Final report

A novel amino acid approach to lamb survival

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Prepared by: Dr Mariana Caetano, Leesa-Joy Flanagan, Dr Michelle Hebart, Associate Prof Forbes Brien, Charlotte Allen, and Lillian Carlyon

The University of Adelaide

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Abstract

This project developed novel methods to improve lamb survival, particularly twins/multiples between conception and weaning. Twin-bearing ewes sustain higher nutritional demands during gestation with nutritional recommendations focussed on meeting higher energy requirements. Amino acids including glutamine, glutamate, arginine, histidine and leucine have been identified as limiting in twin fetuses development. These amino acids are important for fetal growth and contribute significantly to hormone production required for fetal development. The first experiment in this project aimed to determine the role of Non-Essential (glutamine and glutamate) and Essential (arginine, histidine and leucine) amino acid supply in twin-bearing ewes on foetal development and lamb vigour under controlled animal house conditions approved by animal ethics. The results indicated that twin lambs born from ewes supplemented with rumen protected glutamine during late gestation showed a positive result, with lambs having similar growth rate as single-born lambs at weaning, despite having lower weights at birth, although there was no statistical significance between treatments. Based on these findings several nutrition companies demonstrated interest in exploring opportunity to develop a rumen protected glutamine product that could be utilised to achieve the remaining project objectives and improve lamb vigour and survival metrics. Unfortunately a consistent supply of rumen protected glutamine could not be sourced for the remaining trails as the method for developing the product is not commercially available and requires further R&D development. Discussions with one commercial global nutrition company have shown promise although reaching a definitive agreement may take time, the company remains optimistic about potential collaboration and its positive impact for the sheep and red meat industry.

Executive summary

Background

While recommendations for optimal metabolisable energy intake and condition score have been established for single and twin-bearing ewes, when these are adhered to the difference in survival between single and twin/multiple lambs is still high. Twin and triple-bearing ewes are considered to be under greater nutritional challenge during gestation. There is evidence that amino acids (AA) generally synthesised by the ewe, and therefore identified as non-essential, become limiting when carrying multiple foetuses. Optimising AA supply during critical periods of gestation may have significant effects on foetal development. The proposed investigation of AA aim to provide a cost-effective nutritional strategy to improve the development and growth of the fetus, resulting in stronger and healthier lambs at both birth and weaning.

Aims/objectives

The current project aims to: 1) Determine the role of Non-Essential (glutamine and glutamate) and Essential (arginine, histidine and leucine) amino acid supply in twin-bearing ewes on foetal development and lamb vigour; 2) Determine if oxygen is required in combination with amino acid supplementation to optimise amino acid metabolism; 3) Determine the timing of amino acids supplementation during pregnancy of twin-bearing ewes based on cost-effectiveness; 4) Evaluate the cost-effectiveness and practicalities of supplementing amino acids on-farm under two different feeding regimes to evaluate foetal loss, lamb survival at birth and weaning.

The aims (1) and (2) were delivered before the termination of this contract. The outstanding project objectives were unable to be achieved due to the difficulties in obtaining a consistent pilot rumen protected glutamine product produced by a nutritional company that aligns to this project timeline for moving forward with the research.

Methodology

The first experiment was designed to test 6 maternal supplementation treatments including; Blend of amino acids, Blend of amino acids + sildenafil citrate (SC), Glutamine, Glutamine + sildenafil citrate, Twin control, and Single control and the beneficial effect in the progeny. Due to the difficulties in obtaining a pilot rumen protected glutamine produced by a nutritional company, the second and third trials to evaluate the time of maternal glutamine supplementation (early, mid, late and entire gestation) and validate under farm conditions, respectively, could not be conducted by our research group.

Results/key findings

- The combination of SC and arginine used during the safety trial demonstrated to be safe for pregnant ewes and did not cause adverse effects. However, further trials should investigate the effects of supplementation during pregnancy on the fetal and neonatal lambs to ensure no negative consequences.
- Twin lambs born from ewes supplemented with glutamine during late gestation were able to grow at a similar rate as single-born lambs by weaning, showing similar liveweights (25.2, 27.6 and 18.5

kg, respectively for glutamine, single and twin control groups). It demonstrates the potential benefit in using maternal amino acid supplementation to reduce mortality rate of twin lambs.

- The combination of amino acids with sildenafil citrate did not provide any beneficial effect on the progeny at birth and weaning.
- There is a potential in using CT scanning to evaluate the nutritional requirements of live animals at different physiological stages without the need for euthanasia, however further validation studies are needed.
- Natural substances 1 and 2 can be used in a cost-effective way to prevent partial microbial degradation of glutamine in the rumen. However, further investigation is needed to determine if these substances are effective in increasing glutamine levels in the small intestine and bloodstream of ruminants for metabolism.

Recommendations

The results generated from the first experiment demonstrate that further R&D into this area is required once a method has been developed to produce a consistent rumen protected glutamate product. The involvement of commercial companies in developing a pilot supplement has introduced challenges and inefficiencies in the project moving forward with the research to date and hindered the project continuing to completion. These obstacles have the potential to prevent the continuation of further research and hinder the progress of any HDR student involved in the project. Therefore, its recommended that exploratory R&D of this nature includes commercialisation strategies (including benefit cost analysis and pathways to adoption) once core R&D objectives have been achieved.

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1. Background

Average mortality rate in twins is estimated to be around 30% or more across Australia (Hinch and Brien, 2014). Consequently, the peri-natal mortalities in sheep is estimated to cost AU$850.6M in Australia (Shephard et al., 2022). One of the primary factors influencing lamb survival and life-long performance is maternal nutrition. Current feeding recommendations for twin-bearing ewes focus on meeting energy requirements. Single-bearing ewes at condition score 2.3 presented lamb survival rates of 85%, and 91% at a condition score of 3.2. Comparatively, twin-bearing ewes had survival rates of only 57% at a condition score of 2.2 and 71% at a condition score of 3.2 (Behrendt et al. 2011). While this practice improves birth weight and lamb survival, the difference between single and twin lambs is dramatic (Hocking Edwards et al. 2011).

Multiple-bearing ewes are under a greater nutritional challenge during pregnancy with minimal consideration given to protein requirements. Under normal circumstances sheep synthesise specific AA to levels sufficient for normal physiological function, hence are deemed “non-essential”. We propose these “non-essential” AA become “essential” under specific conditions (i.e. carrying multiple foetus), a result of the ewes inability to synthesise sufficient quantities required during gestation. A recent study demonstrated twin foetuses present with decreased plasma glutamine (non-essential AA), arginine, leucine and histidine (essential AA) concentrations compared with singletons (van der Linden et al., 2013) suggesting both non-essential and essential AA may be limiting during gestation in twin-bearing ewes.

The foetus requires AA’s for normal physiological development (i.e. skeletal muscle synthesis; thermoregulation capacity and organ development). Leucine increases muscle protein synthesis during foetal and postnatal life. Arginine increases nitric oxide (potent vasodilator; Hefler et al., 2001; Winer et al., 2009), skeletal muscle (Harding et al., 1997; Sales et al., 2014), brown fat tissue and core-body temperature (McCoard et al., 2013; McCoard et al., 2014) and is also an important substrate for polyamine production during rapid placental growth (Kwon et al., 2004). Glutamine (primary source), arginine and histidine can be used as precursors of glutamate, the major energy source for the placenta (Lemons et al. 1976). A lack of glutamine in the foetal liver results in less glutamate oxidised in the placenta as an energy source. Leucine, arginine and histidine increase insulin secretion and consequently increase insulin-like growth factors (IGF). Furthermore, these maternally-derived AA’s are required during foetal programming events for signalling pathways and hormone synthesis representing 26% to 34% of the total AA in hormone molecules (IGF-I, IGF-II, insulin and erythropoietin; Figure 1).

Oxygen supply is important to be considered when evaluating AA as a supplement due to its role in AA metabolism and transport. Sildenafil citrate is proven to increase vasodilation and consequently oxygen supply. Previous study using Sildenafil citrate increased total AA and polyamine concentration in amniotic fluid, allantoic fluid and foetal serum without affecting values in maternal serum (Satterfield et al., 2010).

Current findings suggest the strategic feeding of “non-essential” and “essential” AA during gestation will provide a highly cost effective and practical tool to decrease lamb mortality and improve lamb vigour at birth and overall improve lifetime performance of twins.

2. Objectives

The project aims to:

i. Determine the role of Non-Essential (glutamine and glutamate) and Essential (arginine, histidine and leucine) amino acid supply in twin-bearing ewes on foetal development and lamb vigour.
ii. Determine if oxygen is required in combination with amino acid supplementation to optimise amino acid metabolism.
iii. Determine the timing of amino acids supplementation during pregnancy of twin-bearing ewes based on cost-effectiveness.
iv. Evaluate the cost-effectiveness and practicalities of supplementing amino acids on-farm under two different feeding regimes to evaluate foetal loss, lamb survival at birth and weaning.

The aims (i) and (ii) were met before the termination of this contract. The termination of the contract occurred due to the difficulties in obtaining a pilot rumen protected glutamine produce that could be fed to sheep via non-invasive methods (eg via feed) produced by a nutritional company that aligns to this project timeline for moving forward with the research.

Experiment one was completed successfully with proof of concept results and additional information was generated prior to running into challenges obtaining larger quantities of the amino acid which showed the most promise.

The results of this final report will focus on the findings generated from the first project and potential opportunities according to the following topics:

- Safety Trial - to investigate the effects of supplementing sildenafil citrate (SC) and arginine, in an amino acid blend infusion, since the combination of both compounds increases vasodilation and the cumulative effect is not known in pregnant sheep, and may be detrimental.
- Experiment 1 – to evaluate the effect of maternal amino acid supplementation on the development and growth of twin fetuses, to understand if this nutritional approach resulted in stronger and healthier lambs at birth and weaning; and to investigate the effect of sildenafil citrate in combination with maternal amino acid supplementation to optimise amino acid metabolism and the effects on the progeny.
- Estimating organ size and body composition – to validate Computer Tomography (CT) scan analysis for evaluating organ size and body composition in neonatal lambs, by comparison with standard methods of water displacement and weighing.
- Experiment 1 – CT scanning – to evaluate organ size and body composition of a subset of progeny from MLA Experiment 1 using CT scan analysis.
- Substances to rumen protect glutamine - to determine the efficacy of two cost-effective natural substances (NS) in protecting glutamine from microbial degradation in the rumen through *in vitro* analysis.

3. Methodology

3.1 Safety trial

This trial was conducted in accordance with the guidelines provided by the Australian Code for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 2013). The use of animals was approved by The University of Adelaide Animal Ethics Committee (S-2020-061). Experimental work was conducted at the University of Adelaide, Roseworthy Campus, Roseworthy, South Australia.

3.1.1 Animals, housing and nutrition

Multiparous Merino ewes (n=280) were synchronised with progesterone controlled intrauterine drug release devices (CIDR) in mid-February. Following removal of the CIDRs, ewes were mated naturally over approximately two oestrus cycles (21 days). Thirty-two twin bearing ewes were identified via transabdominal ultrasonography and managed under commercial grazing conditions until entering the indoor animal house at day 125 of gestation.

From day 125 to 135 of gestation, thirty-two twin bearing ewes were moved to the animal house for acclimatisation to individual pens, a total mixed ration (TMR) diet and daily handling. On day 129 all ewes were fitted with intravenous jugular catheters for treatment administration and blood sample collection. During the 10-day acclimatisation period heart rate (bpm), respiratory rate (bpm) and rectal temperature (°C) were recorded for each ewe.

A total mixed ration was provided to meet 100% of National Research Council requirements for twin bearing ewes, based on bodyweight (NRC, 1985). Ewes were fed daily at 09:00 and daily refusals were recorded (Custom sheep mix, Hills Farm Supplies, Mount Barker South Australia; Table 1.1).

Table 1.1. Nutritional composition of the total mixed ration feed supplied to pregnant ewes per kg of dry matter.

Nutrients	Analysis
Dry matter (%)	100
Me (MJ/kg)	10.94
Crude protein (%)	14.90
NDF (%)	36.16
Fat (%)	2.51
Calcium (%)	0.79
Phosphorus (%)	0.33
Magnesium (%)	0.21
Potassium (%)	1.41
Sulphur (%)	0.19
Sodium (%)	0.08
Chloride (%)	0.36
Zinc (mg/kg)	60
Cobalt (mg/kg)	0.5
Copper (mg/kg)	0
Iodine (mg/kg)	0.5

Manganese (mg/kg)	20
Selenium (mg/kg)	0.1
Vitamin A KIU/kg	4.5
Vitamin D KIU/kg	0.5
Vitamin E KIU/kg	40
Vitamin B1 (mg/kg)	1000
Bovatec (mg/kg)	33

3.1.2 Treatments and sampling

Ewes (n = 8 per group) were randomly allocated into four treatment groups (amino acid blend and sildenafil citrate (AA+SC), amino acid blend (AA), sildenafil citrate (SC) and control (saline infusion and reverse osmosis (RO) water injection; Table 2). Each treatment group was balanced for age (4.5 years $\pm$ 0.8), live weight (78 kg ± 9.5) and body condition score (3.7 $\pm$ 0.4). Control saline and AA was administered via the jugular catheter. Control RO and SC water injections were administered on alternating injection sites on the ewe's rump. Treatment began at day 135 of gestation. Treatments were administered thrice daily (06:00, 14:00 and 22:00). Between treatments, catheters were flushed with heparinised saline to maintain patency (heparinised saline (0.9% sodium chloride, Baxters Healthcare Pty Ltd, Old Toongabbie, Australia: 10 U/mL sodium heparin).

Table 1.2. Treatment composition summary.

Treatment	Injection	Infusion	Number of ewes
AA+ SC	SC	AA	8
AA	RO	AA	8
SC	SC	Saline	8
Control	RO	Saline	8

Each AA blend consisted of 0.4g of L-Arginine, 0.37g of L-Histidine, 0.17g of L-Leucine, 0.47g of L-Glutamine and 0.23g of L-Glutamic (Sigma Aldrich Pty Ltd, St Louis, Missouri USA) in a 20 mL saline bolus thrice daily. Injections followed infusions. SC (50 mg; Pfizer, New York, NY) was administered in a 9 mL RO water bolus. Dosage of SC was determined as the highest concentration previously given to multiparous ewes to produce effects (Satterfield *et al.* 2010). Both the AA blend and SC were prepared no more than two days in advance, in bulk for daily administration. The SC was prepared by dissolving 2.4 g of SC in 432 mL of reverse osmosis water on a stirrer at 600 rpm at 25°C. The AA blend solution was prepared using 19.7 g of L-Arginine, 18.2 g of L-Histidine, 7.8 g of L-Leucine, 22.9 g of L-Glutamine and 11.4 g of L-Glutamic and dissolved in 960 ml of sterilised RO water at 600 rpm at 25°C. Baseline rectal temperature, heart rate, respiratory rate, ketones (mmol/L) and blood gas parameters were collected on day 129 of gestation. Rectal temperature, heart rate, respiratory rate and all blood parameters and gases were collected two (D2), six- (D6) and ten-days (D10) post commencement of treatment, at 14:00.

Blood was collected via intravenous jugular catheters. Ketones were measured using a LifeSmart blood glucose plus ketone monitor system, with use hydroxybutyrate dehydrogenase ketone strips (LS-946; Borkstraβe, Münster, Germany). Whole blood collected D137, D141 and D144 was analysed for blood gas concentrations immediately following collection using an i-Stat Alinity point-of-care analyser and

CG8+ cartridges to measure blood pH, partial pressure of carbon dioxide (pCO_2), partial pressure of oxygen (pO_2), bicarbonate (HCO_3), base excess (BE), saturated oxygen (SO_2), total carbon dioxide (TCO_2), sodium (Na), potassium (K), calcium (Ca), i-Stat glucose (iglucose; mmol/L), i-Stat haematocrit (iHct; L/L) and haemoglobin (Hgb; Abbott Point of Care, i-Stat Alinity, Abbott Park, Illinois, USA).

3.1.3 Statistical analysis

Ewe data was analysed using a Linear Mixed model in SPSS (IBM SPSS, version 27, Armonk, NY, USA). The final model included treatment (AA+SC vs AA vs SC vs control) and day (baseline, D2, D6, and D10) and the interaction between treatment and day as fixed factors. Ewe ID was fit as a random effect to account for both within and across animal variability. The interaction between treatment and day has not been reported as it was not statistically significant for any variable.

3.2 Experiment 1

This trial was approved by The University of Adelaide Animal Ethics Committee (approval number S-2020-005) and conducted in accordance with the Australian code of Practice for the Care and Use of Animals for Scientific Purposes (2013).

3.2.1 Animals and experimental design

Pre-breeding, 280 multiparous Merino ewes housed under commercial grazing conditions, were divided into two groups, and mated 30 days apart. All ewes were synchronised using progesterone-controlled internal drug release devices (CIDR) for 14 days. During this period each ewe was supplemented with 0.6kg of lupins to maintain the daily protein intake of 16% in the period immediately prior to mating. Immediately after the removal of the CIDRs, ewes were naturally mated over 21 days. Over the mating period rams were fit with crayon harnesses and daily conception was recorded, with a ram to ewe ratio of 1:10. At day 60 of gestation, litter size and stage of gestation (mated in the early or late in the joining period) were determined via transabdominal ultrasonography. Prior to individual housing and commencement of treatment, ewes were acclimatised to grain for 10 days with 0.44kg of barley, per ewe, per day.

Thirty-six pregnant Merino ewes were placed in individual pens and allocated to one of six treatment groups: 1) Blend (B; n=6 twin-bearing ewes), 2) B + sildenafil citrate (BSC; n=6 twin-bearing ewes), 3) Glutamine (Gln; n=6 twin-bearing ewes), 4) Gln + sildenafil citrate (GlnSC; n=6 twin-bearing ewes), 5) twin control (T; n=6 twin-bearing ewes) and 6) single control (S; n=6 single-bearing ewes). Ewes were split into two replicates based on gestation age at the time of entering the trial (day 80 and 110 of gestation) and stratified within and across each treatment group based on age (mean $\pm$ SD; 4.5 years $\pm$ 0.8), liveweight (72.9 $\pm$ 7.1) and body condition score (3.7 $\pm$ 0.4) measured at day 60 of gestation.
Ewe diets were formulated to meet 100% NRC requirements for either twin or single bearing ewes (Table 1; National Research Council, 2007). All ewes had free access to clean water throughout the trial. Ewes were transitioned onto the experimental diet over 15 days. The final diet that was supplied until parturition consisted of 0.59 kg of concentrate and 0.65 kg of roughage for twin bearing ewes and 0.51 kg of concentrate and 0.45 kg of roughage for single bearing ewes, as fed. Diets were fed over two feeding periods (50% at 1000h and the remaining 50% at 1600h).

Table 2.1. Composition of concentrate (on a DM basis).

Item	%
Oats	39.05
Barley	38.92
Lupins	17.35
Buffer	1.50
Vegetable oil	1.08
Urea	0.64
Calciprill, 36% Ca	0.55
Salt	0.22
Mineral & vitamin mix plus copper	0.22
Vitamin E, 5%	0.43
Bovatec, 20%	0.04
Metabolisable energy, MJ/kg feed	12.4
Crude protein, %	15.20
NDF, %	19.76
Fat, %	4.25

Treatments were administered daily for either 40- or 70-days during gestation, until parturition. Treatments provided to Gln ewes contained 5 g of L-Glutamine (Amtrade International) and blend ewes contained 2.53 g of L-Arginine (Amtrade International); 2.34 g of L-Histidine Monohydrochloride Monohydrate (Ajinomoto Co., Inc); 1.01 g of L-Leucine (Ajinomoto Co., Inc); 2.94 g of L-Glutamine (Amtrade International) and 1.18 g of L-Glutamic Acid (Shijiazhuang Shixing Amino Acid Co., Ltd). As part of the amino acid treatment, 10g of zein was solubilised in 85% ethanol solution and used to protect the amino acids from microbial degradation. Zein was also used as a source of amino acids as ruminal bacteria have inadequate ability to degrade zein. Zein is particularly rich in glutamic acid/glutamine, leucine, proline and alanine, and also contains other amino acids including arginine and histidine (Gianazza et al., 1977; Geraghty et al. 1981). Treatments were coated with 20g of melted coconut oil and stored at -20°C until required to prevent lipid degradation and the development of oxidative rancidity products. During the trial, treatments were thawed prior to inclusion into daily total feed ration.

Ewes received either a bolus of 75 mg of sildenafil citrate (SC) in 3 mL of sterile water per day or the same volume of sterile water via subcutaneous injection, twice daily (1000h and 1600h) for 30 days. The sildenafil citrate dose rate used was based on the previous study by Scatterfield (2010). Injections were rotated over 9 positions per administration sites, which were alternated between side (left or right) of the animal and location (2 sites per side; total of 4 sites; total of 36 injection points).

3.2.2 Data collection

3.2.2.1 Ewe measurements

Blood samples of pregnant ewes (n=35) were collected on day one of treatment, day 30 of treatment, and 72 h post parturition. Blood samples, collected in lithium heparin tubes, were centrifuged (3000 rpm / 10min) and stored at -80°C for plasma amino acid analysis. Blood gases (n=25) were measured at day 30 of treatment using an i-Stat Alinity point-of-care analyser and CG8+ cartridges (Abbott Point of Care, i-Stat Alinity, Abbott Park, Illinois, USA) to measure blood pH, partial pressure of carbon dioxide (pCO_2), partial pressure of oxygen (pO_2), bicarbonate (HCO_3), base excess (BE), saturated

oxygen (SO_2), total carbon dioxide (TCO_2), sodium (Na), potassium (K), calcium (Ca), i-Stat glucose (iglucose; mmol/L), i-Stat haematocrit (iHct; L/L) and haemoglobin (Hgb). From this test blood pH, pCO_2, pO_2, HCO_3, SO_2, TCO_3, Hct and Hgb were reported as related to the physiological mechanisms of targeted results. Liveweight (kg) and BCS were measured before commencement of treatment and at 72 h post parturition. In addition, glucose (mmol/L), Hct (L/L) and ketones (mmol/L) were also measures at 72 h post parturition.

3.2.2.2 Lamb measurements

At birth, within 30 minutes and prior to colostrum ingestion, liveweight (kg), rectal temperature (°C), blood gases, blood glucose (mmol/L), blood ketones (mmol/L), total serum protein (g/dL) and blood samples were collected (n=55). Blood samples collected at birth, in lithium heparin tubes, were centrifuged (3000 rpm / 10min) and plasma aliquots were stored at -80°C prior amino acid concentration and blood hormone analysis. Liveweight, rectal temperature, blood gases, blood glucose, blood ketones and total serum protein were measured at 72 h post birth. After 72 h ewes and lambs were managed under a commercial system, offered oaten hay and a hay-based pellet (JT Johnston and Sons, ewe and lamb pellet; Table 2). Lambs were weighed monthly up until weaning at 16 weeks of age.

Table 2.2. Dry matter and nutrient composition of oaten hay and ewe and lamb pellets offered from 72 h until weaning.

Nutrient	Composition, %	
	Hay	Pellet*
Dry Matter	90.50	90.60
Crude Protein	13.00	8.70
ADF	17.70	36.00
NDF	36.10	55.20
Lignin	4.78	5.18
Starch	33.00	3.30
Crude Fat	2.26	1.60
Ash	6.56	5.00
Calcium	0.38	0.31
Phosphorus	0.73	0.22
Magnesium	0.32	0.16
Potassium	1.53	1.00
Sulphur	0.25	0.19
ME (MJ/kg)	10.84	9.58

* Ewe and Lamb Pellets *(*JT Johnston and Sons, Kapunda, South Australia, Australia)

3.2.3 Amino acid and hormone analysis

Plasma samples taken from ewes after 30 days of treatment (n=35) and from lambs at birth (n=52) were analysed for amino acid concentrations using a method adapted from Agilent Technologies' AdvanceBio Amino Acid Analysis system authored by Lucas Willmann, Agilent Technologies; 'Automation of Sample Derivatization Using the Agilent 1260 Infinity II Prime LC System for Amino Acid Analysis'. The Agilent 1260 liquid chromatography system (Agilent Technologies, Santa Clara, CA,

USA) consisted of a quaternary pump (G1311B), HiP sampler (G1367E), a temperature-controlled column compartment (G1316A) and a UV-VIS diode array detector (G4212B). Calibration was performed using amino acid standards (Sigma-Aldrich, St. Louis, MO, USA) using 17 hydrolysate amino acids in 0.1M hydrochloric acid at 2.5mM, and supplemented with tryptophan, asparagine and glutamine (Agilent Technologies, Santa Clara, CA, USA). Samples were calibrated on four levels, 1600, 400, 100 and 15μM and internal standards norvaline and sarcosine at 500μM. Instrument control and peak integration was done with Agilent chemstation software (Santa Clara, CA, USA). Amino acids glutamic acid, asparagine, serine, glutamine, histidine, threonine, arginine, alanine, tyrosine, valine, isoleucine, leucine and lysine were analysed for total concentrations. However, proline, cystine, glycine, methionine, phenylalanine, tryptophan and aspartic acid could not be reported due to analytic imprecision.

Lamb plasma insulin and IGF-I concentrations (n=55) were measured at the Adelaide Research Assay Facility, Adelaide, South Australia, Australia. IGF-I (EIA-4140R, DRG Instruments, Marburg, DE, Germany) was measured by Enzyme Linked Immunosorbent Assay (ELISA), as per manufacturers' protocol. Insulin (Hi-14K, Millipore, Billerica, USA) was measured by radioimmunoassay (RIA) as per manufacturers' protocol.

3.2.4 Statistical analysis

3.2.4.1 Ewe analysis

Ewe data were analysed using the MIXED procedure of SAS (ver. 9.4; SAS Inst. Inc., Cary, NC). Treatment (B, BSC, G, GSC, T and S) and the days treated (40 days or 70days) were fit as fixed effects. Data are presented as least-squares means with SEM. To test the hypotheses, $P<0.05$ was considered significant.

3.2.4.2 Lamb analysis

Lamb data were also analysed using the MIXED procedure of SAS (ver. 9.4; SAS Inst. Inc., Cary, NC). Treatment (B, BSC, G, GSC, T and S), the days ewes were treated (40 days or 70 days), time point (birth, 72 h post birth and/or weaning, n=up to 2) and sex (male or female) were fitted as fixed effects. Ewe ID was included as random effects. Birth weight was fitted as a covariate. All, two-way interactions were tested for the fixed effects. Glucose, ketones, rectal temperature, and total serum protein were analysed as repeated measures for the time points of birth and 72 h post birth. Body weight was analysed for the time points of birth, 72 h post birth and weaning. Results are presented as least-squares means with SEM. To test the hypotheses, $P<0.05$ was considered significant.

3.3 Estimating organ size and body composition

This trial and associated procedures was approved by The University of Adelaide Animal Ethics Committee (approval number S-2020-005) and conducted in accordance with the Australian code of Practice for the Care and Use of Animals for Scientific Purposes (2013).

3.3.1 Animals and computer tomography (CT) scanning procedure

Ten naturally deceased neonatal lambs, from either Merino or Border-Leicester x Merino dams, were scavenged from The University of Adelaide research farm, Roseworthy, South Australia. The lambs were weighed, then stored in a cool room at –4^{o}C until they could be CT scanned; for 26.40 ± 52.41 hours on average (± s.d.).

Whole body CT scans of the lambs were obtained using a 16-slice scanner (Alexion, Toshiba Medical Systems Co Ltd., Otowara, Japan), at the Equine Health and Performance Centre (The University of Adelaide, School of Animal and Veterinary Sciences, Roseworthy, South Australia). The X-ray tube operated at a voltage of 100 kV and a current of 100–120 mA. The lambs were placed on their sternum with their fore and hind limbs extended, supported by foam pads and straps. Cross-sectional slices of 0.5 mm thickness were taken at 0.4 mm slice intervals, resulting in an average (± s.d.) of 2047 ± 179 images per lamb. Following CT scanning, the lambs were stored in a cool room at –4^{o}C until the *post-mortem* could be performed; for 40.80 ± 30.04 hours on average (± s.d.).

3.3.2 *Post mortem* measurements

Deceased lambs were placed on their backs with fore and hind limbs spread laterally to stabilise the body for internal examination. To expose the abdominal and thoracic cavities, secateurs were used to make an incision approximately 3 cm caudal to the navel, before proceeding to cut upwards towards the neck, through skin, muscle, peritoneum, and the sternum. Care was taken to angle the secateurs upwards while cutting, to avoid injuring internal viscera. Then, the left and right kidneys, spleen, and liver were dissected away from surrounding tissues. These organs were weighed using gravimetric scales, then their volumes were measured by water displacement in glass volumetric cylinders.

3.3.3 Organ volumes, weights and body composition

For organ volumes, the CT scans were imported into 3D Slicer software (version 5.0.3, https://www.slicer.org/, accessed March 2022; (Fedorov et al., 2012) as Digital Imaging and Communications in Medicine (DICOM) files. The method used to estimate organ volumes is described, in full, in the supplementary material. Briefly, the locations of the left and right kidneys, spleen, and liver were firstly identified using reference CT images of sheep (Davies et al., 1987). Each organ was reconstructed using a semiautomatic segmentation tool in 3D Slicer, which applied mathematical computerised models to define the organ boundaries, based on differences in pixel grey values (i.e., Hounsfield units). The volumes of the resulting reconstructed organs were automatically calculated using the *Quantitative Statistics* module in 3D Slicer. This analysis was completed twice for each lamb, by a single operator.

For organ weights, the reconstructed organs were isolated in 3D Slicer using the *Masking* effect, then saved as DICOM files which were imported into ImageJ (version 1.53, https://imagej.nih.gov/ij/, accessed August 2022). The average HU of each organ was determined from a histogram of the pixel distribution. Tissue density was calculated (Eq 1; (Fullerton, 1980), then multiplied by the previously determined organ volume to give the CT estimated organ weight (Eq. 2; (Roberts et al., 1993).

$$\text{Tissue density (g/mm}^3\text{)} = (\text{HU} \times 0.00106 + 1.0062)/1000. \quad (1)$$

$$\text{Tissue weight (g)} = \text{total tissue volume (mm}^3\text{)} \times \text{tissue density (g/mm}^3\text{)}. \quad (2)$$

The proportional weight of each organ was calculated in relation to bodyweight (Burrin et al., 1990; Arce-Recinos et al., 2022). The CT estimated bodyweight was used for CT estimated organ weights, and measured bodyweight used for measured organ weights. To estimate body composition, the CT scans were edited in 3D Slicer to remove the CT table from the images, then saved as new DICOM files. The *Threshold* effect in 3D Slicer was used to define HU ranges of – 65 to 0 for fat, 0 to 100 for muscle, and 100 to 2000 for bone. The edited CT scans were then imported into ImageJ to generate a histogram of the number of pixels distributed within the HU range of – 65 to 2000, including all of the images for each lamb. The raw histogram data was then transferred to Excel to calculate body composition.

To estimate the fat, muscle, and bone weights (kg) of each lamb, calculations similar to those used by (Geraldo et al., 2020) were applied. Firstly, the total area of each tissue was calculated from the number of pixels within each range, multiplied by the area of one pixel (0.22 mm^2) (Eq. 3). Total tissue area was then multiplied by the slice interval (0.40 mm) to give the total tissue volume (Eq. 4; (Gundersen et al., 1988).

Total tissue area (mm^2) = total pixel count × area of one pixel (mm^2). (3)
Total tissue volume (mm^3) = total tissue area (mm^2) × slice interval (mm). (4)

Tissue densities of fat, muscle, and bone were estimated using the average HU within their respective ranges (Eq. 1; (Fullerton, 1980). Then, tissue weights were calculated by multiplying volume and density (Eq. 2; (Roberts et al., 1993). The CT estimated fat, muscle, and bone weights were then combined to represent CT estimated bodyweight for comparison with measured bodyweight.

3.3.4 Statistical analysis

The organ and body composition data were analysed for descriptive statistics, and normality using normal quantile-quantile plots, in GraphPad Prism software (version 9.4.1 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com). The methods used to determine organ volume, organ weight, and body composition by CT scan analysis were compared to the associated standard methods of water displacement, weighing and body weight. Correlation was used to measure the linear association between the results obtained from the two methods being compared. Spearman's rank correlation (r_s) was used instead of Pearson correlation (r) when normality could not be assumed. Correlations from 0 to 0.3 were considered to be weak, 0.4 to 0.7 were considered moderate, and 0.8 to 1.0 were considered strong. Correlations were considered significant when $P < 0.05$, obtained from a two-tailed test (Giavarina, 2015). Also, the coefficient of determination (r^2) was used to describe how much of the variability in CT estimations of organ volume, organ weight, and bodyweight could be explained by the associated standard measurements.
Bias was calculated as a measure of the agreement between the two methods, along with precision and limits of agreement for the body composition data, following the Bland-Altman procedure (Giavarina, 2015).

3.4 Experiment 1 – CT scanning

3.4.1 Animals and treatments

A subset of progeny from experiment one (n = 34; initial liveweight = 31.1 ± 1.6kg, mean ± s.e.m.; 5 months of age) were scanned via computed tomography (CT) to investigate the effect of maternal amino acid supplementation on twin lamb growth and composition. Treatments included 1) AA blend (Blend), 2) Blend + sildenafil citrate (B + SC), 3) Glutamine (Gln), 4) Glutamine + sildenafil citrate (G + SC) and 5) twin control (twins). Single-bearing ewes were included in the trial as a positive control. The blend treatment contained 2.53 g of arginine, 2.34 g of histidine, 1.01 g of leucine, 1.18 g of glutamic acid, 2.94 g of glutamine and 10 g of zein. Lambs were then managed under commercial grazing systems until CT scanning.

3.4.2 Computer tomography scanning

Lambs were prepared for CT scanning at the Equine Health and Performance Centre (The University of Adelaide, School of Animal and Veterinary Sciences, Roseworthy, South Australia). Body weight (kg), heart rate (bpm), and respiration rate (bpm) were measured prior to scanning for monitoring

purposes. Sedation and general anaesthesia were used to keep the lambs still and prevent respiratory movement during the scan. Midazolam was administered for sedation (0.2 – 0.5 mg/kg of live body weight, intravenously or intramuscularly), alfaxalone to induce anaesthesia (2 – 5 mg/kg), and isoflurane to maintain anaesthesia.

The lambs were positioned on their sternum, supported with bolsters, and stabilised using wide Velcro straps before being CT scanned (16-slice, Alexion, Toshiba Medical Systems Co Ltd., Otowara, Japan; operating at 100 kV and 100-120 mA). The scanned region included all internal organs, excluding the head and the hooves. Cross-sectional slices of either 0.5 mm or 1.0 mm thickness were taken at respective slice intervals of 0.4 mm and 0.8 mm, resulting in an average (± s.d.) of 1,357 ± 433 slices per lamb.

Plain CT scans were performed before and after Omnipaque®, an intravenous contrast agent, was administered. Contrast was administered at 2 mL per kg through a previously placed cephalic vein catheter. Both scans were completed within five minutes. Lambs were then monitored for two hours during recovery post-scanning and then returned to normal husbandry conditions.
For organ volumes and body composition, both methodologies remain the same as previously presented under item 3.3. The analysis was both blinded and randomised in terms of the treatment group, treatment length, sex, and weight of each lamb.

3.4.3 Statistical analysis

The organ and body composition data were analysed using the MIXED procedure of SAS (ver. 9.4; SAS Inst. Inc., Cary, NC). Treatment (B, BSC, G, GSC, T and S), the days ewes were treated (40 days or 70 days) and sex (male or female) were fitted as fixed effects. Ewe ID was included as random effects. Birth weight was fitted as a covariate. Results are presented as least-squares means with SEM. To test the hypotheses, $P<0.05$ was considered significant.

3.5 Substances to rumen protect glutamine

The rumen-protected glutamine substances, natural substance 1 (NS1) and natural substance 2 (NS2), were prepared under proprietary protocols. The protocols considered labour intensity and preparation time in manufacturing the rumen-protected glutamine to obtain a feasible and cost-effective process in a small-scale setting.

3.5.1 *In vitro* analysis

The rumen fluid was collected from an abattoir (TPL Meat Exports Pty Ltd) and transferred into a pre-warmed thermos. The *in vitro* methodology was adapted from Goering and Van Soest (1970) and Suh et al. (2022) with minor adjustments. The incubated bottles contained a negative control (blank), positive controls (glutamine only, NS1 only, and NS2 only), and the food-grade substance-encapsulated glutamine; all replicated in triplicate. The *in vitro* trial was conducted over a total of 480 minutes and sampling was conducted at 10 time points: 30, 60, 90, 120, 180, 240, 300, 360, 420 and 480 minutes in duplicate.

3.5.2 Ammonium analysis

Nitrogen levels from the *in vitro* samples were indirectly measured using a colorimetric assay for ammonium determination. For this, 1 mL of each sample was added to microtubes containing 20 µL of 6 N HCl solution and then stored at –20°C prior to ammonium determination. Once defrosted, sample tubes are centrifuged at 1,107 g force or 5 minutes, and an ammonium standard solution

(1,000 mg/L ammonium) was used to develop a standard curve. The ammoniacal nitrogen was determined by reacting the sample with sodium-salicylate and sodium nitroprusside. Then samples were exposed to a dichloroisocyanurate solution and the colorimetric detection was measured at 650nm by using the Multiskan plate reader (Bio-Rad Laboratories Inc., xMark™ Microplate Absorbance Spectrophotometer; Hercules, California, USA), 1 hour after all reagents were added (ammonium determination 11732; International Standards ISO 1997).

3.5.3 Statistical analysis

Descriptive statistics and the area under the curve for each of glutamine-containing combination were calculated in SAS 9.4 using the PROC MEANS function.

4. Project outcomes

4.1 Safety trial

4.1.1 Results

Treatment had no effect on the average rectal temperature, heart rate or respiratory rate measured over the trial period (Table 1.3). While heart rate and respiratory rate remained unaffected by sampling day, rectal temperature fluctuated lower at baseline and sampling on D6, and slightly higher, by less than 1°C, on D2 and D10.

Treatment had a significant effect on PO_2, where the control group had 1.09 to 5.26 mmHg lower than other treatment groups (Table 1.4). The AA+SC had the highest average PO_2 concentration, followed by the AA and SC groups. A similar trend was seen in sO_2 concentrations where control had the lowest followed by SC, AA and then AA+SC. In regard to sampling day, PCO_2 was significantly affected, being lower at D2 and D10 compared to the baseline and D6 samples.

Blood glucose was significantly higher in the SC group compared to other average glucose concentrations, where the AA group had the lowest glucose (Table 1.5). There was a trending effect on blood potassium in which the control group had a higher average concentration of 4.10 mmol/L compared to 4.01, 3.95 and 3.86 mmol/L for SC, AA and AA+SC, respectively. Serum biochemistries, including average ketone, sodium, potassium, calcium, Hct and Hb were all significantly affected by sampling day. Ketone concentrations increased from baseline to D6 before lowering again at D10. A similar trend was seen for potassium, calcium and Hct. Sodium fluctuated between 149.06 and 150.18 mmol/L over the course of the trial period and Hb reduced from baseline to D10.

Table 1.3. Rectal temperature, heart rate and respiratory rate values (mean ± SD) and probabilities for each treatment, sampling day and treatment by sampling day interaction.

	Treatment					Day				
	AA+SC	AA	SC	Control	*P-value*	Baseline	D2	D6	D10	*P-value*
Rectal temperature (°C)	39.0 ± 0.12	38.9 ± 0.12	39.1 ± 0.12	39.0 ± 0.12	0.96	38.6 ± 0.10	39.3 ± 0.10	38.9 ± 0.10	39.3 ± 0.13	<0.001
Heart rate (bpm)	74.2 ± 4.21	74.1 ± 4.21	77.9 ± 4.21	81.5 ± 4.21	0.56	74.4 ± 3.87	82.3 ± 3.87	79.6± 3.87	71.5 ± 4.91	0.27
Respiratory rate (bpm)	53.7 ± 5.42	65.4±5.42	54.2 ± 5.42	59.5 ± 5.42	0.35	54.5 ± 3.70	59.8 ± 3.70	62.4± 3.70	56.0 ± 4.49	0.26

D2 = two days of treatment; D6 = six days of treatment; D10 = ten days of treatment
AA+SC = amino acid and blend sildenafil citrate; AA = amino acid blend and RO water; SC = sildenafil citrate and saline; Control = saline and RO water

Table 1.4. Blood gas values (mean ± SD) and probabilities including pH, partial pressure of carbon dioxide (PCO_2), partial pressure of oxygen (PO_2), bicarbonate (HCO_3), base excess (BE), sulphur dioxide (sO_2) and total carbon dioxide (TCO_2) for each treatment and sample.

	Treatment					Day				
	AA+SC	AA	SC	Control	*P-value*	Baseline	2	6	10	*P-value*
pH	7.46 ± 0.01	7.48 ± 0.01	7.48 ± 0.01	7.48 ± 0.01	0.34	7.47 ± 0.01	7.48 ± 0.01	7.47 ± 0.007	7.48 ± 0.01	0.25
PCO_2 (mmHg)	34.77 ± 0.89	33.97 ± 0.88	35.68 ± 0.89	36.17 ± 0.89	0.41	35.99 ± 0.51	34.68 ± 0.55	35.16 ± 0.53	34.77 ± 0.57	0.02
PO_2 (mmHg)	46.50 ± 1.36	45.82 ± 1.42	42.33 ± 1.41	41.24 ± 1.41	0.04	43.44 ± 0.95	43.77 ± 1.07	44.41 ± 1.02	44.27 ± 1.55	0.84
HCO_3 (mmol/L)	24.71 ± 0.86	26.35 ± 0.88	27.07 ± 0.88	26.98 ± 0.88	0.28	26.45 ± 0.52	26.33 ± 0.56	25.89 ± 0.54	26.45 ± 0.59	0.63
BE, ecf (mmol/L)	0.96 ± 0.95	2.98 ± 0.97	3.63 ± 0.97	3.51 ± 0.97	0.25	2.84 ±0.58	2.96 ± 0.65	2.24 ± 0.62	3.04 ± 0.68	0.63
sO_2 (%)	84.49 ± 1.48	84.34 ± 1.55	81.43 ± 1.54	79.24 ± 1.54	0.07	81.76 ± 1.06	82.48 ± 1.19	82.66 ± 1.14	82.58 ± 1.28	0.88
TCO_2 (mmol/L)	25.73 ± 0.87	27.33 ± 0.89	28.15 ± 0.89	28.15 ± 0.89	0.26	27.55 ± 0.53	27.30 ± 0.57	26.99 ± 0.55	27.54 ± 0.60	0.61

D2 = two days of treatment; D6 = six days of treatment; D10 = ten days of treatment
AA+SC = amino acid and blend sildenafil citrate; AA = amino acid blend and RO water; SC = sildenafil citrate and saline; Control = saline and RO water

Table 1.5. Serum biochemical values (mean ± SD) and probabilities including blood ketone, sodium, potassium, calcium, glucose, haematocrit (Hct) and haemoglobin (Hb) for each treatment and day.

	Treatment					Day				
	AA+SC	AA	SC	Control	*P-value*	Baseline	2	6	10	*P-value*
Ketone (mmol/L)	1.27 ± 0.10	1.17 ± 0.11	0.94 ± 0.11	1.20 ± 0.10	0.16	1.07 ± 0.12	1.36 ± 0.13	1.50 ± 0.13	1.00 ± 0.15	0.02
Sodium (mmol/L)	149.79 ± 0.32	149.58 ± 0.33	149.33 ± 0.24	149.36 ± 0.33	0.62	149.06 ± 0.22	150.16 ± 0.25	149.33 ± 0.24	150.18 ± 0.26	<0.001
Potassium (mmol/L)	3.86 ± 0.06	3.95 ± 0.06	4.01 ± 0.06	4.10 ± 0.06	0.07	3.87 ± 0.05	3.95 ± 0.05	4.06 ± 0.05	4.05 ± 0.05	0.02
Calcium (mmol/L)	1.22 ± 0.02	1.19 ± 0.02	1.24 ± 0.02	1.18 ± 0.02	0.12	1.16 ± 0.02	1.21 ± 0.02	1.24 ± 0.02	1.22 ± 0.02	0.01
Glucose (mmol/L)	3.06 ± 0.08	2.76 ± 0.09	3.32 ± 0.08	3.21 ± 0.08	<0.001	3.20 ± 0.09	2.96 ± 0.11	2.95 ± 0.10	3.12 ± 0.12	0.22
Hct (L/L)	0.29 ± 0.01	0.29 ± 0.01	0.28 ± 0.01	0.28 ± 0.01	0.54	0.31 ± 0.01	0.28 ± 0.01	0.28 ± 0.01	0.27 ± 0.01	<0.001
Hbˆ (g/L)	100.95 ± 3.14	98.93 ± 3.18	94.36 ± 3.19	96.32 ± 3.19	0.50	104.25 ± 1.80	95.33 ± 1.93	96.75 ± 1.87	94.25 ± 2.01	<0.001

via Hct

D2 = two days of treatment; D6 = six days of treatment; D10 = ten days of treatment

AA+SC = amino acid and blend sildenafil citrate; AA = amino acid blend and RO water; SC = sildenafil citrate and saline; Control = saline and RO water

4.1.2 Discussion

The objective of this study was to determine the effect of supplementation of 150mg/day of sildenafil citrate with 1.2g/day of arginine, in a blend, on blood gases in multiparous ewes. Overall, the findings of the study indicated that sildenafil citrate can be safely used in a blend with arginine, causing no adverse effects in physiological measures, blood gases or serum biochemical variables, although there were significant differences between treatments for the variables measured.

Sildenafil citrate enhances NO mediated vasodilation by inhibiting cGMP breakdown (Dishy *et al.* 2001). This messenger molecule is responsible for the regulation of downstream effects on vasodilation, as well as retinal phototransduction, calcium homeostasis and neurotransmission (Zbrojkiewicz and Śliwiński 2016). In both animal (Zoma *et al.* 2004) and human (Wareing *et al.* 2005; Panda *et al.* 2014) studies, SC has been shown to improve uterine blood flow in pregnancies compromised by IUGR. Arginine, however, acts to increase NO circulation through a direct role as an essential substrate (Wu *et al.* 2021). As it is hypothesised that the increased demand and reduced supply of nutrients, including the specific amino acids supplemented in this study, cause twin lambs to be born physiologically immature compared to singles, a IUGR model can be followed, and the supply of NO may have a beneficial effect. The effects described, as a result of this study, are assumed to be as a combination of both SC and arginine as the direct influence from each compound has not been determined.

Treatment had no statistical effect on physiological measurements, although temperate was statistically affected by the sample (Table 3). Average temperature across all treatments was slightly higher at D2 and D10 of sampling compared to the baseline and D6 samples. While this was a statistical difference, the range of 38.6–39.3°C from the current study is within the normal range for core body temperature (38.5-39.5°C) in sheep (Kearton *et al.* 2020). Previous research has noted SC to increase the maternal heart rate as a reaction to systematic vasodilation (Miller *et al.* 2009), which would be expected in this study, yet was not observed. Average heart rates for each treatment group were within the reference range of 65-80 bpm (Reece 2004), except for day two where rates were slightly elevated. This is not considered concerning as heart rates can increase by 50% during handling, without the incorporation of treatment (Scott 2015).

Respiratory rate was much higher in the current study than in the noted reference range of 16-34 bpm (Reece *et al.* 2004), although control respiratory rates of 50 bpm have been recorded in Merinos (Srikandakumar *et al.* 2003). Studies in humans have observed insignificant changes in respiratory rate after taking SC (Zusman *et al.* 2000; Snyder *et al.* 2008). In the current study, while high, average respiratory rate did not statistically differ between treatment groups or over time. High rates may be due to high stress during handling as an addition to being housed indoors and individually, despite acclimatisation and regular handling. The PO_2 describes the amount of oxygen that is in the blood at the time of collection, and reflects the respiratory effectiveness of the animal to breath in oxygen from the surrounding atmosphere and into the blood (Reece and Rowe 2017). For this trial, it was investigated as an indicator of respiratory distress or respiratory acid-base unbalances due to SC and arginine combined. The SC + AA group containing both arginine and SC had the highest average PO_2 value, potentially indicating an increased response due to the increased production of NO (Table 4). This effect was predicted due to the vasodilation functions of NO (Kourembanas *et al.* 1993). The control and SC group had lower PO_2, but all average PO_2 values from each treatment group correspond with other levels reported in conscious, untreated sheep (Sobiech *et al.* 2005; Onmaz *et al.* 2009).
Blood glucose concentrations were significantly lower in those ewes supplemented with amino acids (both AA and AA+SC; Table 5). This decrease in glucose may have occurred through the regulation of serum glucose concentrations by glucokinase, triggering a rise in insulin simultaneously as a product of amino acid metabolism, and therefore upregulation of glucose depositing in the liver as glycogen

(Norton *et al.* 2022). Previous studies in humans have varying results when investigating the impact of single amino acids on blood glucose. Research supplementing arginine resulted in a sustained serum insulin, yet blood glucose concentration did not significantly change (Dupré *et al.* 1968). A similar result was found when orally supplementing glutamic acid (Fernstrom *et al.* 1996) and glutamine (Greenfield *et al.* 2009). Oral administration of leucine, however, significantly increased serum insulin and simultaneously decreased glucose concentrations in 75% of experiments (Fajans *et al.* 1962). Timing of sampling may have coincided with the regulation of high blood glucose from amino acid metabolism, by insulin and the liver. It should be noted that while treatment was found to have a statistically significant effect on glucose, levels were within reported ranges of 2.4 - 4.5 mmol/L (Gardner *et al.* 2003).

Blood calcium concentrations were significantly low in all treatment groups (Table 5). Normal reported calcium levels are 2.2-2.7 mmol/L (Pardi *et al.* 2004). As the demand for calcium is considerably high during twin pregnancies compared to singles, particularly during late gestation, it is expected that calcium levels may be low. Low levels may also be attributed to the supply of NO as without the inhibition of cGMP, the activation of cGMP-dependant protein kinase (PKG) leads to a release of intracellular calcium (Wang *et al.* 2005). In hypoxic rats, SC administration decreases resting calcium concentrations (Pauvert *et al.* 2004), but reports of low calcium following SC or NO supply has not occurred in other species. Ketone levels were measured as an indicator of pregnancy toxaemia during late pregnancy and although were not statistically significant between treatment groups, levels indicate subclinical ketosis (Marutsova and Marutsov 2017). Pregnancy toxaemia is often noticeable through high ketone and low glucose concentrations. Although glucose levels were not outside the lower limits of reference, levels were not particularly high. Pregnancy toxaemia is common in multiple bearing ewes in late gestation when glucose demands are high (Schlumbohm and Harmeyer 2008). The pregnancy status and twice daily feeds, rather than *ad libitum* grazing, may have contributed to the subclinical ketosis ketone levels observed.

Although statistically effected by either treatment or sampling day, variables such as pH, PCO_2, HCO_3, BE, SO_2, TCO_2, sodium, potassium, Hct, and Hb were observed within reported references ranges and therefore are not considered to be of biological significance. No adverse effects on the pregnant ewe were recorded following administration of SC in combination with arginine in a blend of amino acids, but following parturition, measurements were not taken on the lamb. There seems to be confliction regarding the safety or benefit of SC on the progeny of maternally supplemented animals. While several studies have found beneficial outcomes for fetuses following SC administration, detrimental effects on uteroplacental perfusion has been recorded in a study where fetal growth restriction was induced in fetal lambs (Miller *et al.* 2009).

4.2 Experiment 1

4.2.1 Results

4.2.1.1 Ewe measurements

The supplementation of amino acids during parturition had no effect on amino acid concentrations (Table 2.3), blood gas concentrations (Table 2.4), liveweight, BCS or blood biochemistry. Blood gases and biochemistry lie within range of healthy animals, as referenced in previous research.

Table 2.3. Ewe plasma amino acid concentrations after 30 days of supplementation.

	Treatment							
Amino acid	B	BSC	G	GSC	T	S	SEM	P-value
Glutamic Acid	129.98	91.94	93.16	94.46	114.81	94.89	15.65	0.22
Asparagine	27.38	24.02	27.63	26.10	25.36	28.40	1.63	0.88
Serine	80.85	87.99	83.94	89.69	73.17	101.73	9.59	0.34
Glutamine	351.97	330.62	368.88	333.89	360.37	337.56	15.56	0.88
Histidine	39.34	41.71	37.93	41.32	33.63	48.34	4.87	0.32
Threonine	47.79	30.97	39.18	49.05	34.03	52.59	8.81	0.18
Arginine	95.10	79.97	95.17	90.77	92.18	105.97	8.36	0.65
Alanine	201.89	145.10	169.88	162.15	165.40	182.76	19.36	0.56
Tyrosine	46.33	37.77	43.87	44.74	41.61	44.23	3.02	0.73
Valine	99.35	73.71	78.30	95.83	85.75	103.13	11.93	0.36
Isoleucine	57.65	49.00	47.73	51.13	53.81	54.15	3.67	0.74
Leucine	82.84	63.53	65.53	72.35	63.75	67.85	7.39	0.83
Lysine	102.08	100.35	99.63	99.53	102.09	109.16	5.67	0.92

B = Blend of rumen-protected amino acids (2.53g of arginine, 2.34g of histidine, 1.01g of leucine, 1.18g of glutamic acid and 2.94g of glutamine); BSC = Blend of rumen-protected amino acids (2.53g of arginine, 2.34g of histidine, 1.01g of leucine, 1.18g of glutamic acid and 2.94g of glutamine) + 150mg of sildenafil citrate; Gln = 5g of rumen-protected glutamine; GlnSC = 5g of rumen-protected glutamine + 150 mg of sildenafil citrate; T = Negative control (twin-bearing ewes); and S = Positive control (single-bearing ewes).

Table 2.4. Blood gas concentrations in pregnant ewes after 30 days supplementation.

	Treatment							
Variable	B	BSC	G	GSC	T	S	SEM	P-value
pH	7.43	7.45	7.45	7.46	7.43	7.35	0.04	0.052
PCO_2, mmHg	39.25	37.05	36.54	38.24	36.97	45.55	2.25	0.14
PO_2, mmHg	43.68	47.89	45.95	45.52	45.55	40.83	2.41	0.95
HCO_3, mmol/L	25.66	25.59	25.77	27.35	24.49	25.44	0.93	0.73
SO_2, %	74.62	81.85	75.96	81.45	80.22	69.32	4.88	0.56
TCO_2, mmol/L	26.71	26.56	27.15	28.45	25.73	26.72	0.89	0.75
Hct, L/L	0.27	0.24	0.25	0.24	0.25	0.28	0.02	0.35
Hgb, g/L	92.50	80.00	86.33	82.50	87.00	96.40	6.12	0.37

B = Blend of rumen-protected amino acids (2.53g of arginine, 2.34g of histidine, 1.01g of leucine, 1.18g of glutamic acid and 2.94g of glutamine); BSC = Blend of rumen-protected amino acids (2.53g of arginine, 2.34g of histidine, 1.01g of leucine, 1.18g of glutamic acid and 2.94g of glutamine) + 150mg of sildenafil citrate; Gln = 5g of rumen-protected glutamine; GlnSC = 5g of rumen-protected glutamine + 150 mg of sildenafil citrate; T = Negative control (twin-bearing ewes); and S = Positive control (single-bearing ewes). pCO_2 = partial pressure of carbon dioxide; pO_2 = partial pressure of oxygen; HCO3 = bicarbonate; SO_2 = saturated oxygen; TCO_2 = total carbon dioxide; Hct = haematocrit; and Hgb = haemoglobin.

Ewe weight and body condition measured 72 h post parturition were not affected by treatment. Over all 35 ewes, average liveweight was 67.56 kg ± 1.94 and BCS 2.9 ± 0.2. Blood parameters, including glucose (2.57 ± 0.18 mmol/L), HCT 23.28 ± 2.11 L/L) and ketones (0.37 ± 0.09 mmol/L) were recorded as a routine health check and did not differ between treatment groups.

4.2.1.2 Lamb measurements

Treatment affected birth (P=0.03) and weaning weight (P=0.05) statistically, with single control lambs heaviest at 5.60 kg followed by glutamine twin lambs at 5.58 kg and no statistical difference between treatment groups (P=0.46; Figure 1A).

At this timepoint single and glutamine supplemented lambs were both statistically heavier than the BSC (0.56 and 0.54 kg respectively) and the GSC (0.55 and 0.53 kg respectively). Although the mean birth weight of glutamine twin lambs was higher, it did not differ statistically from the twin control treatment group (5.58 vs. 5.39 kg; P=0.31). By weaning, liveweights of single (27.55 kg) and glutamine (25.25 kg) lambs were not statistically different (P=0.48), but glutamine lambs were 6.75 kg heavier than twin control lambs (18.50 kg; P=0.04; Figure 1B). Glutamine lambs were also statistically heavier than lambs from the BSC (17.17 kg; P=0.04) and showed a trending difference, heavier that the GSC lambs (18.69 kg; P=0.09). Average plasma amino acid concentrations from 52 lambs presented varying trends between treatment groups and were not significantly affected by maternal amino acid supplementation, similarly to that of the ewes (Table 2.5).

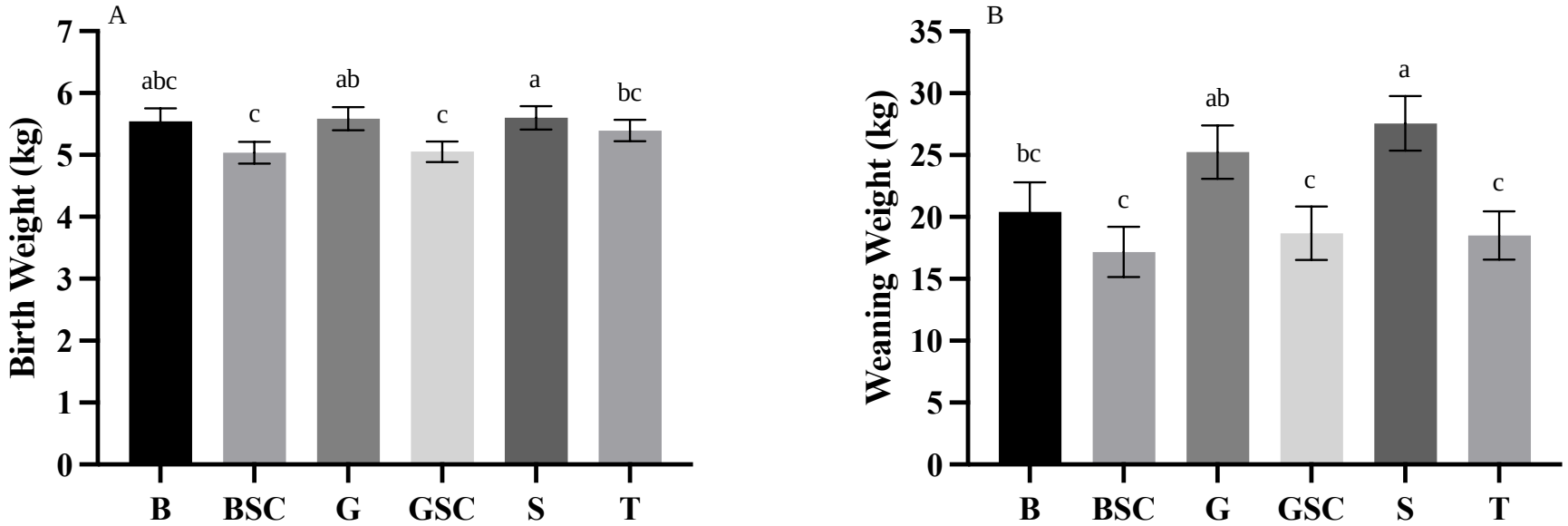

Figure 2.1. Average birth (A) and weaning weight (B; kg) for each treatment group. Error bars are presented as ± SEM. Different letters indicate statistical difference between groups. B = Blend of rumen-protected amino acids (2.53g of arginine, 2.34g of histidine, 1.01g of leucine, 1.18g of glutamic acid and 2.94g of glutamine); BSC = Blend of rumen-protected amino acids (2.53g of arginine, 2.34g of histidine, 1.01g of leucine, 1.18g of glutamic acid and 2.94g of glutamine) + 150mg of sildenafil citrate; Gln = 5g of rumen-protected glutamine; GlnSC = 5g of rumen-protected glutamine + 150 mg of sildenafil citrate; T = Negative control (twin-bearing ewes); and S = Positive control (single-bearing ewes).

Table 2.5. Plasma amino acid concentrations (mM) in newborn lambs immediately after birth.

Variable	Treatment								Duration			
	B	BSC	G	GSC	T	S	SEM	P-value	40d	70d	SEM	P-value
Glutamic Acid	124.22	124.56	142.92	111.61	106.04	110.88	24.21	0.59	130.88	109.20	9.22	0.12
Asparagine	49.12	35.68	40.37	42.22	33.77	30.09	10.63	0.61	35.82	41.26	4.55	0.42
Serine	245.68	176.12	248.33	346.70	310.89	217.89	66.16	0.37	288.84	226.36	35.06	0.23
Glutamine	591.67	424.10	456.75	501.59	429.50	382.34	107.06	0.37	416.16	512.50	39.37	0.11
Histidine	58.95	48.68	54.61	55.51	44.72	48.45	8.93	0.85	53.19	50.45	5.19	0.72
Threonine	219.38	125.45	159.83	191.36	132.97	193.80	45.73	0.47	171.16	169.78	36.18	0.97
Arginine	55.47	53.65	43.24	44.89	59.65	42.01	4.47	0.85	46.71	52.93	7.12	0.55
Alanine	609.90	438.94	457.98	542.85	397.27	350.14	167.03	0.66	425.75	506.61	135.48	0.42
Tyrosine	81.15	71.71	81.74	87.41	62.47	55.91	13.69	0.43	69.39	77.40	7.55	0.45
Valine	168.02	154.41	134.85	123.88	106.85	159.74	29.41	0.83	134.72	147.86	21.54	0.68
Isoleucine	62.45	44.59	38.58	40.65	29.88	25.98	19.14	0.35	36.51	44.21	6.64	0.43
Leucine	118.61	93.66	86.03	84.62	59.80	53.68	34.78	0.36	74.80	90.61	12.49	0.39
Lysine	95.83	60.32	51.72	57.06	36.13	32.32	36.77	0.23	46.82	64.31	13.20	0.37

B = Blend of rumen-protected amino acids (2.53g of arginine, 2.34g of histidine, 1.01g of leucine, 1.18g of glutamic acid and 2.94g of glutamine); BSC = Blend of rumen-protected amino acids (2.53g of arginine, 2.34g of histidine, 1.01g of leucine, 1.18g of glutamic acid and 2.94g of glutamine) + 150mg of sildenafil citrate; Gln = 5g of rumen-protected glutamine; GlnSC = 5g of rumen-protected glutamine + 150 mg of sildenafil citrate; T = Negative control (twin-bearing ewes); and S = Positive control (single-bearing ewes). 40d = dams supplemented for 40 days before parturition; 70d = dams supplemented for 70 days before parturition.

Rectal temperature was higher at birth at 39.16°C, compared to 39.06°C at 72 h post birth (P=0.04). However, treatment did not have a significant effect on rectal temperature at either timepoints, nor was there an effect of duration of treatment or treatment by timepoint interaction. Blood ketone levels (0.48 ± 0.08 mmol/L) were not affected by either variable or the interaction. Total serum protein was higher at birth at 3.73 g/dl, compared to 5.48 g/dl at 72 h post birth (P<0.0001). In addition to an effect on timepoint, treatment also had a significant effect (Figure 2), with blend having a higher concentration at birth (4.06 g/dl) and singles having the lowest (3.55g/dl). At 72 h post birth, singles had the highest concentration (6.09g/dl), at least 0.33g/dl higher than other treatment groups. Hormones IGF-1 (100.11 ± 29.64 ng/mL) and insulin (3.48 ± 0.04 ng/mL), from 55 lambs at birth, were unaffected by treatment or duration of treatment.

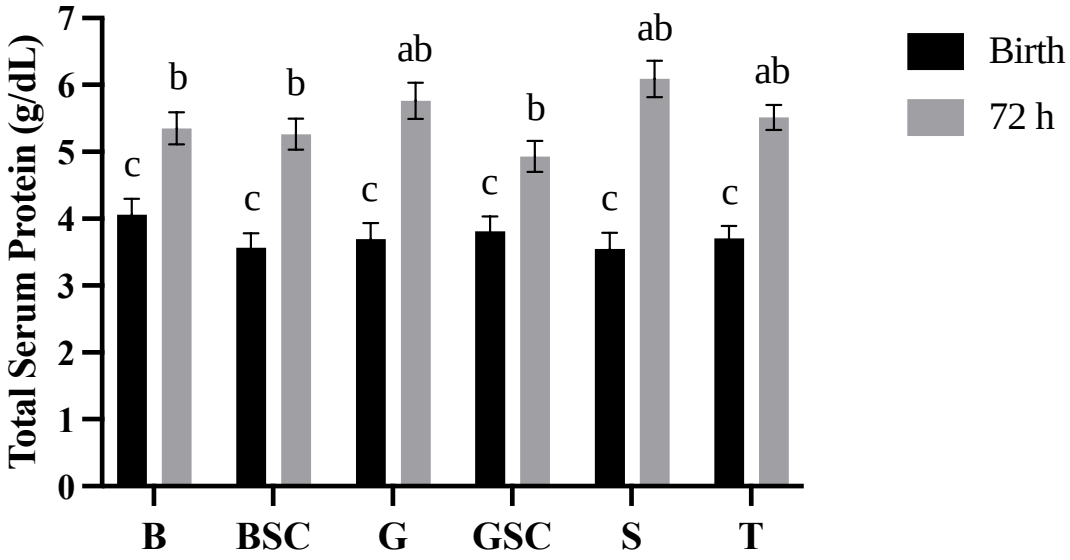

Figure 2.2. Total serum protein (g/dL) for each treatment group at birth and 72 h post birth (P=0.02). Values are presented as mean ± SEM. Different letters indicate statistical difference between groups. B = Blend of rumen-protected amino acids (2.53g of arginine, 2.34g of histidine, 1.01g of leucine, 1.18g of glutamic acid and 2.94g of glutamine); BSC = Blend of rumen-protected amino acids (2.53g of arginine, 2.34g of histidine, 1.01g of leucine, 1.18g of glutamic acid and 2.94g of glutamine) + 150mg of sildenafil citrate; Gln = 5g of rumen-protected glutamine; GlnSC = 5g of rumen-protected glutamine + 150 mg of sildenafil citrate; T = Negative control (twin-bearing ewes); and S = Positive control (single-bearing ewes).

The interaction between treatment and timepoint was not significant for pH, pO_2, SO_2 and HCT and Hgb in newborn lambs. The main effect of treatment and time point were statistically significant for blood pH and pCO_2 of lambs, while timepoint only significantly affected the averages of SO_2, HCT and Hgb between birth and 72 h post birth (Table 2.6). Hct and Hgb were both significantly affected by time point. At birth, Hct levels were significantly higher at 0.39 L/L, compared to 0.34 L/L at 72 h post birth (P<0.0001). Hgb was similar in that levels at birth (134.53 G/L) were higher that at 72 h post birth (114.75 g/L; P<0.0001) Overall, pO_2 (50.43 ± 19.55 mmHg) was not affected by either treatment or timepoint.

Table 2.6. Blood gases, including pH, pCO_2 (mmHg), HCO_3 (mmol/L), SO_2 (%), TCO_2 (mmol/L), Hct (L/L) and Hgb (g/L), measured at birth and 72 h post birth. Averages are presented for each treatment group, duration of maternal treatment, and timepoints.

Variable	Treatment								Duration				Time Point			
	B	BSC	G	GSC	T	S	SEM	P-value	40d	70d	SEM	P-value	Birth	72 h	SEM	P-value
pH	7.31	7.38	7.38	7.36	7.39	7.42	0.03	0.04	7.38	7.37	0.02	0.45	7.33	7.41	0.02	<0.001
pCO_2, mmHg	49.36	42.99	46.07	47.43	45.17	40.88	3.00	<0.001	45.36	45.27	2.95	0.98	48.25	42.38	2.16	<0.001
HCO_3, mmol/L	25.23	25.26	27.16	26.92	27.09	26.96	1.52	0.66	26.73	26.15	1.12	0.72	25.92	27.36	0.86	0.01
SO_2, %	51.61	64.64	65.79	59.00	63.92	73.01	7.81	0.31	65.11	60.88	7.92	0.71	52.51	73.48	6.02	<0.001
TCO_2, mmol/L	26.71	26.52	28.57	28.39	28.43	28.21	1.56	0.66	28.12	27.49	1.18	0.71	26.92	28.68	0.91	0.02
Hct, L/L	0.39	0.36	0.36	0.36	0.34	0.37	0.02	0.23	0.35	0.37	0.009	0.27	0.39	0.34	0.008	<0.001
Hgb, g/L	135.22	122.72	122.71	124.32	115.39	127.49	5.63	0.21	121.94	127.35	3.27	0.25	134.53	114.75	2.71	<0.001

B = Blend of rumen-protected amino acids (2.53g of arginine, 2.34g of histidine, 1.01g of leucine, 1.18g of glutamic acid and 2.94g of glutamine); BSC = Blend of rumen-protected amino acids (2.53g of arginine, 2.34g of histidine, 1.01g of leucine, 1.18g of glutamic acid and 2.94g of glutamine) + 150mg of sildenafil citrate; Gln = 5g of rumen-protected glutamine; GlnSC = 5g of rumen-protected glutamine + 150 mg of sildenafil citrate; T = Negative control (twin-bearing ewes); and S = Positive control (single-bearing ewes). pCO_2 = partial pressure of carbon dioxide; HCO_3 = bicarbonate; SO_2 = saturated oxygen; TCO_2 = total carbon dioxide; Hct = haematocrit; and Hgb = haemoglobin. 40d = dams supplemented for 40 days before parturition; 70d = dams supplemented for 70 days before parturition.

The main effect of treatment was statistically significant (P=0.04) where single lambs had the highest blood pH (7.42), followed by the twin control lambs (7.39) and the Gln treatment group (7.38; Table 6). The blend group had a significantly lower pH (7.31) that others, except for GlnSC (7.36; P=0.10). All other between group comparisons were not statistically different. The pH at 72 h post birth was significantly different than at birth, 7.41 and 7.33, respectively (P=0.001). Depending on the duration of treatment, there was no effect of supplementation observed at 72 h post birth on lamb blood pH. At 72 h post birth SO_2 was higher (73.48%) compared to at birth (52.51%; P=0.0001). While SO_2 wasn't statistically affected by treatment, singles had a blood concentration of at least 5.37% higher than other treatment groups.

At birth PCO_2 was higher for the lambs born of the blend and the twin control treatment groups compared to other groups (Figure 2.3). Between time points, the blend and twin groups were statistically higher at birth than 72 h (P=0.001). Generally, concentrations were higher at birth than at 72 h post birth, except for singles, although values were not significantly different.

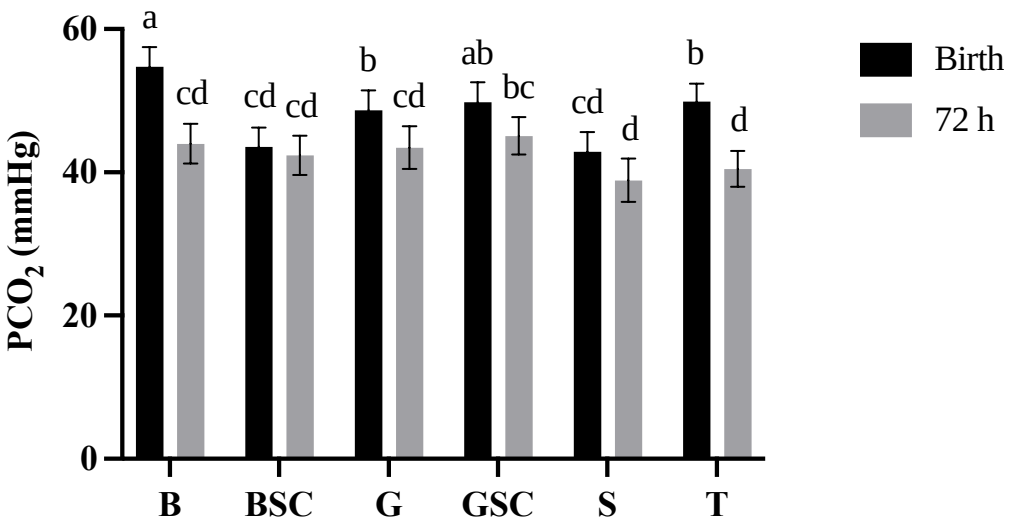

Figure 2.3. Blood PCO_2 (mmHg) of lambs at birth and 72 h post birth (P=0.05). Error bar are ±SEM. B= Blend of rumen-protected amino acids (2.53g of arginine, 2.34g of histidine, 1.01g of leucine, 1.18g of glutamic acid and 2.94g of glutamine); BSC = Blend of rumen-protected amino acids (2.53g of arginine, 2.34g of histidine, 1.01g of leucine, 1.18g of glutamic acid and 2.94g of glutamine) + 150mg of sildenafil citrate; Gln = 5g of rumen-protected glutamine; GlnSC = 5g of rumen-protected glutamine + 150 mg of sildenafil citrate; T = Negative control (twin-bearing ewes); and S = Positive control (single-bearing ewes).

Average blood HCO_3 tended to be higher after 72 h than at birth for the treatment groups, but higher at birth for both control groups, although not statistically significant (Figure 2.4). At 72 h post birth, all treatment groups were not statistically different. Within treatment groups between time points, Gln + SC (P=0.00) had lower concentrations at birth than 72 h. Blend + SC was also lower at birth (P=0.01).

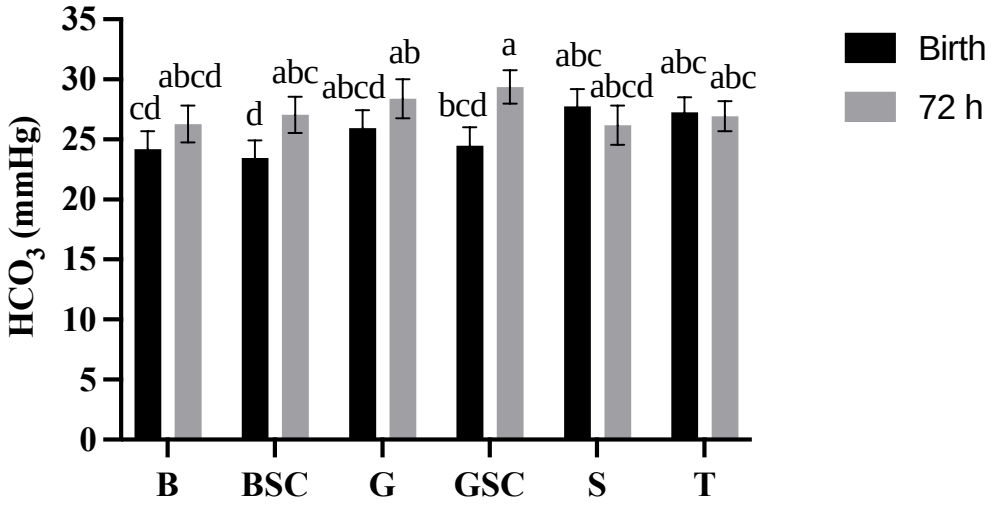

Figure 2.4. Blood HCO_3 (mmol/L) of lambs at birth and 72 h post birth (P=0.01). Error bar are ±SEM. B = Blend of rumen-protected amino acids (2.53g of arginine, 2.34g of histidine, 1.01g of leucine, 1.18g of glutamic acid and 2.94g of glutamine); BSC = Blend of rumen-protected amino acids (2.53g of arginine, 2.34g of histidine, 1.01g of leucine, 1.18g of glutamic acid and 2.94g of glutamine) + 150mg of sildenafil citrate; Gln = 5g of rumen-protected glutamine; GlnSC = 5g of rumen-protected glutamine + 150 mg of sildenafil citrate; T = Negative control (twin-bearing ewes); and S = Positive control (single-bearing ewes).

Concentrations of TCO_2 tended to be lower for the treatment groups at birth compared to after 72 h, but not for the control singles or twins (Figure 5). There was also a statistical difference between blend + SC and the single control (P=0.02) and twin group (P=0.02), where the controls had higher levels. By 72 h, levels had surpassed that at birth, excluding the control groups, and the Gln + SC group had higher levels than that of the blend and singles.

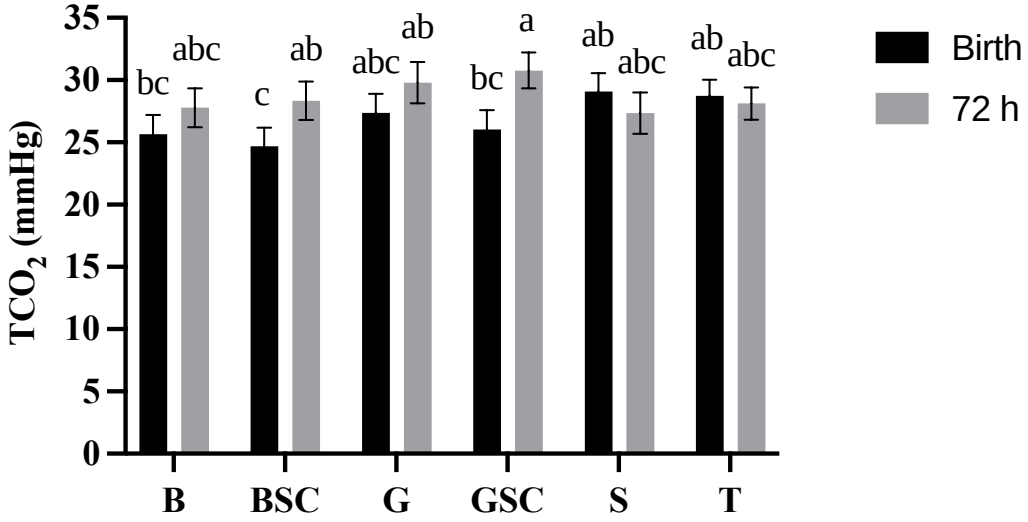

Figure 2.5. Blood TCO_2 (mmol/L) of lambs at birth and 72 h post birth (P=0.01). Error bar are ±SEM. B = Blend of rumen-protected amino acids (2.53g of arginine, 2.34g of histidine, 1.01g of leucine, 1.18g of glutamic acid and 2.94g of glutamine); BSC = Blend of rumen-protected amino acids (2.53g of arginine, 2.34g of histidine, 1.01g of leucine, 1.18g of glutamic acid and 2.94g of glutamine) + 150mg of sildenafil citrate; Gln = 5g of rumen-protected glutamine; GlnSC = 5g of rumen-protected glutamine + 150 mg of sildenafil citrate; T = Negative control (twin-bearing ewes); and S = Positive control (single-bearing ewes).

4.2.2 Discussion

4.2.2.1 Liveweight

The amino acids supplemented in this study all contribute to the growth and development of a growing fetus or neonatal lamb. It was hypothesised that supplementation would increase growth rate compared to the control animals, specifically, that ewes supplemented with amino acids would show higher growth rates compared to other treatments and the control groups, however this was not observed. Instead, we observed that twins from the glutamine-supplemented ewes grew faster from birth to weaning than twins from untreated ewes to the extent that they had similar weaning weights to single-born lambs. The blend lambs also reached a higher live weight than the twin control at weaning, although the difference was not significant, while being significantly lighter at weaning than the singles. This finding highlights the importance of continuing research on progeny beyond the neonatal period. Multiple studies in sheep have demonstrated that even when no difference is seen at birth after maternal intervention, maternal nutrition has already had a lasting impact on the development of the fetus of which effects are not seen until later in life (Ford, et al., 2007; Jaquiery, et al., 2011).

The concept that multiple born lambs are physiologically immature as they start their neonatal life having been supplied insufficient concentrations of crucial amino acids provides the opportunity to improve overall fetal programming effects by ensuring the pregnant dam has sufficient levels of amino acids to supply their lambs. The supplementation with both the blend, and glutamine by itself may have allowed the ewe to supply the growing fetus with the amino acids and nutrients required for efficient growth, without compromising levels required to maintain her own requirements, at a time of consequential development for the lamb. The supply of glutamine, and both glutamine and glutamate in the blend, may have provided the necessary nutrients for twin lambs to be physiologically ready for growth beyond birth through an efficient gastrointestinal system as both glutamine and glutamate are major metabolic sources for the digestive system, specifically the small intestine and are required for healthy function (Wu, 2014). The small intestine is the primary site for nutrient absorption for what has by-passed the rumen, and therefore must maintain integrity to ensure the animal is not being deprived of necessary nutrients (Meyer & Caton, 2016). The supply of amino acids that specifically ensure the healthy function of the digestive tract may have future applications to prevent loss of production or decrease the severity of impact of weaning and transitioning from milk to forage or feed exclusively, on digestion (Indrio et al., 2022). Glutamine also plays a significant role in milk yield and quality. As a precursor for casein, glutamine contributes to improved milk yield and nutritional value (Luo et al., 2021). If milk supply was improved in the treated ewes, twin lambs may have endured less competition for resources and allowed to grow without nutrition restriction.

Although glutamine has not been supplemented to pregnant ewes or lambs as an application for performance previously, it has been investigated in other livestock species, especially pigs. In pigs, maternal glutamine supplementation increased the jejunal villus height and surface area but did not affect the growth of other organs such as the liver, kidney or brain as hypothesised (Burrin, Shulman, Langston, & Storm, 1994). While maternal supplementation in pigs did not completely prevent total intestinal mucosal atrophy in the neonatal piglets, associated with total parenteral nutrition, it did improve villus morphology.

Glutamine has been shown to have application in human medicine to attenuate effects of alcohol induced fetal cardiovascular adaptations. A study exposed pregnant sheep to a simulated binge drinking scenario, and caused maternal acidaemia, hypercapnia and hypoxemia, reducing blood flow to the fetus by 40% (Onkar B. Sawant, Ramadoss, Hankins, Wu, & Washburn, 2014). Maternal

supplementation with infused glutamine (100mg/kg of body weight) was able to attenuate the maternal effects of alcohol and improve fetal blood flow in the brain and mitigated the alcohol induced acid-base imbalances seen in the maternal and fetal units. The results of this study are supported by that of Washburn, Sawant, Lunde, Wu, and Cudd (2013) and Onkar B Sawant, Wu, and Washburn (2015).

Glutamine has also been shown to have benefits when supplemented in early life, with a large interest in the addition of glutamine in the diets of pig. Inclusion of glutamine in the diet of piglets from weaning significantly increased feed efficiency and altered small intestine length and morphology to have high villus to crypt depth ratio in animals subjected to cold stress (da Silva et al., 2019). This finding was supported by a similar study in weaned piglets where glutamine was supplemented in varying percentages in the diet (0, 0.5, 1 or 1.5%) for 28 days, that also found an improvement in villus height and villus height to crypt depth ratio in the duodenum (Lee et al., 2003). It has also been shown that glutamine can alter the gene expression in the small intestine of weaned piglets to prevent atrophy and a reduction in nutrient absorption efficiency, avoiding a lull in growth (Wang et al., 2008; Wu, Meier, & Knabe, 1996).

4.2.2.2 Blood parameters

Amino acid supplementation had an interactive effect, with time point between birth and 72 post birth, on blood pH, where pH was higher 72 h post birth, except for single lambs. Blood pH may be expected to change with the supplementation of amino acids as they are metabolised, although due to the ability of amino acids to act as both an acid and a base, it may have also been expected for there to be no changes in pH (Ahn Jin, 2007).

The blood pH of lambs in the blend treatment group (7.24 ± 0.03) at birth had levels lower than often reported in other studies (Bessho, Taira, & Koyama, 1997; Peiró, Borges, Gonçalves, & Mendes, 2010), similarly for levels at birth for those lambs born to ewes supplemented for 40 days, although few studies look at lambs at birth. Mitchell and Williams (1975) reported a range of 7.34-7.40 as an average measurement in lambs from birth to six weeks old, receiving no treatment, and Bartko, Vrzgula, and Chyla (1975) a range of 7.30-7.48 in sheep sampled over a year period for baseline blood pH. In other species, no changes in blood pH were found in infants infused with amino acids (Van Goudoever et al., 1995), or in healthy dogs after pre-anaesthetic amino acid infusion (Clark-Price et al., 2015). The reduced blood pH in the blend treatment group indicates an imbalance in the acid-base status of those lambs. This is confirmed with the results of PCO_2 being slightly high in the blend lambs.

The administration of sildenafil citrate was intended to increase blood flow in the maternal circulation, and therefore oxygen supply to the placenta (Inocencio et al., 2019; Oyston, Stanley, Oliver, Bloomfield, & Baker, 2016; Satterfield, Bazer, Spencer, & Wu, 2010). This supply allows for efficient transportation and metabolism of the amino acids provided as a treatment (Satterfield et al., 2010). Sildenafil citrate administration causes a release of oxygen from erythrocytes to increase blood oxygen (Ellis & Pepple, 2015). With this shift in the blood oxygen status, it could be expected that blood gas concentrations could be altered in the new-born lamb. As PCO_2 increases, blood pH will decrease, and HCO3 is expected to also increase (Kellum, 2000). For lambs, PCO_2 is expected to be between 40-50 mmHg (Strand, Ikegami, & Jobe, 2003). All values for lambs, except from the blend treatment group, were within this expected range. For the blend group, the average PCO_2 concentration was 55.75 mmHg. This correlates with the low blood pH also seen at birth for this treatment group.

Bicarbonate (HCO_3) in the blood plays an important role in the release of carbon dioxide from the body and regulating the acidity in the blood (Costill, Verstappen, Kuipers, Janssen, & Fink, 1984). Following the trend of results, specifically for the blend treatment group, with low blood pH and high PCO_2, it

could be expected that the average HCO_3 would higher for these animals, but this was not observed in the current study. Values for blood HCO_3 in sheep should be within 21-28 mmol/L (Jackson & Cockcroft, 2002). All measures of HCO_3 in this study fit within this reference range, so although there was a significant difference found between treatment groups, this difference is not of biological significance. Another indicator of good blood health is TCO_2. Reported ranges for TCO_2 are 23-27 (House & Gunn, 2009) and 24.58-28.78 (Peiró et al., 2010). At birth, levels were elevated in the singles, and at 72 h post birth, both glutamine and glutamine SC groups had elevated levels, indicating an excess of bicarbonate in the blood, or metabolic alkalosis (Oren, Wasserman, Davis, & Whipp, 1981). Due to the mechanisms of SC, and consequently the role of oxygen in the blood, it was expected that TCO_2 may have been higher in the blend and glutamine group, and lower, or within range in the SC treated groups, however this was not observed in the current study. The percentage of Hgb that is fully combined with oxygen is indicated via SO_2 concentration, with a low percentage indicating respiratory issues or an issue with blood flow (Shepherd & Pearse, 2009). In the current study, lambs born after 70 days maternal supplementation had significantly low SO_2 levels at 45.23%, while a normal value in a mature sheep's blood has been reported as 60-100% (Saini et al., 2020). The same study did report a lower range of 1-60% in the fetal circulation, but as lambs were measured post birth in the current study, this is not a comparable reference. The low SO_2 value, although not statistically effected by treatment, was lowest in the blend lambs at 50.66% compared to 62 – 70% in the other treatment groups. As both PO_2, as the indicator of the rate of oxygen diffusion in the blood, and SO_2, which is the measure of oxygen availability, are low in the blend group can be observed that there is an issue with blood flow in that group of lambs. This effect is not observed in the Gln or sildenafil citrate, or control groups. This may be due to the supplementation of multiple amino acids that require oxygen for metabolism being supplemented, without the supply of oxygen compensating sildenafil citrate.

Although sildenafil citrate increases the synthesis of nitric oxide to increase blood oxygen (Satterfield et al., 2010; Wareing, Myers, O'Hara, & Baker, 2005), no changes, or advances in performance, were seen in the current trial, in lambs born to dams administered sildenafil citrate compared to those supplemented amino acids without sildenafil citrate. The changes seen in various blood gas parameters may be explained by individual lambing circumstances, such as lambing time, difficulty, and time to draw the first breath, which were not considered in this study.

Throughout the trial period, supplementation with amino acids, along with the administration of SC, did not influence pregnant ewe blood amino acid concentrations or blood gas values. Intravenous arginine supplementation has been found to increase circulating amino acids at a dose of 465 μmol/kg per day (Lassala et al., 2010), but no change was seen supplementing arginine in a blend orally. In the current trial a dosage of 2.53g of arginine was supplied to ewes in the blend treatment groups Considering an average liveweight of 72.9 kg that would be a concentration of 37 μmol/kg per day. Differences may also have been difficult to observe as amino acids were fed as part of a total mixed ration provided throughout the day, rather than to all animals to be consumed at once.

4.3 Estimating organ size and body composition

4.3.1 Results

The CT estimated organ volumes were, on average, less than the measured organ volumes (Table 3.1). There was a strong correlation between measured and estimated right kidney volumes, and a moderate correlation between combined kidney volumes, both of which were statistically significant for the linear relationship between the two methods. The measured and estimated volumes of both the liver and left kidney had moderate correlations, and there was a weak correlation between spleen volumes. However, these were not statistically significant for the linear relationship between the two methods. The bias between the measured and CT estimated organ volumes was lowest for the right

kidneys, and highest for both kidneys combined. The bias between the measured and estimated volumes was relatively similar for the liver, left kidney and spleen. The precision (i.e., s.d. of the difference between measured and estimated volume) was 47.13 mL for the liver, 3.56 mL for the left kidney, 1.77 mL for the right kidney, 4.6 mL for both kidneys, and 2.56 mL for the spleen.

Table 3.1. Mean (± s.d.) organ volumes (mL) of deceased neonatal lambs, measured by water displacement and estimated by repeated CT scan analysis. Correlation coefficient (Pearson *r* or Spearman r_s), associated *P* value, and bias (mL) between the two methods are presented.

Organ	Mean organ volume (mL)		Correlation		*P* value	Bias (mL)
	Measured	CT estimated	*r*	r_s		
Liver	99.8 ± 53.46	96.9 ± 24.85		0.38	0.29	2.903
Left kidney	12.6 ± 3.69	9.1 ± 2.97		0.46	0.18	3.487
Right kidney	11.2 ± 2.94	9.7 ± 3.51	0.86		0.0013	1.501
Both kidneys	23.8 ± 5.49	18.81 ± 6.32	0.583		0.0832	4.987
Spleen	9.2 ± 2.15	5.3 ± 1.86	0.19		0.5967	3.871

4.3.1.1 Organ weights

The CT estimated organ weights (absolute and relative) were, on average, less than the measured organ weights except for the absolute liver weight (Table 3.2). There were strong correlations between the measured and estimated absolute weights for all organs, all of which were statistically significant for the linear relationship between the two methods. The bias between the measured and CT estimated organ weights (absolute) was lowest for the spleen, highest for the liver, and relatively similar between the kidneys. The precision (i.e., s.d. of the difference between measured and estimated absolute weight) was 5.24 mL for the liver, 0.81 mL for the left kidney, 1.53 mL for the right kidney, 1.97 mL for both kidneys, and 1.01 mL for the spleen.

For the organs as relative weights, the precision (i.e., s.d. of the difference between measured and estimated weight) was 1.23 mL for the liver, 0.094 mL for the left kidney, 0.109 mL for the right kidney, 1.99 mL for both kidneys, and 0.0705 mL for the spleen.

Table 3.2. Mean (± s.d.) organ weights (g) of deceased neonatal lambs, measured by scales and estimated by CT scan analysis, as absolute weights (g) and relative to bodyweight (%). Correlation coefficient (Pearson *r*), associated *P* value, and bias (g) between the two methods are presented.

Organ		Mean organ weight		Correlation	*P* value	Bias (g)
		Measured	CT estimated	r_s		
Liver	Absolute, g	98.31 ± 28.69	103.5 ± 26.25	0.9356	<0.0001	5.24
	Relative, %	3.09 ± 1.16	2.75 ± 0.719	0.7091	0.0268	0.339
Left kidney	Absolute, g	11.66 ± 2.663	9.599 ± 3.11	0.9725	<0.0001	2.06
	Relative, %	0.37 ± 0.113	0.25 ± 0.0752	0.8061	0.0072	0.113
Right kidney	Absolute, g	11.51 ± 2.58	10.22 ± 3.687	0.9412	<0.0001	1.29
	Relative, %	0.36 ± 0.108	0.27 ± 0.104	0.8061	0.0072	0.0891
Both kidneys	Absolute, g	23.17 ± 5.202	19.82 ± 6.629	0.9734	<0.0001	3.35
	Relative, %	0.729 ± 0.221	0.527 ± 0.176	0.8061	0.0072	0.203
Spleen	Absolute, g	6.061 ± 1.972	5.635 ± 1.959	0.8677	0.0011	0.426
	Relative, %	0.189 ± 0.0738	0.15 ± 0.0544	0.3333	0.3487	0.0392

The relationships between the organ volumes measured by water displacement and estimated by CT analysis were evaluated (Figure 3.1). For the right kidney, the coefficient of determination for the relationship between estimated and measured volume was r^2 = 0.67, and this was larger than the other organs. For the left kidney, liver, and spleen the coefficients of determination were r^2 = 0.20, r^2 = 0.19, r^2 = 0.015, respectively. The relationship between the estimated and measured volume of both kidneys was also evaluated and had a coefficient of determination of r^2 = 0.44 (figure not presented). The relationships between measured and CT estimated organ weights were evaluated and high coeficient of determination (0.75 – 0.95) were obtained for the organs measured, including kidney (right and left), liver and spleen (Figure 3.2).

The relationships between measured and CT estimated organ weights, relative to bodyweight, were evaluated and low to moderate coeficient of determination were observed for the right and the left kidneys, followed by low correlation observed for spleen and poor correlation for the liver (Figure 3.3).

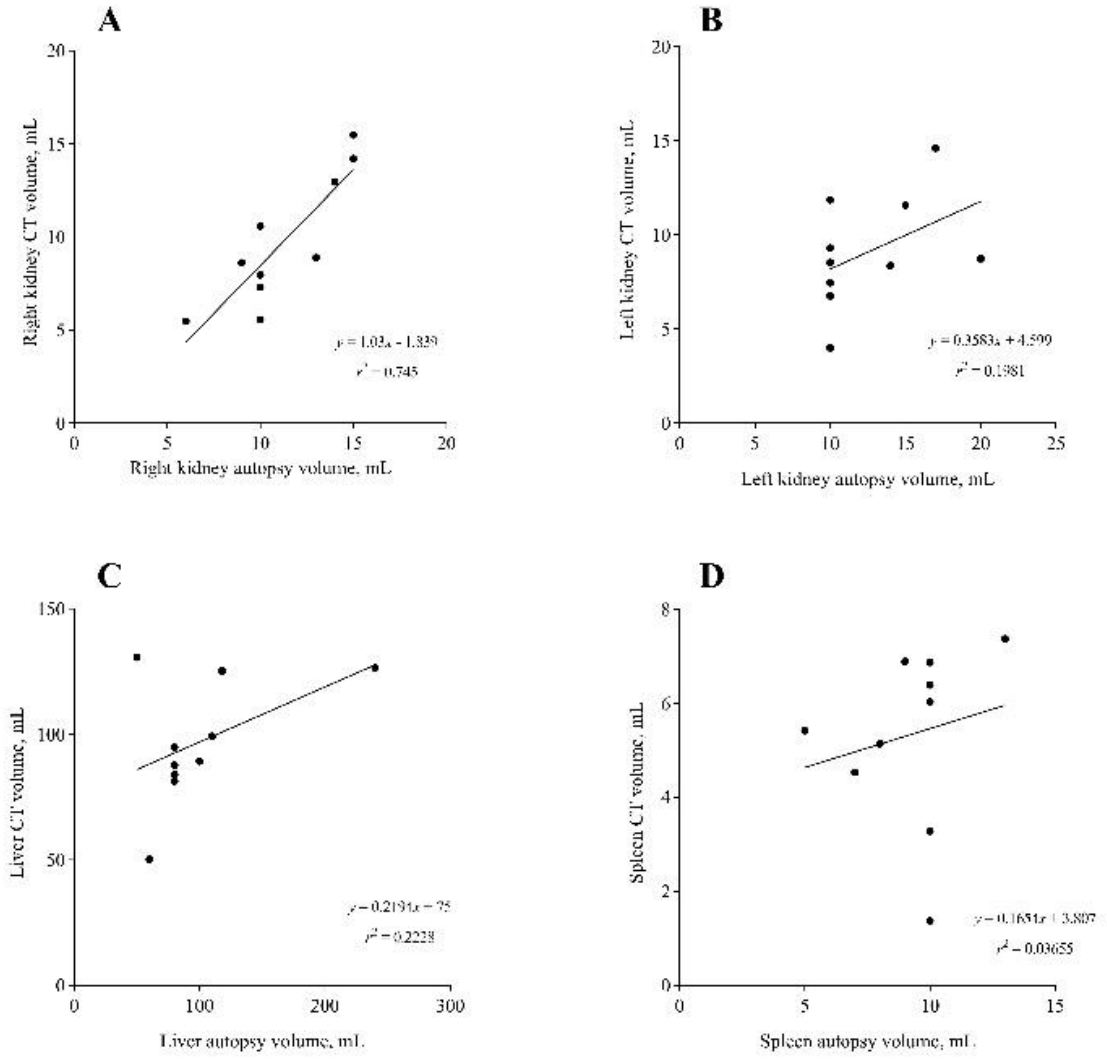

Figure 3.1. Relationships between organ volumes measured by water displacement and estimated by CT scan analysis, for the right kidney (A); left kidney (B); liver (C); and spleen (D) volumes of deceased neonatal lambs (*n* = 10).

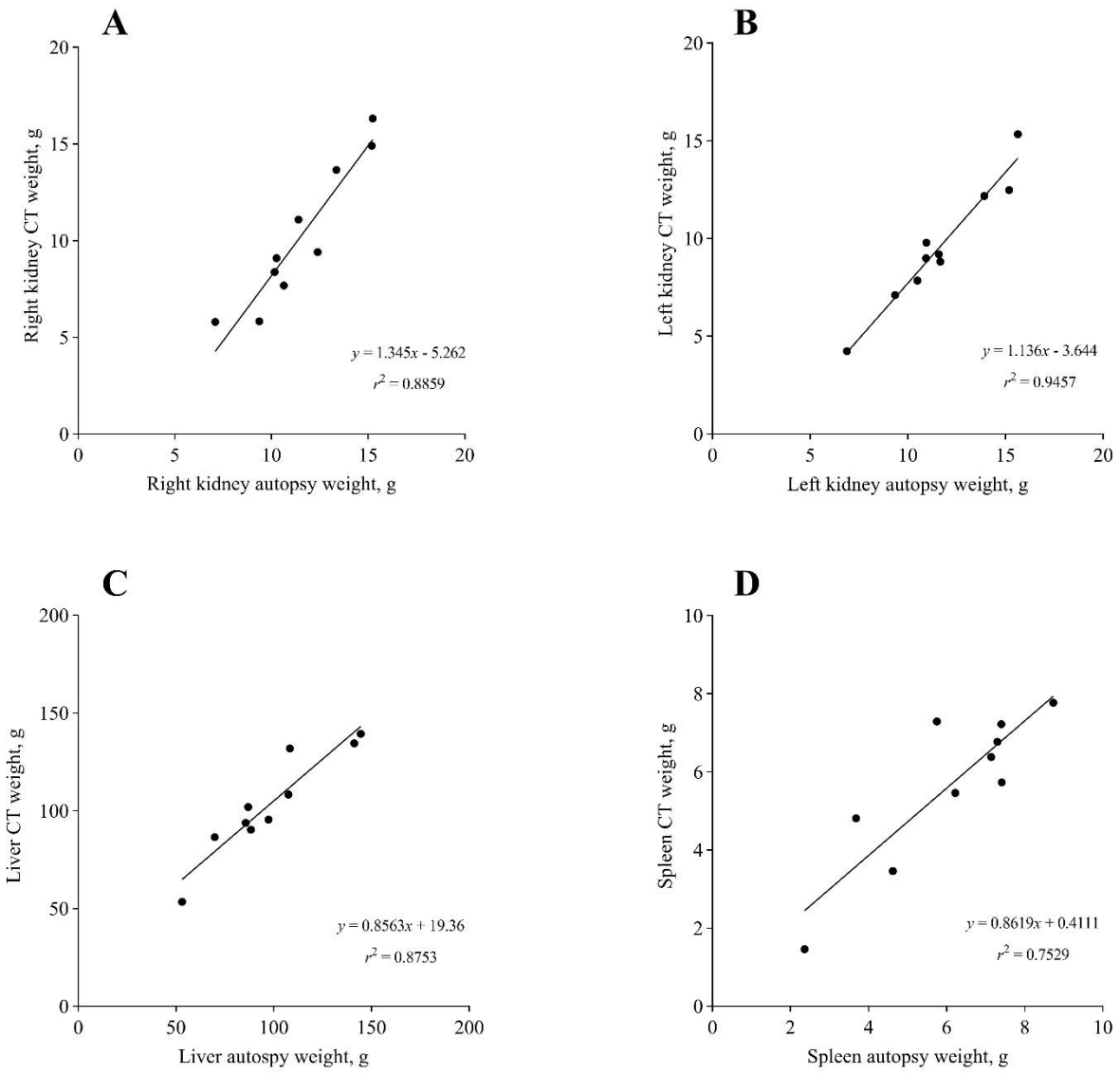

Figure 3.2. Relationships between measured and CT estimated organ weights, for the right kidney (A); left kidney (B); liver (C); and spleen (D) of deceased neonatal lambs (*n* = 10).

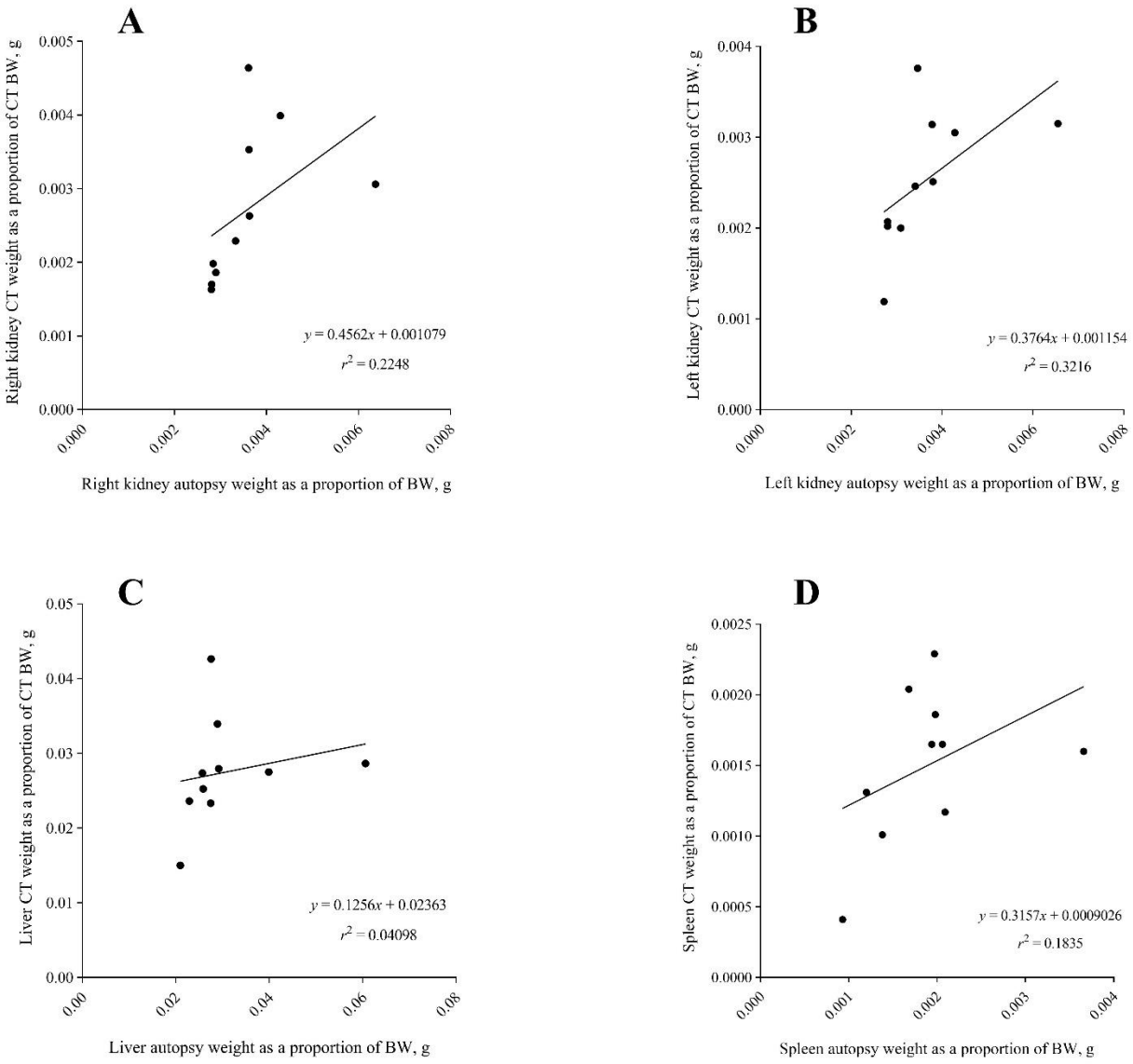

Figure 3.3. Relationships between measured and CT estimated organ weights, relative to bodyweight, for the right kidney (A); left kidney (B); liver (C); and spleen (D) of deceased neonatal lambs (*n* = 10).

4.3.1.2 Body composition

The mean (± s.d.) CT estimated weights of fat, muscle, and bone for the ten deceased lambs were 0.13 ± 0.021 kg, 2.47 ± 0.51 kg, and 1.23 ± 0.25 kg, respectively. The mean (± s.d.) CT estimated bodyweight was 3.82 ± 0.72 kg. The mean (± s.d.) measured bodyweight was 3.24 ± 0.47 kg. The measured bodyweight and CT estimated bodyweight data had a Pearson correlation of $r = –0.06$, which was not significant ($P = 0.87$) for the relationship between the two methods (Figure 3.4).

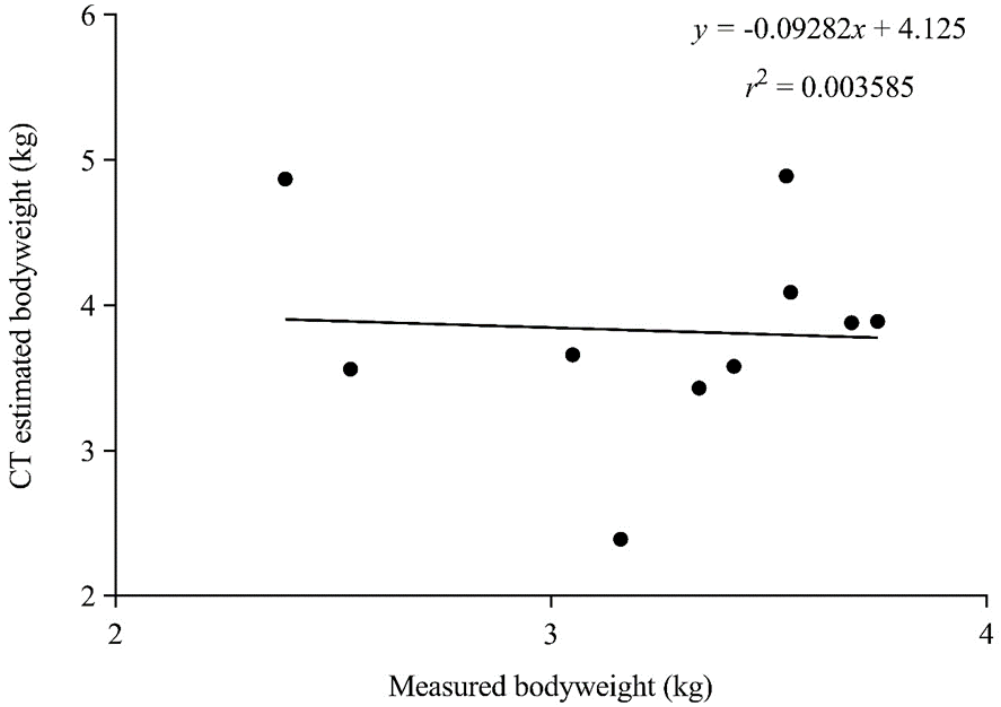

Figure 3.4. Relationship between measured and CT estimated bodyweight (kg) of deceased neonatal lambs (*n* = 10).

The Bland-Altman plot represents the difference between the two methods used to determine bodyweight, as a function of the mean of the two methods (Figure 3.5). The bias (± s.d.) for CT bodyweight estimation was 0.58 ± 0.88 kg. The limits of agreement were between –1.15 and +2.31 kg.

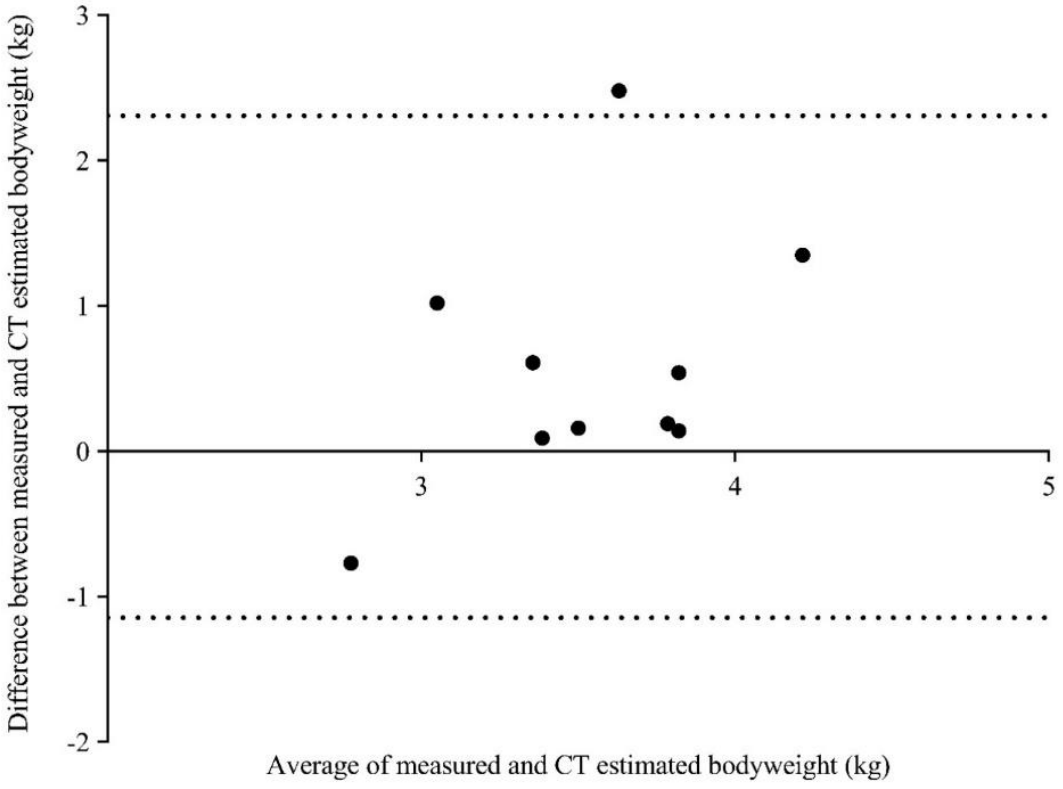

Figure 3.5. Bland-Altman plot for measured and CT estimated bodyweight (kg) data of deceased neonatal lambs (*n* = 10). The dotted lines represent the limits of agreement.

4.3.2 Discussion

4.3.2.1 Organs

This study aimed to firstly determine whether CT estimation of organ size is representative of *post-mortem* organ size in neonatal lambs. There was a strong relationship between the volume of the right kidney estimated by CT scan analysis and measured by water displacement. The absence of statistical significance for the positive correlations between the two methods for the liver, left kidney, and spleen volumes implies that the associated moderate and weak relationships were inconclusive. This suggests that the CT scan analysis method is suitable when estimating the right kidney volume.

These results should be considered in the context of the methods used. Firstly, the CT scans were obtained from neonatal lambs which were deceased, and had been for indeterminate lengths of time. This resulted in the absence of blood flow to the organs and the presence of tissue autolysis, which occurs naturally after death. The CT scan images are constructed as different grey values along the Hounsfield unit scale, which are defined by X-rays that detect differences in tissues throughout the body during CT scanning. This is based on tissue attenuation properties related to the tissue density. Tissue autolysis likely altered tissue density, thereby limiting the capacity for organ boundaries to be defined by differences in grey values during CT scanning. Consequently, the CT scan analysis method was limited by inaccuracies associated with locating and defining organs in the CT scans.

Consideration should also be given to potential sources of error contributing to variations between organ volume estimated by CT and measured by water displacement. Firstly, the period between CT scanning and organ volume measurement *post-mortem* exceeded 24 hours on average, which increased the likelihood of tissue autolysis and organ fluid loss. Additionally, blood loss from open vessels during *post-mortem* measurements may have contributed to disparities between the two methods. Given this, it could be expected that the volumes measured by water displacement may be less than those estimated by CT analysis. Given that organ volumes were, on average, underestimated by CT, this was not apparent in these results.

In contrast to the results of this study, Jani *et al.* (2005) found that fetal sheep organ volumes were overestimated by MRI when compared to water displacement, despite measurements being taken within 24 hours of each other (Jani *et al.* 2005). The authors suggested that vessels should ideally be tied off before organ removal to prevent blood loss. This, along with minimising the time between death, CT scanning, and *post-mortem*, could help maintain consistent experimental conditions so that the methods are more comparable for validation.

However, evaluating the bias provides insight into the agreement between measurements obtained from the two methods (Altman and Bland, 1983). The absence of a trend in bias for the left and right kidney, liver, and spleen volumes suggests that a calibration value equal to the bias for each organ might be employed. For example, given the bias of 2.903 mL for liver, an adjustment of 2.903 mL could be applied for future CT estimated liver volumes of deceased neonatal lambs. Calibration values would need to be validated for a range of organ sizes, and established based on variations in CT scanner settings.

The calibration of CT is dependent on the accuracy of the reference method, water displacement in this case. The precision of the water displacement measurements were dependent on the graduation of the volumetric cylinders used, and observing the changes in volume. Weight using digital scales gives a less subjective measurement than visually approximating the slight change in water level when using a cylinder to determine volume.

4.3.1.1 Body composition

The CT estimated weights of fat, muscle, and bone were combined to represent CT estimated bodyweight, for comparison with measured bodyweight, in the absence of chemical analysis of the body composition components. This approach has been researched and found to be effective in pigs, with a correlation of 0.99 between CT estimated bodyweight and measured bodyweight at CT scanning (Giles et al., 2009). Further, a body composition study in dogs found the methods to be interchangeable when using CT derived bodyweight compared to measured bodyweight, given all data points were within the mean +/- 1.96 s.d. and BA ranges were not wide (Purushothaman et al., 2013). Consistent underestimation of CT derived bodyweight was observed for the dog study.

Evaluation of the negative correlation between CT estimated and measured bodyweight suggests that there was a lack of a statistically significant linear relationship between the methods. However, further analysis using the Bland-Altman approach indicates that there was good agreement between bodyweight measurements when comparing the CT estimation method to the standard of weighing. For CT estimated bodyweights to be acceptable, the limits of agreement required that they did not exceed the standard measure by more than 2.31 kg, or fall below it by more than 1.15 kg. The suitability of these limits in future applications of CT will depend on the context of the research; the limits of agreement must be small enough to provide assurance that the new method of CT estimation can replace the standard method with sufficient accuracy (Altman and Bland, 1986). However, the sample size of ten lambs limits the interpretation of these results. Sample size calculations using a two-sided test comparing the means suggested that at least 29 lambs would be required to obtain statistical significance at $P < 0.05$ with the 95% confidence interval, so this should be considered for further validation studies.

Another limitation was that the CT estimation method did not include wool, as it was not detected within the Hounsfield unit ranges applied. Further, the ranges of Hounsfield units were applied to the entire body, resulting in regions such as the intestines and stomach being considered equivalent in density to either fat, muscle, or bone. Other research has accounted for rumen content using CT scan analysis (Goopy et al., 2014; Bond et al., 2017; Oddy et al., 2019), however this has not been applied to neonatal lambs where milk is the main component of their stomach contents.

Additionally, the appearance of certain tissues in the CT scans caused areas such as the gut to be wrongly identified as bone. This likely contributed to error in the body composition calculations; since bone has the highest density, an overestimation of bone has a higher impact on the final weight than an overestimation of muscle or fat. The previously mentioned time intervals between collection (i.e., when bodyweight was measured) and CT scanning may explain the CT scan appearance and inaccurate tissue assignment in the body composition analysis. Weighing the lambs at the same time as CT scanning could reduce this error, by reducing potential discrepancies in the physiological composition of the deceased lambs between measurements.

Further validation of CT scan analysis for individual weights of fat, muscle, and bone should ideally involve comparative chemical composition analysis, for direct validation of the CT method. This approach for the body composition of neonatal lambs was not apparent in the literature, however strong correlations were found (r = 0.90, 0.98, and 0.92 for fat, muscle, and bone, respectively) in a study comparing virtual dissection of carcasses using CT to manual dissection (Kongsro et al., 2008). Comparative analysis could also quantify how inaccuracies in tissue assignment during scan analysis contribute to the error in CT tissue weight estimation. Further, the standard method excludes the stomach and gut content from analysis (Hegarty et al., 1999; Gonzalez et al., 2019). As such, the contribution of milk, which was detected as fat in these scans, and intestinal contents to CT estimated body composition of neonatal lambs could be quantified. Therefore, further development of the CT

scan method for body composition of neonatal lambs requires validation in terms of the wool, skin, stomach, gut, and contents in the stomach and gut, and whether removal of these from CT images is necessary for comparable results.

Future validation research should ideally utilise live lambs, given the occurrence of tissue autolysis and the absence of blood flow to organs during scanning evident when using deceased lambs to validate the CT scan analysis method. Preferably, live lambs within an existing trial should be used, where euthanasia is already necessary for other sampling purposes. This could involve CT scanning the live lambs immediately prior to euthanasia, then taking *post-mortem* organ volume measurements immediately after, followed by chemical analysis of body composition. Assessment of intra and inter observer reliability should also be incorporated into future validations of the CT scan analysis method, particularly for organ volume estimations given that there may be subjectivity associated with the image analysis. Additionally, using animals across a range of ages may be a consideration for further validation. Previous research assessing human organ volumes by CT used water-filled balloons as a phantom validation, and found that CT volume estimates were accurate for volumes above approximately 10 mL (Geraghty et al., 2004). Therefore, older lambs with organs above this 10mL may be more accurately defined by CT analysis than neonatal lambs, however more research is needed to confirm this.

4.4 MLA Experiment 1 – CT scanning

4.4.1 Results

The effect of treatment on organ volume was not significant, except for the spleen which trended towards significance ($P = 0.082$; Table 4.1). The estimated least squares mean spleen volume of single lambs was significantly larger than the blend ($P = 0.021$) and B + SC lambs ($P = 0.046$). Significant differences between treatment groups, and trends towards a significant difference, were also found for the other organs, however the effect of treatment itself was not significant. For the right kidney volumes, there was a significant difference between single and G + SC lambs ($P = 0.043$), a trend for blend and single lambs ($P = 0.069$), and a trend for B + SC and single lambs ($P = 0.071$). For the left kidney, single and G + SC lambs had significantly different volumes ($P = 0.017$), and there were trends for blend and single lambs ($P = 0.078$), along with B + SC and single lambs ($P = 0.065$). There was a trend towards a significant difference in the liver volumes of B + SC and single lambs ($P = 0.079$).

Table 4.1. Least squares means estimates (± s.e.m.) for the organ volumes (mL) estimated by CT scan of five-month-old lambs ($n = 34$) from different treatment groups (Blend, B + SC, Gln, G + SC, twin control and single control). The *n* values are animals per treatment. The *P* values are given for the effect of treatment on organ volume. Different letter superscripts within a row indicate differences between least square means, ($P < 0.05$) due to treatment group.

	CT estimated organ volume (mL)						
Organ	Blend *n* = 6	B + SC *n* = 4	Gln *n* = 4	G + SC *n* = 4	Twin Control *n* = 8	Single Control *n* = 8	*P* value
Liver	711.48 ± 49.32	634.27 ± 58.68	685.39 ± 63.03	666.29 ± 62.40	705.00 ± 41.83	777.22 ± 40.10	0.47
Spleen	246.91 ± 31.36^{a}	253.11 ± 37.25^{a}	313.95 ± 40.10^{ab}	257.07 ± 39.77^{ab}	230.69 ± 26.56^{a}	360.22 ± 26.33^{b}	0.082
Right kidney	63.54 ± 5.57	61.55 ± 6.65	65.53 ± 7.12	59.37 ± 7.02	69.70 ± 4.74	77.99 ± 4.31	0.22
Left kidney	65.48	62.77	67.44	56.65	69.38	79.18	0.15

	± 5.43	± 6.47	± 6.93	± 6.85	± 4.61	± 4.28	
Both kidneys	129.03 ± 10.82	124.35 ± 12.91	132.96 ± 13.82	116.02 ± 13.64	139.09 ± 9.19	157.12 ± 8.46	0.18

Body composition was measured in a subset of lambs (Table 4.2), with the remaining lambs to be analysed and results published in a high impact peer reviewed journal.

Table 4.2. Least squares mean estimates (± s.e.m.) for the CT estimated fat muscle and bone (as proportions of body weight, kg) of five-month-old lambs (*n* = 12) from different treatment groups (Blend, B + SC, Gln, G + SC, twin control and single control).

	CT estimated body composition as a proportion of body weight (kg)					
	Blend *n* = 2	B + SC *n* = 2	Gln *n* = 2	G + SC *n* = 2	Twin Control *n* = 2	Single Control *n* = 2
Fat (%)	24.09 ± 3.16	21.57 ± 3.14	23.70 ± 1.27	19.67 ± 2.51	18.93 ± 0.69	18.11 ± 6.35
Muscle (%)	46.56 ± 3.45	45.58 ± 4.22	47.19 ± 4.30	41.17 ± 2.42	47.67 ± 2.10	30.03 ± 10.72
Bone (%)	7.88 ± 0.41	8.38 ± 1.41	9.25 ± 0.10	7.00 ± .068	7.12 ± 1.75	5.01 ± 1.75

4.4.2 Discussion

4.4.2.1 Organs

Using CT analysis to estimate the organ volumes, as an indicator of growth and development, from the CT scans of live, five-month-old lambs allowed for the detection of statistically significant differences between treatment groups. The overall effect of treatment on organ volume was not significant, except for a trend towards significance for the spleen volume. Regardless, the ability of CT scan analysis to detect differences in organ volume between treatment groups contributes to the applicability of CT scanning as a replacement for the standard methods that require euthanasia. Further, these results were limited by the sample sizes within each treatment group. Sample size calculations using a two-sided test comparing the means suggested that a sample size of at least 13 animals per group would be required for statistical significance to be detected at $P < 0.05$ with a 95% confidence level. As such, the sample size should be increased for future studies applying CT scan analysis as a protocol to detect differences in organ volumes between treatment groups.

Further validation of the organ volumes estimated from the existing CT scans should ideally include repeated analysis. This could involve having the original operator repeat the CT organ volume estimations, in addition to a second operator analysing the CT scans. Thus, providing insight into the repeatability of the CT scan analysis method, by including intra- and inter-observer measures. Also, the mean of repeated measures may reduce any potential error in the results. Thus, incorporating repeated analysis may reinforce the validity of CT scan analysis to detect differences in organ size between treatments.

Another aspect observed is that the organs highlighted by the contrast agent appear as white in contrast enhanced scans (Appendix 10.1; Figure 1), so would likely be detected as bone during body composition analysis, making contrast enhanced scans unsuitable for this. Further, only having one set of scans to analyse reduces 'handling', and removes the need to administer contrast agent at the time of scanning. As such, plain scans should be validated against contrast enhanced scans for determining organ volume.

Ultimately, the context that CT scan analysis is applied to determines the suitability of the technology to establish organ volumes of lambs. Evaluation of treatment effects on organ size may only require the insight that there are significant differences between treatment groups, in order to quantify an increase or decrease in organ size in response to the treatments. As such, validating CT estimated organ size with the standard methods may be redundant, given that precision is less crucial when evaluating the effect of treatment on specific organ size.

4.4.2.2 Body composition

Reducing the time spent to manually edit images could expand the application of CT scanning as an analysis approach, given that extensive time requirements may limit the uptake of the technology. However, this should be considered in relation to the labour intensive, and perhaps equally time consuming, standard method of dissection and grinding to prepare the carcass for chemical analysis. Further, the automation of CT scan analysis through machine learning and artificial intelligence is already being explored in sheep, with accuracies of 98% for the fully automated removal of structures, such as the CT table and testes, from the images (Robson et al. 2021).

4.5 Substances to rumen protect glutamine

4.5.1 Results

Glutamine (non-protected) had a stable release throughout the 480-minute incubation, while NS1 had prolonged efficacy in glutamine protection compared to NS2 (Figure 4.1). At 360 minutes of incubation, there was no difference between non-protected glutamine and glutamine protected with NS2.

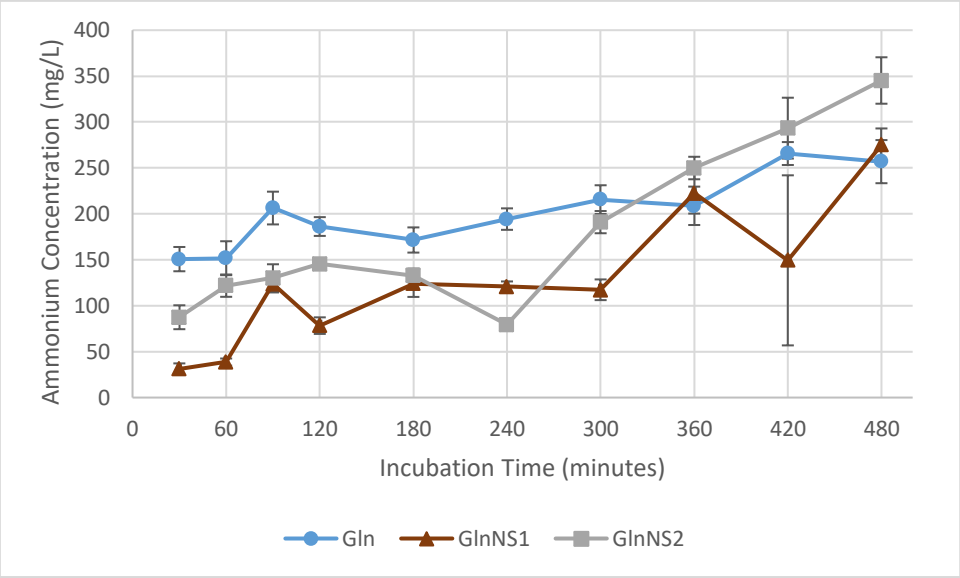

Figure 4.1. Ammonium concentration (mg/L) of the incubated glutamine (Gln), encapsulated glutamine with natural substance 1 (GlnNS1), and encapsulated glutamine with natural substance 2 (GlnNS2) over 480 minutes *in vitro* incubation (mean ± SEM).

During 480 minutes of fermentation, the glutamine group (non-protected) released 9.24g of ammonia. During incubation, the bottle with glutamine protected with NS1 released 6.12g of

ammonia that equates to 33.83% decrease in ammonia release. The bottle with glutamine protected with NS2 released 8.26g of ammonia that equates to 10.66% decrease in ammonia release.

4.5.2 Discussion

The study completed the objective to establish the efficacy of NS1 and NS2 to protect glutamine from microbial degradation in rumen fluid. NS1 resulted in a higher protective efficacy than NS2 as it had a 23.17% less ammonia released during the *in vitro* incubation. Both natural substances were economically feasible to manufacture, requiring minimal preparation and time-effective techniques. There is dearth of information around the use of rumen-protected glutamine in ruminants and on methodologies and substances used to produce a rumen-protected glutamine supplement. A previous study investigated the use of formaldehyde 2% sprayed on glutamine and dried at room temperature to protect the AA from microbial degradation (Nemati et al. 2018). Depending on the volume of formaldehyde used, methodology is cost-effective being an important factor to consider for future commercial applications. Neither the efficacy nor the bioavailability of this supplement was revealed, therefore conclusions around its protection efficiency cannot be drawn.

Furthermore, current substances used to rumen-protect glutamine usually involve intellectual property and remain undisclosed. A rumen-protected glutamine product made by an Italian company, Ascor Chimici, was used in a study conducted by Caroprese et al. (2012) evaluating glutamine's effect on Friesian cow immune function. The glutamine supplement had a 40% bioavailability. This product is no longer commercially available, bringing into question the rumen protective efficacy and whether the cost of production was feasible. Additionally, rumen-protected glutamine produced by Wansheng Biological Co. Ltd. was used in a study conducted by Wang et al. (2022). This product has a high bioavailability of 65% and on average 25.3% rumen degradability (figures derived from their unpublished research).

The current study measured the efficacy of two natural substances in rumen-protecting glutamine therefore, to determine the bioavailability of the more efficient substance, NS1, an *in vivo* trial would be required. One third of the glutamine intake reaches blood circulation and the residual glutamine is metabolised by intestinal cells (Häussinger & Sies 1984). Therefore, blood glutamine analysis post feeding is a potential measurement for the evaluation of the amount absorbed through the small intestine and available to the body for use. Digesta passage rate and pH would also influence glutamine microbial degradation. Complete degradation of unprotected glutamine in the rumen requires 36 hours (Suh et al. 2022). Different types of feed influence digesta passage rate; high concentrate diets are digested faster than high fibre diets. Differing diets can also influence pH which further impacts rumen microbiome (Vargas et al. 2023).

5 Conclusion

The current project contributed with the following findings:

- The combination of SC and arginine used during the safety trial was safe for pregnant ewes and did not cause adverse effects. However, further trials should investigate the effects of supplementation during pregnancy on the fetal and neonatal lambs to ensure no negative consequences.
- Twin lambs born from ewes supplemented with glutamine during late gestation were able to grow at a similar rate as single-born lambs by weaning, showing similar liveweights. It demonstrates the potential benefit in using maternal amino acid supplementation to reduce mortality rate of twin lambs.

- The combination of amino acids with sildenafil citrate did not provide any beneficial effect on the progeny at birth and weaning.
- There is a potential in using CT scanning to evaluate the nutritional requirements of live animals at different physiological stages without the need for euthanasia, however further validation studies are needed.
- Natural substances 1 and 2 can be used in a cost-effective way to prevent partial microbial degradation of glutamine in the rumen. However, further investigation is needed to determine if these substances are effective in increasing glutamine levels in the small intestine and bloodstream of ruminants for metabolism.

5.1 Benefits to industry

This project aimed to improve lamb survival which would have had significant benefits to industry in producing twin-born lambs at the same weaning weight as single-born lambs. If this can be replicated commercially, with an advantageous benefit cost analysis showcasing value of supplementing twin bearing ewes to producers this could drive improvements in lamb survival on a national scale.

6 Challenges, future research and recommendations

6.1 Challenges in delivering the current project

The major challenges faced to deliver the current project proposal are listed as follows:

1) Covid-19 Pandemic

Due to social distancing measures, restrictions and lockdowns at the University of Adelaide, the research group was not able to conduct the first experiment in early 2020. Instead, another trial (Safety trial) was conducted by our HDR student on the project in order to obtain additional information about the treatments to be tested in Experiment 1.

2) Rumen Protected Amino Acids

Initially, The University of Adelaide research team was tasked with the responsibility of developing rumen-protected amino acids for experiment 1. Unfortunately, the lead of this component was unable to continue working on this task due to medical reasons. The research team explored opportunities to collaborate with the University of South Australia to develop a feasible formula to rumen-protected amino acids but were unable to develop a viable solution or formulate a feasible formula that met the requirements of this project. In light of the above challenges, we had to reassess our options and explore other avenues to address our need to rumen-protect specific amino acids. A published paper describing the use of zein to rumen protect methionine was used. The methodology was improved by solubilising zein obtained from corn in an 85% ethanol solution prior to mixing with the amino acids of interest. However, this approach was not feasible for the continuation of this project given the high cost of zein ($157 per kg) making the cost of production too high.

3) Pilot rumen protected glutamine produced by a nutritional company

Due to the challenges to obtain a feasible formula to protect glutamine from microbial degradation, early on in the project we decided to explore collaboration opportunities with external nutrition companies. We reached out to several companies presenting our research project and explaining our requirements. Fortunately, there was significant interest from multiple companies who recognised the value and importance of rumen-protected amino acids and expressed their willingness to collaborate. However, a challenge arose when each company had its own internal processes,

priorities, and schedules, which made it challenging to find a synchronized timeline that worked for all parties involved. Despite this hurdle, we have continued discussions with one company that has shown particular promise and interest in collaborating in this area.

6.2 Future research

The finding that a rumen protected AA treatment (glutamine in particular) during late pregnancy of multiple bearing ewes can result in twin-born lambs reaching the same weaning weights as single-born lambs begs the question of what effect the AA (glutamine) treatment has on survival in twin and multiple-born lambs and this requires further investigation. As has been well established, the mortality of twin-born lambs is 2 to 2.5 times that of single-born lambs (Kleemann and Walker 2005; Hinch and Brien 2014). There are large economic benefits of improving lamb survival in general (Shepherd 2022). The largest proportion of those benefits derive from improving survival in twin and multiple-born lambs (Young et al. 2014). In addition, there is strong imperative to improve lamb survival from an animal welfare perspective. Indeed, animal welfare groups have recently announced their intention to commence a campaign on improving lamb survival. The findings of this project have given a very strong clue about how the growth performance of twin-born lambs can be made equivalent to single-born lambs and this needs to be extended to seeing whether AA supplementation also can lift twin-lamb survival to equivalent levels as that of single born lambs.

In order to further enhance our efforts in facilitating the adoption of rumen-protected glutamine, it is also imperative to conduct additional research to evaluate the bioavailability of the cost-effective rumen-protected glutamine developed by our research group. Simultaneously, we must engage with a nutritional company to ensure the commercial availability of rumen-protected glutamine in the future. Once the availability of rumen-protected glutamine is determined or a 'commercial pilot product' is developed, it becomes crucial to conduct an evaluation of the optimal timing for its supplementation and the potential outcomes that can be expected from this practice. This assessment will provide valuable insights into the most effective strategies for incorporating rumen-protected glutamine into nutritional protocols, thereby maximising its benefits. This comprehensive approach will contribute significantly to its successful adoption within the industry.

6.3 Recommendations

The involvement of commercial companies in developing a pilot supplement has introduced challenges and inefficiencies in moving forward with the research. These obstacles have the potential to compromise the outcomes of the research and hinder the progress of any HDR student involved in the study. The primary responsibility of the research group must be solely to conduct the necessary research to test the hypothesis presented.

7 References

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