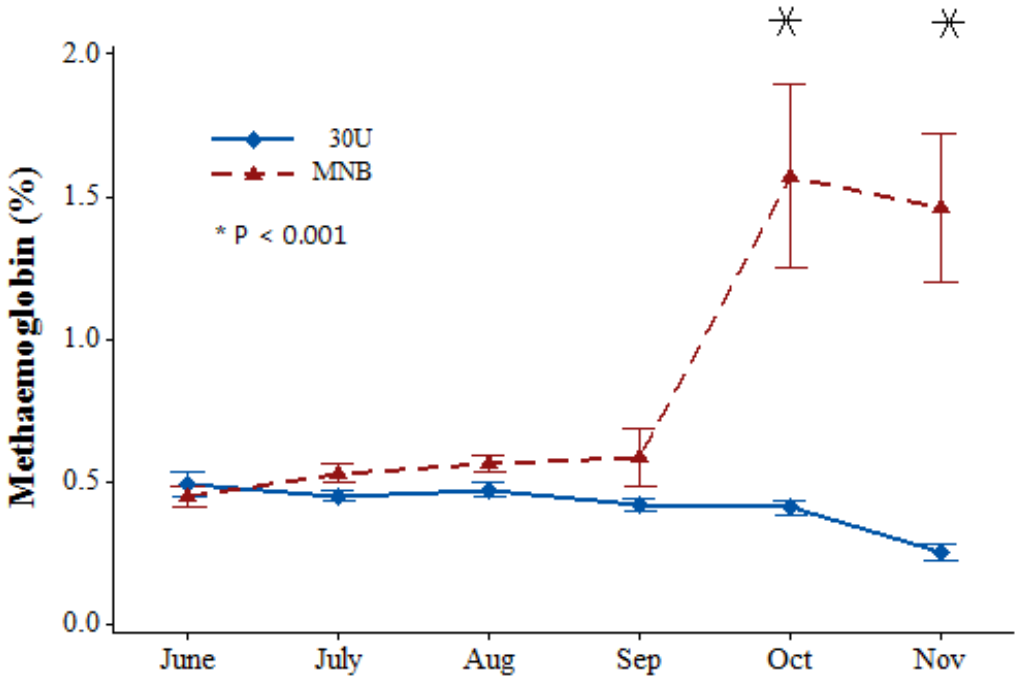

Fig 5.5. Mean blood methaemoglobin concentrations (%) of *Bos indicus* heifers and cows grazing tropical pastures during the 2014 dry season with unrestricted access to either molten urea (30U) or molasses nitrate (MNB) blocks at Fletcherview, Charters Towers.

Diet quality

FNIRS estimates of diet quality at the commencement of Y1 were (g/kg DM); 63 CP, 554 DMD and 154 dietary non-grass. Dietary CP increased (P < 0.001) at week 2, peaking at 83 g/kg. However it declined thereafter (P < 0.001) to 75 and 62 g/kg DM at weeks 3 and 4 respectively. There were no differences in DMD over the first 3 weeks of the experiment. At week 4 DMD declined (P <0.001) to 536 g/kg DM. Both dietary CP and DMD in control cows (89 ± 2; 557 ± 3 g/kg) was higher (P < 0.001) than that of MNB cows (81 ± 2; 548 ± 3 g/kg DM) after 2 weeks. However the opposite effect was observed after 4 weeks; where the dietary CP and DMD of control cows (55 ± 3; 525 ± 5 g/kg DM) was lower (P < 0.001) than MNB cows (68 ± 3; 548 ± 3 g/kg DM). Dietary non-grass composition of the diet remained unchanged at approximately 1555 ± 1 g/kg DM during Y1.

FNIRS estimates of diet quality at the commencement of Y2 were (g/kg DM); 67 CP, 574 DMD and 294 dietary non-grass. Dietary CP declined ($P < 0.001$) between August and September, increasing in October before falling again in November (Fig 5.6). During July, 30U animals had greater dietary CP concentrations (42 *vs.* 40 g/kg DM) than MNB. However during both October and November, 30U animals had lower concentrations of dietary CP. The DMD of the diet also declined over the experimental period ($P<0.001$). There was an initial decline in June (525 ± 3 g/kg DM), then declining again in September (503 ± 3 g/kg DM). There were no DMD differences between treatment groups during Y2. Concentrations of dietary non-grass declined across the experimental period, reaching a nadir in October of 239 ± 7 g/kg DM.

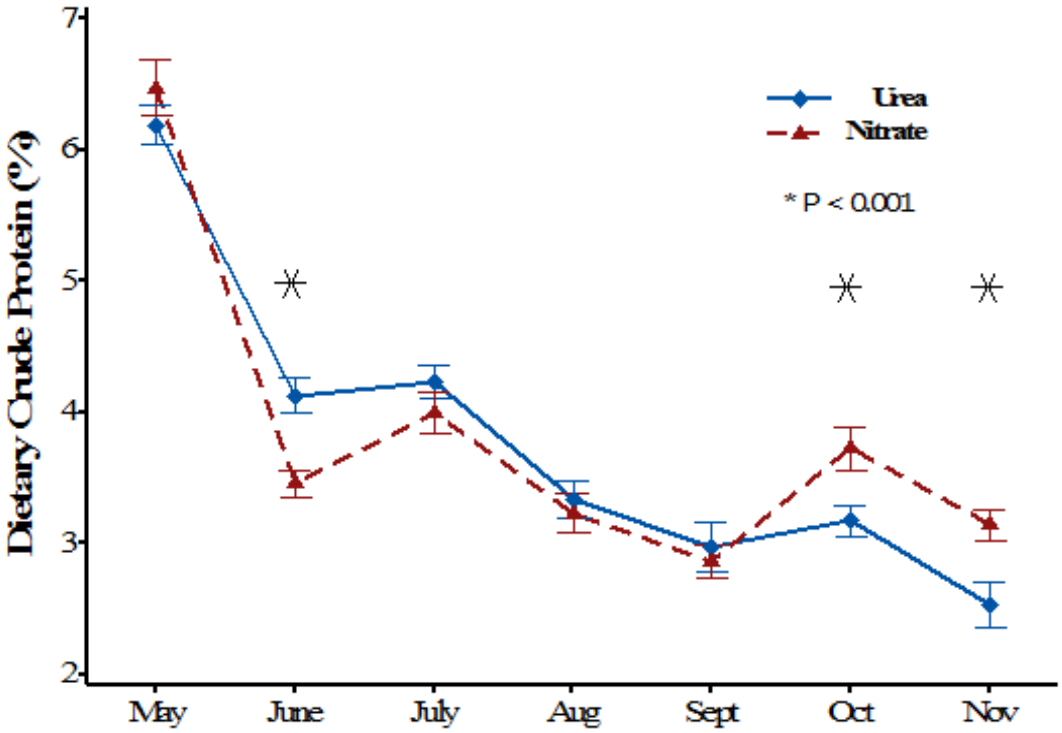

Fig 5.6. Mean dietary crude protein (CP g/kg DM) of *Bos indicus* heifers and cows grazing tropical pastures during the 2014 dry season with unrestricted access to either molten urea (30U) or molasses nitrate (MNB) blocks at Fletcherview, Charters Towers.

Methane emissions

In Year 1, only 11 and 8 days of data could be modelled to determine flux estimates for MNB and CON animals, respectively. The field work could not be extended in duration and what data was available generated an output from WindTrax with a high degree of uncertainty. After final filtering criteria was applied < 10 %, and approximately 30 % of 10 min averaged data for the MNB and CON group, respectively, could be interrogated. Comparisons could not be drawn from the Year1 final flux results due to the high degree of uncertainty, small sample size and the overestimation of emissions from the CON group.

Estimates of herd scale methane emissions from open path laser measurements, expressed on an average individual basis across the measurement period, during Y2 are summarised in Table 5.5. The estimated methane emissions of the urea supplemented cattle were consistent between the June and November measurement period. However estimated methane emissions from the June period appear greater than September in nitrate supplemented animals. When comparing nitrate methane production across both dry season measurements, mean emissions were similar between the treatment groups.

Table 5.5. Methane emissions (mean ± s.e.m) from herd scale measurements of *Bos indicus* heifers and cows grazing tropical pastures during the 2014 dry season with unrestricted access to either molten urea (30U) or molasses nitrate (MNB) blocks at Fletcherview, Charters Towers.

	Methane emissions (g/animal.d)		
	June	September	Mean
Urea	215 ± 3.7	200 ± 2.8	207 ± 2.2
Nitrate	281 ± 3.5	156 ± 2.7	219 ± 2.1

GPS Collars

Animals supplemented with either urea or nitrate walked similar distances in July or October. Mean distances travelled on a daily basis were less, regardless of the NPN supplement available, in October, during the late dry season, compared with distances travelled earlier in the year (July).

The maximum distance travelled from the water/supplement point for each animal, regardless of day, was used as an indication of grazing range across the paddock. Regardless of time of year (July *v*. October) or supplement group (MNB *v.* 30U) the maximum range from the single water and supplement point in this experimental paddock was similar and did not exceed 2.7 km.

Table 5.6. GPS performance, mean (± sem) daily distance travelled, and range from water/supplement point for *Bos indicus* heifers and cows grazing tropical pastures during the 2014 dry season with unrestricted access to either molten urea (30U) or molasses nitrate (MNB) blocks at Fletcherview, Charters Towers.

	MNB		30U	
	July	October	July	October
n	*28*	*23*	*27*	*29*
Days %	53	77	51	72
Distance (m)	8019 ± 153	7534 ± 225	8232 ± 218	7523 ± 180
Range (m)	2536	2673	2549	2580

n, number of animals fitted with GPS collars; Distance is the mean daily distance (m) travelled by animals for each supplement group; Days % is the number of days when individual GPS devices collected locational data divided by the number of days deployed; Range is the the maximum distance (m) from the water/supplement point for each animal (regardless of day), then averaged over the GPS group for each supplement treatment

Distinct and repeatable patterns of daily travel were observed for both time of year (July v. October) and supplement group (MNB v. 30U) (Fig 5.7). Cattle exhibited peaks of travel (distance travelled per hour) for approximately three hours in the morning, from 0600 and for four hours, from 1600 every afternoon. A distinct degree of inactivity was observed between 2300 and 0200 for all deployments.

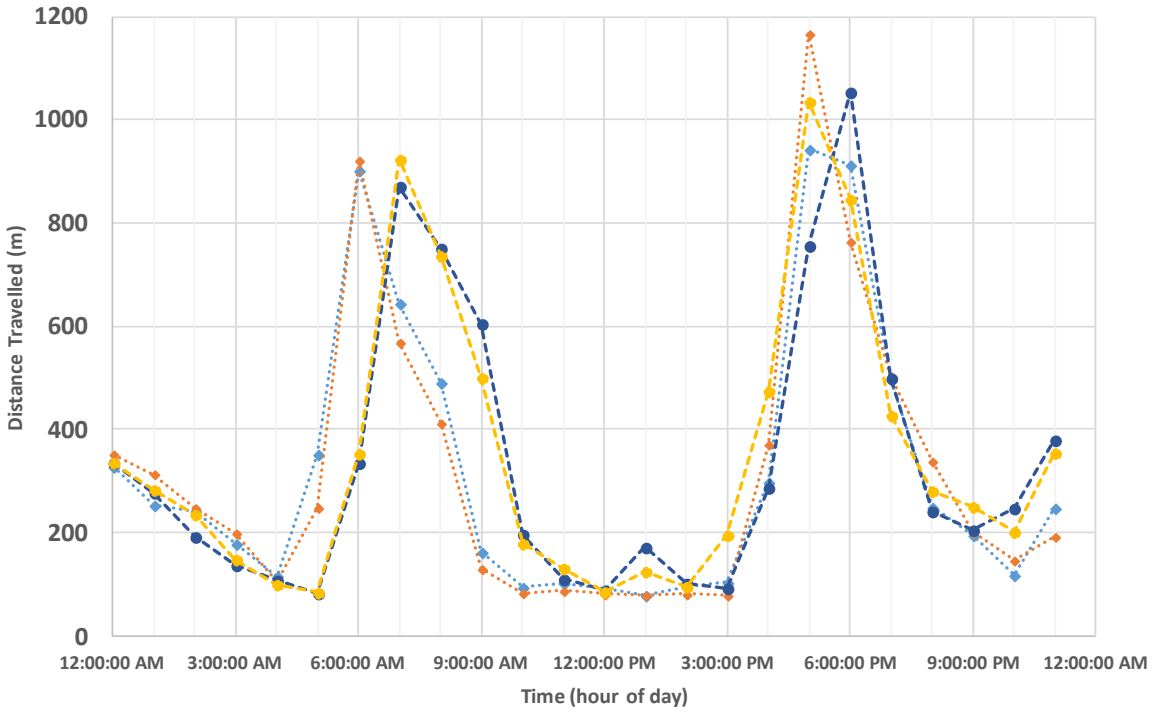

Fig 5.7. Mean distance travelled per hour for *Bos indicus* heifers and cows grazing tropical pastures during the 2014 dry season with unrestricted access to either molten urea (30U; ♦ October; ●July 2014) or molasses nitrate (MNB; (♦ October; ● July 2014) blocks at Fletcherview, Charters Towers.

4. Discussion

Experiment 1: Methane emissions, dry matter intake, rumen fermentation and methaemoglobin concentrations of *Bos indicus* steers fed Flinders grass (*Iseilemia spp*.) hay supplemented with either urea or nitrate

This experiment is the first to measure the methane abatement potential of nitrate in the context of forage quality and supplements characteristic of extensively managed beef herds in Australia. Both daily methane production and methane yield were similar to levels reported by Kennedy and Charmley (2012), where *Bos indicus* steers were fed a range of tropical grass hays, including forage of comparable quality used in this experiment. Although a number of studies in sheep (Nolan *et al*. 2010, van Zijderveld *et al*. 2010, Li *et al*. 2012), dairy (van Zijderveld *et al*. 2011; Lund *et al*. 2014) and beef cattle (Hulshof *et al*. 2012; Velazco *et al*. 2014) have previously validated the potential of nitrate to reduce enteric methane production, diets fed in those studies were typical of intensive animal production systems. The replacement of urea with nitrate, as an alternative NPN source in free choice loose mixes or lick blocks, would be attractive to the cattle industry in northern Australia because there are few abatement technologies that could be applied to extensively grazed livestock (Waghorn and Clark 2006). However this study was unable to demonstrate a significant reduction in daily methane production through the use of dietary nitrate. Stoichiometrically, 1 mol of nitrate (approx 62 g) reduces methane production by 1 mol (approx 16 g) during the reduction of

nitrate, via nitrite, to ammonia. In this experiment, CaN2 treatments consumed 50 g nitrate daily and therefore methane production could be expected to decrease by 13 g daily. When compared against urea, which had the same DMI as CaN2, the measured decrease in enteric methane was 66% of the expected value. This reduction efficiency is lower than the previously reported values of 87% in beef cattle (Hulshof *et al*. 2012) and between 78 and 98% in sheep (Nolan *et al*. 2010, van Zijderveld *et al*. 2010, Li *et al*. 2012). The discrepancy may be partially explained by differences in diet quality. Burrows *et al*. (1987) demonstrated that both the rate of reduction of nitrate and nitrite in the rumen was increased when fermentable substrates were added to the diet. The reduction of nitrate and nitrite by rumen microbes is also pH dependant, with levels of 6.5 and 5.6 considered optimal for the reduction of nitrate and nitrite, respectively (Tillman et al 1965). Cattle in this experiment were fed a low digestibility forage, supplemented with NPN and minerals only and rumen pH values were ≥ 6.5 at all measurements. Previous experiments in beef cattle fed nitrates have included significant amounts of concentrates, or, in the absence of concentrates, utilised moderate to high digestibility forages. These experiments also fed nitrate in the form of a total mixed ration, where small meals of nitrate could be consumed over the course of 24 h. In the current study, supplements were totally consumed (or the balance intra-ruminally dosed) within 1 h of feeding to ensure all animals received their allocated treatments within a similar time period. This concentrated pattern of dosing may have exceeded the capacity of the rumen microbial population to fully reduce nitrate (or nitrite) to ammonia, particularly in the absence of fermentable energy, thereby contributing to a lower efficiency of methane abatement. The alternative approach of mixing supplements with the forage and presenting the diet in a total mixed ration was considered a less informative method, especially in the context of feeding nitrate to extensively managed beef cattle in northern Australia. Typically supplements containing NPN in extensive grazing systems are fed *ad libitum* manner. Minimal intervention often results in variable intake patterns by animals (Eggington *et al.* 1990; Dixon *et al*. 2003). In addition, when supplements are self-fed, it is common for an animal's daily supplement consumption to occur at one feeding event in any one 24 h period (Cockwill *et al*. 2000).

In this experiment, the equivalent, elevated DMI and rumen ammonia (NH_3-N) concentrations of the isonitrogenous treatments (urea, CaN1 and CaN2) confirmed that nitrate salts could be an alternative to urea as an NPN source for cattle consuming low quality forage diets. In a short-term feeding study Benu *et al*. (2015) also found no difference in DMI between *Bos indicus* steers fed low quality Flinders grass hay and isonitrogenous supplements containing either urea only, or a combination of urea and calcium nitrate. In contrast, some previous studies with beef cattle have reported negative associations between dietary nitrate and DMI. (Hulshof *et al*. 2012; Hegarty *et al*. 2013; Newbold *et al*. 2014). Dietary concentrations of nitrate in the current experiment were 4.6 and 7.9 g NO_3/kg DM for CaN1 and CaN2 respectively. Concentrations did not exceed 10 g NO_3/kg DM, the level at which nitrate may cause a reduction in DMI of beef cattle (Bruning-Fann and Kaneene 1993). The findings from this experiment also do not support suggestions that nitrate is more likely to reduce DMI in diets containing high levels of NDF (Lichtenwalner *et al*. 1973).

The elevated DMI induced by the isonitrogenous treatments relative to control (10-14%) in this study was consistent with other work investigating the effect of the addition of an NPN source to low protein forages. This DMI response to NPN supplementation is at

the lower end of those reported in other studies where cattle are fed urea with low quality grass hays (15-65%; Ernst 1975; Romero *et al*. 1976). Calculations incorporating the measured forage analysis bulked over the experiment, intake of the control treatment and application of assumptions outlined earlier, confirms that the basal diet was deficient in RDN. Recycling of urea nitrogen to the rumen would have reduced this deficit and may explain the limited DMI response. Further evidence of a limited response to NPN supplementation is provided by mean rumen NH_3-N concentrations for the control treatment, which were close to 50 mg NH_3-N/L, the threshold concentration required to support maximum growth rates of rumen bacteria and MCP supply (Satter and Slyter 1974). Intake responses may also have been limited by the once daily dosing with NPN, which is associated with a reduced efficiency of utilisation compared to the same dose provided in incremental doses across the day when cattle are fed low quality roughages (Romero *et al*. 1976).

The combined influence of numerically lower daily methane production and increased DMI due to NPN supplementation resulted in an effective decrease in methane yield (g CH_4/kg DM) when comparing CaN2 to control and urea treatments. Reductions in methane yield due to nitrate addition in the diets of beef cattle have been previously reported (Hulshof *et al*. 2012; Newbold *et al*. 2014). The reduction in methane yield of CaN2 during the current experiment represents approximately 15% and 7.5% compared to control and urea treatments respectively. However given that supplementation with urea during the dry season in the northern Australian beef cattle industry is already common practice (Bortolussi *et al*. 2005), the scope for methane yield abatement appears limited (< 10 %) especially when the ceiling for nitrate feeding is set to correct an underlying deficiency of RDN in the forage diet.

A review of *in vivo* studies by Lee and Beauchemin (2014) concluded that enteric methane emissions are linearly decreased as nitrate consumption is increased. It could therefore be expected that greater methane abatement could have been achieved had greater concentrations of nitrate been fed. This experiment intended only to supplement the shortage of RDN in the forage diet because the feeding of NPN in excess of microbial N requirements does not impart additional productivity gain. This is an important consideration because the costs associated with using supplements containing nitrates are significantly greater than supplements containing urea (Callaghan *et al*. 2014). Furthermore, where excess dietary nitrogen is fed to grazing livestock, a higher proportion would be excreted in urine and this may result in increased nitrous oxide emissions. Increases in nitrous oxide on farm could effectively negate the benefits associated with methane mitigation from the animal.

During the course of the experiment, there were no clinical signs of nitrate toxicity for steers supplemented with either level of calcium nitrate. Despite the lack of overt signs, CaN2 treatments had much greater mean blood methaemoglobin concentrations relative to other treatments. The higher concentrations measured at 3 h, compared to 6 h in the CaN2 treatments, is consistent with previous research demonstrating that daily peak methaemoglobin concentration usually occurs in cattle at 3 to 5 h after a single dose of nitrate (Holtenius 1957; Kemp *et al.* 1977; Benu *et al.* 2015). Although daily peaks were below 20-30% methaemoglobin, the threshold at which clinical signs of hypoxia may develop in cattle (Parkinson 2010), the concentrations reported here are greater than in other studies where nitrate has been fed with the purpose of methane abatement. Newbold *et al*. (2014) fed a total mixed ration (65:35 forage to concentrate

DM basis) containing increasing concentrations of nitrate to Holstein steers and reported methaemoglobin concentrations at 3 h post feeding. At dietary nitrate concentrations between 6 and 22 g/kg DM, methaemoglobin concentrations did not appear to exceed 3%. Velazco *et al*. (2014) reported maximum mean methaemoglobin levels of 1.3% for Angus steers consuming a feedlot ration (> 80% concentrate DM basis) containing 19 g NO_3/kg DM. The apparent discrepancy between greater values reported in this experiment, at lesser inclusions of dietary nitrate, may be a consequence of a number of factors including the lack of fermentable substrates in the low quality forage diet, a single daily dose of nitrate delivered in a concentrated period of time and a sampling regimen designed to capture peak daily blood methaemoglobin. In contrast, Benu *et al*. (2015) demonstrated a close agreement for peak methaemoglobin concentrations with the current study. Daily peak blood methaemoglobin concentrations approached 10% when *Bos indicus* steers fed Flinders grass hay *ad libitum* were dosed once daily at 7 g NO_3/kg DM, a similar dose to the CaN2 treatment in this experiment. The mean methaemoglobin concentrations of CaN1, urea and control treatments were all within the physiologically normal range for cattle of < 1%. Thus despite the small increase in daily methaemoglobin peak concentrations (3 h) for the CaN1 treatment, it is likely that nitrate fed at this dietary concentration could be considered safe even if consumed rapidly by cattle consuming a low quality forage diet. Without any effective on methane abatement, the safety imparted by this dietary concentration of nitrate is of limited practical importance for northern Australian beef herds.

Observed molar proportions and total VFA concentrations were within the range of expectation for cattle fed low quality tropical grass hay (Bowen *et al*. 2008; Panjaitan *et al*. 2014). Similar total VFA concentrations between isonitrogenous treatments comprised of either nitrate or urea have also been previously reported (Hulshof *et al*. 2012; Li *et al*. 2012). Although Nolan *et al.* (2010) reported greater total VFA concentrations in sheep fed nitrate compared to an isonitrogenous urea treatment consumed at the same DMI, this difference may be a consequence of fermentation patterns driven by changes in feeding frequency and meal size when nitrate is included in the diet (Velazco *et al*. 2014). The findings that nitrate supplementation resulted in an increase of acetate production and the acetate:propionate ratio is consistent with other studies in both sheep and cattle (Nolan *et al*. 2010; Hulshof *et al*. 2012). The redirection of H_2 in the rumen to the energetically favourable pathway of nitrate reduction competes with methanogenesis and propiogenesis (Ungerfeld and Kohn 2006). However in this study there was no evidence of decreased propionate being associated with nitrate based treatments. A reduction in butyrate production without a decrease of propionate, when feeding nitrates to sheep, has been previously reported (Li *et al*. 2012). The competition for electrons from nitrate reduction causes Acetyl-CoA to favour acetate formation at the expense of butyrate synthesis (Alaboudi and Jones 1985).

Experiment 2: 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

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*. 2102) 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 grams of nitrate into the rumen of *Bos indicus* steers caused a greater increase in the methaemoglobin 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 methaemoglobin 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 methaemoglobin 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 and 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 methaemoglobin (Parkinson *et al.* 2010). The steers receiving 40 or 50 grams 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 grams of nitrate. In addition, the steers treated with 50 grams divided into two doses also had their peak MetHb concentrations double over seven days. Moreover the rate of incline (% MetHb/h) of MetHb for once a day dosing of 40 and 50 grams 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 methaemoglobin concentration in the blood. The demonstration of cause and effect for this hypothesis is beyond the captured data of this experiment nevertheless our data supports 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 grams of nitrate once a day would suggest that 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 reported previously. Carbon monoxide competes with oxygen for heme sites on the haemoglobin molecule and decreases the ability of haemoglobin to carry and release oxygen to tissues (Guyton and Hall 2011). The only endogenous source of carbon monoxide in the mammalian body results from the metabolism of intravascular haemoglobin to billiverdin catalysed by heme oxygenase-1 (Maines 1988). Heme oxygenase-1 is induced in response to a broad spectrum of

stimuli and agents: heme, metal ions, oxidative stress, bacterial toxins, starvation and haemolytic diseases such as babesiosis (Maines 1984; Taylor *et al.* 1992; Kadinov *et al*. 2002). It is probable that oxidative damage to red blood cells by the once a day treatments of nitrate resulted in an expression of hemeoxygenase-1 thereby increasing carboxyhaemoglobin concentrations. The increase in carboxyhaemoglobin as the experiment progressed would have compounded the hypoxia caused by the methaemoglobin 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 had 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 dealing 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.

Experiment 3: The effect of nitrate supplementation on arterial blood gases, heart rate, respiratory rate and rectal Temperature of *Bos indicus* cattle after exercise

All of the steers appeared to tolerate the exercise regimen however the partial pressure of oxygen (PO_2) for the steers given 50 grams of nitrate a day was alarming (PO_2 = 64.60 mm Hg; Table 3.2). The PO_2, methaemoglobin and the oxyhaemoglobin values for the steers given 50 grams of nitrate indicated a reduction in the oxygen carrying capacity of the blood from these steers. Nitrate is known to cause heme Fe_2 to be oxidised to the Fe_3 state where oxygen cannot bind the haemoglobin sub-units in the blood causing a methaemoglobinaemia (Hammond *et al.* 2005). The steer's response to the hypoxia caused by the 50 grams of nitrate treatment did not involve a greater respiration rate than that seen in the zero and 30 grams of nitrate treatments. All treatments yielded similar PCO_2 values in their respective mean arterial blood samples supporting the lack of difference in respiration rate between treatments at the completion of the exercise regimen. Carbon dioxide controls the breathing centre of the brain until the oxygen concentration of the arterial blood falls below 60 mmHg. The aortic bodies then become the principle regulator of respiration rate (Guyton and Hall 2013). However, the steers dosed with 50 grams of nitrate compensated for the hypoxia in their blood by increasing their heart rate by 60% greater than the steers given the zero nitrate treatment at the completion of exercise. Nitrate and nitrite salts

are known to cause pronounced vasodilation in cattle (Vermunt 1992). Although 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). Vermunt (1992) suggests that vasodilation of the peripheral vessels is possible when cattle consume nitrates in their diet. It is therefore probable that vasodilation of the vascular system and the reduced oxygen carrying capacity of the blood combined to cause a greater heart rate after exercise in the 50 gram of nitrate treatment compared with the 0 and 30 grams of nitrate treatment groups. The untrained steers in this study were exercised for a period of 24 minutes at an average speed of 0.92m/sec. The steers in the 50 grams of nitrate treatment recorded a mean heart rate of 175 beats/min, which is a greater value than other studies published in cattle and buffalo under an exercise load. Kuhlmann *et al.* (1985) exercised 199 kg trained Hereford steers at a speed of 1.0m/sec and attained a heart rate of 140 beats/min. Furthermore, untrained draught buffalo of 363 kg pulling a work load of 11% of their live weight and walking at a speed of 0.69m/sec for three hours have been recorded with a heart rate of 100 beats/min (Martin 1993). Thus we would suggest that the steers in our study were under a considerable stress in maintaining oxygenation to working tissues in the face of supplementation with 50 g nitrate per steer.

The methaemoglobin concentrations of the cattle were similar to the values reported in other studies with increasing dose rates of nitrate resulting in increasing concentrations of methaemoglobin in the blood (Benu *et al*. 2015). The blood samples were taken between 2 and five hours after dosing the steers with their respective treatments. This would have coincided with methaemoglobin concentrations recorded around the peak values for the day from each of the steers treated with nitrate (Callaghan *et al.* 2014).

The fraction of carboxyhaemoglobin in the blood was approximately double the concentration for the 50 grams of nitrate treated steers compared with the other treatment groups. Benu *et al.* (2015) also reported an effect of increasing carboxyhaemoglobin concentrations with time for *Bos indicus* steers treated with varying dose rates of nitrate over seven days. This reflects a further inhibition of the oxygen carrying capacity of the blood of cattle treated with nitrate. We would also argue that the increase in carboxyhaemoglobin with the greatest dose of nitrate in this study is a sign of oxidative stress on the animal induced through the production of heme oxygenase-1 (Benu *et al.* 2015).

Arterial blood pH values were similar for all treatments with a slightly alkaline pH demonstrated (pH 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. In comparison, Parker *et al.* (2003) with a similar genotype and body weight as the current study recorded arterial blood pH in penned steers to be 7.44 ± 1.01.

Experiment 4: Effects of urea or nitrate supplements on haemoglobin fractions from *Bos indicus* steers fed Flinders grass (*Iseilemia spp*.) hay for 70 days

We initially hypothesized that increasing the time cattle are on a continuous dose of nitrate salts would enable the rumen microflora to significantly reduce the nitrate and

nitrite in the rumen and therefore slow the development of the expected methaemoglobinaemia in the animals. However, on the data presented in this experiment we reject our hypothesis. The day of sampling (day 10, 30, 50 or 70) demonstrated no difference to the development of methaemoglobinaemia in the cattle treated with nitrate. It is noteworthy that the steers treated with a similar rate of nitrate on a dry matter basis in the study of Benu *et al.* (2015) (7.5 g NO_3/kg DMI) had a similar methaemoglobin profile when sampled as the steers in this study (7.14gNO_3/kg DMI). Benu *et al.* (2015) demonstrated that steers fed nitrate at a rate of 7.5 g NO_3/kg DMI produced the same MetHb profile each day for seven days. It is also apparent that the time zero samples were the same as the no nitrate treated steers suggesting total clearance of the nitrate treatment from the body of the steers each day. It is probable that at doses greater than 7.5gNO_3/kg DMI that the methaemoglobin concentration may continue to increase on a daily basis and total clearance of nitrate from the body may not occur.

The steers in this study were housed in stalls 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. This data is 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 non-protein–nitrogen source (Nolan *et al.*, 2010), (Li *et al.,* 2012) and Benu *et al.* (2015).

Experiment 5: Field experiments for *Bos indicus* cows grazing tropical pastures consuming nitrate lick blocks

When cattle consume low quality forage, responses to supplementary N can be expected when forage CP is less than 60 g/kg DM (Minson 1990). Furthermore, *Bos indicus* cattle have been shown to have a greater ability to conserve nitrogen (Hunter and Siebert 1987; Hennessey *et al* 2000) and generally do not respond to N supplementation until forage CP drops below 50 g/kg DM. Thus the general increase in LWT of non-pregnant *Bos indicus* cows observed during Y1 of this experiment is consistent with the estimates of diet quality from faecal NIRS. The initial increase in forage CP content of the diet occurred following rainfall during late November 2013. However there were no overall treatment differences in F.NIRS diet quality predictions and at time points where differences occurred, these differences were biologically small. Furthermore, although cattle consuming MNB blocks received additional N, the diet quality estimates suggest a response to N supplementation would be unlikely. Under these circumstances, it would be reasonable to expect that both CON and MNB cows during mY1 would have a similar pattern of LWT change. Thus causes of the 8 kg LWT advantage measured in CON cows at the conclusion of Y1 experiment remain unclear.

Supplement intake by MNB cows in both Y1 and Y2 was below the expected consumption rate. Consequently the mean N intake in both years by the MNB treatment was below the target of 20-30 g/d usually recommended for breeding cows grazing senesced tropical pastures (Winks 1984; Dixon and Doyle 1996). To reach the minimum N intake targets to alleviate the likely RDN deficiency, intakes of MNB blocks would need to reach approximately 250 g/hd.d. Cattle on MNB did not reach this threshold despite increasing intakes during the later dry season in Y2. In contrast, supplement intake by 30U animals during Y2 consistently achieved N intake thresholds

between September and November 2014. Supplement intakes observed in 30U cattle during this experiment are similar to previously reported intakes of urea blocks during the dry season at the same location (Callaghan *et al* 2013). During Y2, F.NIRS estimates suggest a response to N supplementation would be observed if sufficient supplementary N was consumed between June and November. Previous experiments have observed that *Bos indicus* steers grazing pastures containing < 50 g CP/kg DM in the seasonally dry tropics have shown liveweight responses of 0.1 – 0.25 kg/d when supplemented with NPN (Coates and Dixon 2008*b*). A response to N supplementation was observed in this study, where 30U cattle consumed adequate supplementary N between September and November, leading to CFLW loss and lower BCS in the MNB treatment.

Animals appeared to be in good health for the duration of the trial and there were no external signs of nitrate toxicity. Despite the lack of external signs, there was evidence that ingested nitrate was not fully reduced to ammonia in the rumen because MNB cows had higher blood MetHb concentrations in both years, which increased over the experimental period. It was not possible to determine when cattle were bled relative to a feeding event or in fact, whether individual cows consumed a daily nitrate dose as a discrete event or over a longer period. The peak of methaemoglobin concentrations usually occur in cattle at three to five hours after ingestion of nitrate (Holtenius 1957; Kemp *et al*. 1977). In this study the experimental design does not allow to state whether these samples represent peaks, troughs or periods of ascending or descending blood MetHb. Furthermore, the methodology used to estimate individual supplement intake did not accommodate sampling to determine whether MetHb concentrations were correlated with individual intake estimates. Regardless, we are not aware of other studies whereby MetHb levels have been measured in grazing cattle given free choice access to nitrate blocks. The data presented here begins to quantify the challenges of attempting to supplement cattle with nitrate at a herd level. The outlying values for MetHb are not necessarily unexpected given that free-choice N supplements fed to beef cattle in northern Australia are characterised by irregular consumption patterns and marked individual variability of supplement intake (Dixon *et al*. 2003).

It is relatively clear from both Y1 and Y2 that the acceptance of MNB blocks appear to be lower than conventional urea blocks. This was unexpected because the inclusion rate of calcium nitrate and urea in the blocks were similar at 35% and 30% respectively. Furthermore, the MNB blocks were molasses based, which often acts as an attractant to cattle and they were deliberately designed to be relatively soft, so that intake was encouraged. In short, such a block would not usually be considered commercially viable to feed to cows in the northern Australian rangelands because of both excessive intake and the potential risk of toxicity. Given the supplements were provided independently in Y2, the lower than expected intakes cannot be attributed to preference. It may be possible that MNB blocks have triggered a feed aversion response, caused by feedback to MetHb, leading to a relatively low supplement intake.

There are few studies that have measured the methane emissions of *Bos indicus* breeding cows consuming low quality forage diets, typical of the northern Australian dry season. Field studies by Tomkins *et al*. (2013) estimated methane production from herds across northern Australia using open path lasers. Values reported include 212 ± 8.9 (mean ± s.e.m) g CH_4/animal.d from *Bos indicus* cows (400 kg LW) grazing early wet season buffel grass (*Cenchrus ciliaris*) and 162 ± 8.9 g CH_4/animal.d from Brahman

X Senepol heifers (317 kg LW) grazing senesced native pastures during the mid-dry season. Methane emissions of mature *Bos indicus* cows grazing mixed buffel and Sabi grass (*Urochloa mosambiensis*) during the pasture growing season were estimated at 281 ± 22 g/animal.d using measurements from open path lasers (Berndt and Tomkins 2013). Thus the mean daily methane production estimates from combined measurements made during Y2 of the current study appear within the range of previously reported values.

The cause of greater methane emissions estimated in nitrate supplemented cattle between June and July are not clear. During this period, mean daily intake from nitrate blocks was 50 g/animal.d, equating to approximately 11g nitrate/animal.d. Based upon findings from Experiment 1, it is unlikely this dose would make a meaningful reduction in methane emissions. It was therefore expected that methane production estimates from 30U and MNB treatments would be similar during this period. Although estimates of daily methane production using open path lasers at the herd scale from cattle consuming tropical pastures are comparable to open circuit respiratory chambers, estimates made using the former method are exposed to multiple sources of error which may contribute to variable results (Tomkins *et al*. 2011). The results between June and July for MNB cattle may therefore be an overestimation of methane production by the nitrate treatment group during that period. Although MNB intakes had increased to an average of 70 g/animal.d during the September methane measurement period, corresponding nitrate intakes (15.5 g/animal.d) remained insufficient to make measureable reductions in methane emissions. A lack of nitrate intake during the methane measurement periods and therefore no expected reduction in methane production confirms the similar dry season methane production estimates of 30U and MNB treatments reported in this study.

Data from Experiment 3 demonstrated that although 400 kg LW *Bos indicus* steers dosed with 50 g nitrate daily tolerated an exercise challenge, the oxygen carrying capacity of the blood was significantly reduced. The current experiment therefore adopted the hypothesis that increased blood methaemoglobin concentrations caused by ingestion of nitrate blocks would decrease the distance walked and grazing range of cattle. In large scale paddocks this may lead to overgrazing around a central point and a piosphere may develop (Lange 1969). During the period of collar deployments in July and October 2014, the corresponding measured nitrate intake from MNB averaged 15 and 30 g nitrate/animal.d respectively. Although MNB cattle had greater blood methaemoglobin concentrations than 30U cattle during October, the mean concentration was relatively low 1.5%. In comparison, the steers dosed with 30 grams nitrate once daily, during Experiment 3, had mean blood methaemoglobin concentrations of 5%. These steers did not display any physiological responses to the exercise challenge. Thus the findings that both distance walked and grazing range were similar between treatments at both deployments is consistent with the low intake of nitrate supplements. The diurnal pattern of animal activity during the early morning and evenings, which coincides with peak grazing activity (Low *et al.* 1981), has been previously reported for *Bos indicus* cattle fitted with GPS collars grazing tropical pasture species in the Charters Towers region (Tomkins and O'Reagain 2007). The shift in animal activity between July and October towards increased animal activity early in the day, followed by a longer period on inactivity during the middle of the day, reflects the increasing daytime temperatures which occur in the late dry season.

5. Significance of findings for Australian agriculture

This project has thoroughly examined whether the concept of feeding nitrate to extensively managed cattle grazing low quality pastures is a feasible methane abatement option for farmers. The project has shown showed that cattle dosed with nitrate can achieve similar feed intake to cattle dosed with urea. However the practice of nitrate supplementation for cattle consuming low quality forages is associated with a significant risk of nitrite toxicity. Importantly, the measured methane production of cattle dosed with nitrate in this project demonstrated only a limited scope for methane abatement. When offered nitrate as free-choice nitrate blocks in the paddock, cattle were unable to consume adequate nitrogen. This finding is of consequence because the consumption of nitrogen is the primary purpose of supplementation and inadequate nitrogen intake may result in lower animal productivity. Thus the limited methane abatement, elevated risk of nitrate toxicity and potential for inadequate nitrogen consumption offers limited incentive for adoption by farmers. This project suggests that the application of nitrate in Australian agriculture may be better directed towards intensive sheep and cattle production systems.

6. Future research needs

In this project there was no reduction in daily methane production when dietary nitrate concentration was capped at supplying adequate RDN on low quality forage diets. This suggests that greater dietary nitrate concentrations are required to make a significant reduction of methane emissions. However based upon the blood methaemoglobin concentrations observed in this project, the lack of fermentable substrates in the diet and variable feeding patterns associated with self-administered supplements in northern Australia, increasing the dose rate is a high risk strategy.

Therefore in the context of the northern Australian beef cattle industry, future research should focus on mechanisms to minimise the risk of nitrite toxicity, allowing greater levels of nitrate to be safely fed and achieving a reduction in methane production. Responses of ‘slow release’ (encapsulated) nitrate should be a high priority for future research.

The comparatively lower level of acceptance of nitrate blocks in the field study should be investigated further. There is ample anecdotal evidence suggesting nitrate salts are unpalatable however this lack of acceptance may be an aversion following negative feedback to nitrite toxicity.

Whilst gradually increasing nitrate concentration in the diet appears logical, it is difficult to achieve using self-fed supplements. There are no published experiments where nitrate has been either fed at a set dose from the outset compared against the gradually increasing nitrate to the same dose over time. An experiment of this nature would help resolve the question of acclimation. It is likely that this experiment would need to be conducted over a range of dietary nitrate concentrations.

7. Publications

Benu I, Callaghan MJ, Tomkins N, Hepworth G, Fitzpatrick LA, 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* (Accepted 30th March 2015).

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