# Effect of Branched-Chain Amino Acid Supplements on Pancreatic Development in Preterm Lambs

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A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy in Biomedical Science.

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# Abstract

**Background:** Babies born preterm frequently receive nutritional supplements after birth to support growth and development. Both preterm birth and rapid early growth are associated with adverse metabolic outcomes. Targeted nutritional supplements are needed to enhance organ development and function without excessive weight gain. Branched-chain amino acids (BCAA) are essential nutrients for the development of the endocrine pancreas, especially β-cells.

**Hypothesis:** Postnatal nutritional supplementation with BCAA will promote pancreatic development after preterm birth without excessive weight gain.

**Methods**: Preterm-born lambs (137-days of gestation, labour induced with dexamethasone) were randomised to receive daily oral BCAA supplements (leucine, isoleucine, and valine, ratio 2:1:1), calorie-equivalent maltodextrin, or volume-equivalent water for two weeks after birth. Term-control lambs (induced at 147-days) received volume-equivalent water. Weight, morphometry, and milk intake were measured. At 12-months of age, an hyperglycaemic clamp (HGC) with arginine-challenge was performed to assess pancreatic function. Plasma concentrations of insulin and glucagon were measured using ELISA. Ten days after HGC, pancreatic tissues were collected for immunohistochemistry to measure islet number and composition. Tissues were multi-labelled with antibodies against insulin, glucagon, somatostatin, Ki67, and caspase-3. Antibodies were visualized using the Vslide scanner system. Groups were compared using ANOVA with Tukey post-hoc test.

**Results:** BCAA supplements did not affect postnatal growth or milk intake. During HCG, baseline plasma glucose concentration was similar among groups; but higher in Maltodextrin-males than Maltodextrin-females (P=0.03). Baseline insulin concentrations were similar among groups, but higher in males than females (P=0.04). BCAA-males had higher insulin-to-glucose ratio (5-minutes post glucose infusion) than BCAA (P=0.01), Term (P=0.02) and Maltodextrin (P=0.04) females. Insulin sensitivity and insulin secretory response to arginine-challenge were similar among groups. Glucagon concentrations were highest in Maltodextrin-females throughout the clamp. There was no difference among groups or between sexes in insulin-, glucagon- or somatostatin-positive-area, β-, α-, or δ-cell number, or islet area and number. β-, α-, δ-cell-mass and islet mass were greater in males than females (P=0.008, P=0.01, P=0.003, and P=0,004, respectively). Ki67-proliferating cells and caspase-3-apoptotic cells were similar amongst groups and between sexes.

**Conclusions**: Preterm birth and BCAA supplements did not alter endocrine pancreas morphology, but sex-specific interactions between nutritional supplements and islet cell function in preterm groups indicate that nutritional interventions after preterm birth may also need to be sex-specific.

**Dedication**

*To My Father*

# Acknowledgments

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My sincere thanks

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# Abbreviations

| | |
|---|---|
| °C | Degrees Centigrade |
| µg | Microgram |
| µL | Microlitre |
| µm | Micrometre |
| × | Times |
| ABDO | Abdominal Girth |
| AC | Arginine Challenge |
| ADP | Adenosine Diphosphate |
| AMP | Adenosine Monophosphate |
| AMPK | Adenosine Monophosphate Protein Kinase |
| ANOVA | Analysis of Variance |
| ATP | Adenosine Triphosphate |
| AU | Australia |
| AUC | Area Under the Curve |
| BCAA | Branched Chain Amino Acid |
| BPD | Biparietal Diameter |
| BSA | Bovine Serum Albumin |
| $Ca^{2+}$ | Calcium Ion |
| cAMP | Cyclic Adenosine Monophosphate |
| Cat # | Catalogue number |
| CCD | Charge-Coupled Device |
| CHEST | Chest Girth |
| CIDR | Controlled Internal Drug Release |
| cm | Centimetre |
| CRL | Crown Rump Length |
| CV | Co-efficient of Variation |
| Cy5 | Cyanine 5 |
| Cy7 | Cyanine 7 |
| $D_2O$ | Deuterium Oxide |
| DAPI | 4',6-diamidino-2-phenylindole |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| F | Female |
| FIR | First Insulin Response |
| FITC | Fluorescein Isothiocyanate |
| g | Gram |
| GLUT | Glucose Transporters |
| GV | Growth Velocity |
| HGC | Hyperglycaemic Clamps |
| HL | Hind Limb Length |
| HT | Hock-to-Toe Length |
| IDAOs | Institutional Drug Administration Orders |
| IgG | Immunoglobulin G |
| IR | Insulin Receptor |
| IRS | Insulin Receptor Substrate |
| IS | Insulin Sensitivity |
| $K^+$ | Potassium Ion |

| | |
|---|---|
| Kg | Kilogram |
| L | Litre |
| LED | Light-Emitting Diode |
| m | Micrometre |
| M | Male |
| MANOVA | Multivariate Analysis of Variance |
| mg | Milligram |
| mL | Millilitre |
| mm | Millimetre |
| mmol | Millimole |
| mTOR | Mammalian Target of Rapamycin |
| n | Number |
| NaOH | Sodium Hydroxide |
| NZ | New Zealand |
| OCT | Optimal Cutting Temperature |
| PBS | Phosphate Buffer Saline |
| QC | Quality Control |
| r | Correlation Coefficient |
| rpm | Revolutions per Minute |
| SEM | Standard Error of the Mean |
| SIR | Steady-state insulin response |
| SOP | Standard Operating Procedures |
| TRITC | Tetramethylrhodamine |
| USA | United State of America |
| WHO | World Health Organization |
| α | Alpha |
| β | Beta |
| δ | Delta |

# Co-Authorship Forms

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| Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.<br><br>Chapter 3 of this thesis examines the effect of BCAA supplements on animal growth. It contains animal weight and morphometric measurements, including growth velocity, measurement of milk intake and organ weight at postmortem at 12 months of age. This chapter will be prepared as a manuscript for peer-review publication. The candidate will be first author on the manuscript. | |
| Nature of contribution by PhD candidate | Conducted the experimental work with support as needed, including induction of labour, lamb care, preparation of nutritional supplements, administration of the supplements, weight and morphometric measures, blood sampling, D2O preparation, administration and blood sampling, and organ collection and processing at postmortem. Performed data analysis and wrote the chapter |
| Extent of contribution by PhD candidate (%) | 80 |

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| Name | Nature of Contribution |
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| Anne Jaquiery | Designed and conducted animal experiments, helped with lamb birth and care, oversaw laboratory work, and critically evaluated the chapter |
| Eric Thorstensen | Advised on laboratory methodology and conducted D2O and amino acid sample analysis |
| Frank Bloomfield | Designed animal experiments, obtained funding, and critically evaluated the chapter |
| Jane Alsweiler | Advised on design, advisor for thesis and reviewed the chapter |
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*Last updated: 19 February 2021*

**Certification by Co-Authors**

The undersigned hereby certify that:

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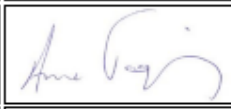
| Anne Jaquiery |  | 14-5-22 |
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| Nature of contribution by PhD candidate | Conducted the experimental work with support as needed, including intravenous catheter insertion, performing clamp test, collecting and processing blood samples, and conducting laboratory analysis of insulin and glucagon; analysed the data, and wrote the chapter |
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| Mark Oliver | Designed and conducted animal experiments, and critically evaluated the chapter |
| Hui Hui Phua | Helped with validation tests for insulin and glucagon ELISA |
| Frank Bloomfield | Designed animal experiments, obtained funding, and critically evaluated the chapter |
| Jane Alsweiler | Advised on design, advisor for thesis and reviewed the chapter |
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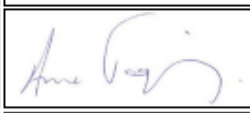
| Anne Jaquiery |  | 14-5-22 |
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|---|---|
| Nature of contribution by PhD candidate | Conducted the experimental work with support as needed, including collection of pancreas tissue and processing it for Immunohistochemistry (sample preparation, paraffin embedding, multiple labelling with insulin, glucagon, somatostatin, DAPI, Ki67, and active-caspase-3), performed imaging, and image / data analysis, and wrote the chapter |
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| Hui Hui Phua | Designed the multiple labelling, conducted image analysis, wrote the macro used in image analysis |
| Frank Bloomfield | Designed animal experiments, obtained funding, and critically evaluated the chapter |
| Jane Alsweiler | Advised on design, advisor for thesis and reviewed the chapter |
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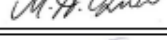
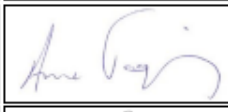
| Mark Oliver |  | 23-05-2022 |
| Anne Jaquiery |  | 14-5-22 |
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| Frank Bloomfield |  | 25/05/22 |
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# Chapter 1. Introduction

## 1.1.Preterm Birth

### 1.1.1. Epidemiology

Every year, an estimated 15 million babies are born preterm, which is more than 1 in 10 babies worldwide, and this number is rising (Figure 1-1) [1]. The causes of preterm birth vary from being iatrogenic (planned preterm birth often in a medical condition or pregnancy complication such as preeclampsia, fetal distress, or fetal growth restriction) to idiopathic (labour occurs spontaneously for unknown reasons) [2]. Around 40–50% of preterm births are idiopathic. There is a strong association between preterm birth complications and death in children under five years, accounting for an estimated 1 million deaths globally in 2015 [1]. Since birth occurs during a critical period for the development of metabolic systems and organ maturation [3], immaturity of multiple organ systems associates preterm birth with increased risk of developing metabolic diseases in adulthood such as type-2 diabetes [3-6], cardiovascular disease [7,8] and adiposity [9,10]. Several studies have reported a 17–43% increased risk of developing type 1 diabetes and a 25-35% of developing type 2 diabetes after preterm birth [11].

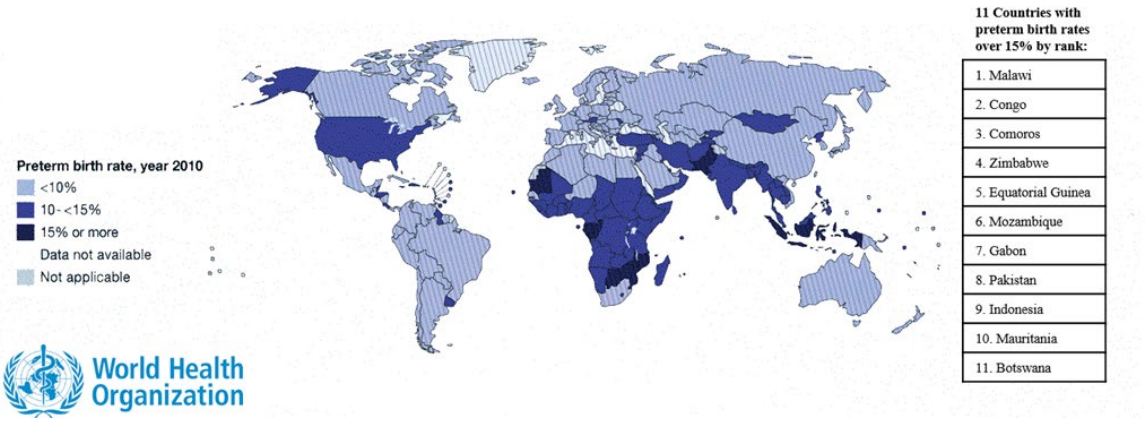


**Figure 1-1. Prevalence of preterm birth in the world, for the year 2010 [12].**
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### 1.1.2. Effect of Preterm Birth on Pancreatic Development and Function

Preterm birth can lead to immature pancreatic development with impaired β-cell function and mass [13]. A wave of β-cell apoptosis in late gestation may signify a change from fetal β-cells to more mature β-cells, preparing the fetus for extrauterine life [11]. Preterm birth is

associated with complications of both hypoglycaemia and hyperglycaemia. For example, in sheep, preterm birth is shown to reduce β-cell mass in lambs at four weeks after birth, a change that persisted until adulthood and was associated with a reduction in insulin secretion in four-week-old lambs and low insulin mRNA expression in the pancreas in adulthood (12 months) [13]. Because of limited glycogen and fat stores and impaired regulation of glucose-insulin metabolism, hypoglycaemia is common in preterm babies [14-16]. Conversely, hyperglycaemia can also occur because of impaired β-cell function and low insulin secretory capacity (inactive insulin or low amount) due to reduced absolute and relative β-cell mass and number, reduced volumes of insulin-sensitive tissues (fat and muscle) [13,16,17], and insulin resistance that is related to receptor defect [18], and/or reduced insulin receptors [19] resulting in alteration of the insulin signalling pathway [20]. In the presence of key nutrients, particularly glucose and amino acids, insulin and other pancreatic hormones are essential for fetal growth, and their homeostasis is necessary for healthy growth [16].

## 1.2. Pancreatic Composition and Development

The pancreas consists of an exocrine and an endocrine component. The exocrine pancreas consists of acinar cells and makes up the bulk of the pancreas, constituting as much as 90% of the organ. These cells secrete digestive juices rich in bicarbonate and contain digestive enzymes such as trypsin, pancreatic lipase, and pancreatic amylase, transported into the digestive tract. All these enzymes play essential roles in digestion and absorption [21]. Scattered between the acinar tissues, the endocrine pancreas is organised into discrete islets of Langerhans, composed of approximately 1000-2000 cells and comprising 1-5% of the pancreas [22].

The islets of Langerhans are responsible for maintaining glucose homeostasis by secreting critical hormones, including insulin and glucagon. Pancreatic islets contain five different endocrine cell types, which react to changes in plasma nutrient concentration by releasing a balanced mixture of hormones into the blood [23]. Each endocrine cell type is characterized by its own typical secretory granule morphology, different peptide hormone content, and specific endocrine, paracrine, and neuronal interactions. Glucagon producing α-cells represent ~15-20% of the islet, insulin-producing β-cells ~60-80%, somatostatin producing δ-cells ~5-10%, pancreatic polypeptide producing PP-cells less than 2%, and ghrelin producing ε-cells ~8% [22,24]. β-cells form a central core in the islet surrounded by other cell types. Glucagon allows glucose to be released from glycogen stored in the liver and muscle, promoting

gluconeogenesis, the *de novo* synthesis of glucose, and preventing cells from utilizing glucose in the circulation [25]. Insulin promotes glucose uptake and storage by insulin-sensitive tissues such as muscle and fat. Somatostatin inhibits the release of insulin and glucagon [26]. Pancreatic polypeptide is stimulated by food intake and pancreatic exocrine secretion [24,27]. It regulates gastrointestinal functions, including gallbladder emptying and exocrine pancreatic secretions [24,27]. Ghrelin is a key hormone involved in appetite regulation [28].

### 1.2.1. Insulin

Insulin plays an essential role in the fuel homeostasis of the entire body. Physiologically, insulin is secreted every five minutes (oscillation) by pancreatic β-cells regardless of blood glucose concentration [29]. Insulin is synthesized in the rough endoplasmic reticulum as a 110-amino acid precursor (pre-pro-insulin), then a 24-residue signal sequence is removed, forming proinsulin [30]. Pro-insulin consists of three chains, A, B, and C, and is stored in the Golgi apparatus in immature secretory granules, where it is converted into its active biological form by removal of C-peptide and stored or released as mature insulin consisting of 51 amino acids in two chains, A and B, connected by di-sulphide bonds [31,32]. (Figure 1-2). Insulin secretion can be stimulated by endogenous or exogenous substances such as carbohydrates, proteins, and lipids [33,34]. Glucose metabolism is activated after food intake leading to an increase in β-cell insulin secretion and suppression of α-cell glucagon secretion. Insulin lowers blood glucose concentration via a synchronized stimulation of glucose uptake in skeletal muscle and adipose tissue and suppression of glucose production in the liver [33,34].

Insulin is stored in vacuoles and released once triggered by increased blood glucose concentration [35]. When insulin is released from the vacuoles, it circulates in an unbound form with a half-life of 6-8 minutes, and it is cleared from the circulation system within 10-15 minutes [35]. Insulin binds to its receptor (insulin receptor, IR) in target tissues such as skeletal muscle, adipose tissue, and liver, which activates insulin receptor tyrosine kinase leading to auto-phosphorylation of insulin receptor substrates (IRSs) on multiple tyrosine residues [36,37]. Phosphorylation activates the mammalian target of rapamycin (mTOR) that mobilizes glucose transporters (GLUT) on the plasma membrane, initiating glucose uptake [37].

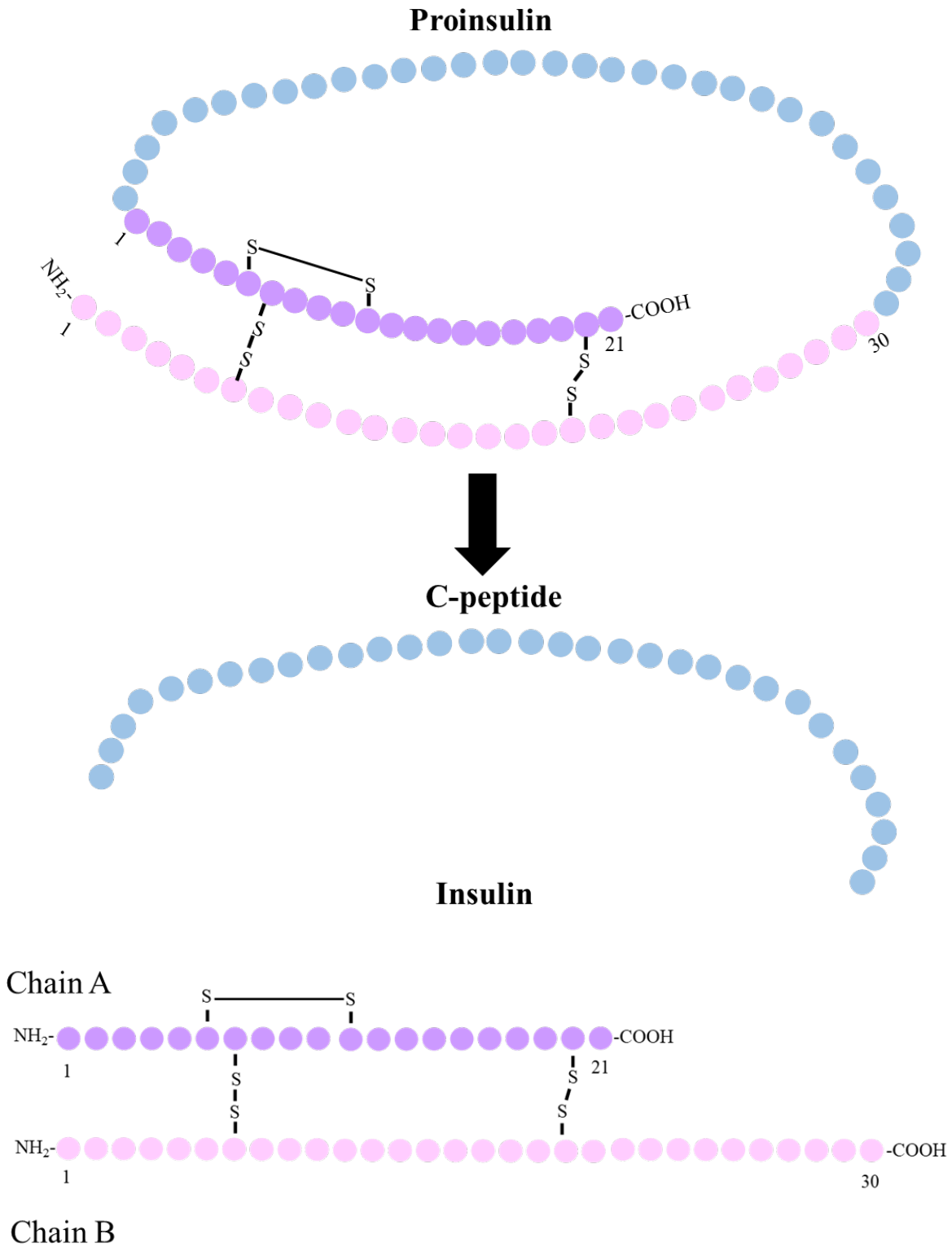



**Figure 1-2. Proinsulin, C-peptide, and insulin structure.**
$NH_2$ is an amine group; COOH: carboxylic group, and S-S: disulphide bond.

#### 1.2.1.1. Insulin Secretion

β-cell function includes insulin production, storage, and secretion. Insulin secretion is a calcium ion ($Ca^{2+}$) dependent process [38]. When the β-cell is electrically excited due to an elevation in blood glucose concentration, the cell membrane potential changes [31]. Glucose metabolism within the cell increases the cytosolic $Ca^{2+}$ fluxes, stimulating insulin granules to translocate and fuse to the cell membrane, secreting insulin into the circulation [39].

##### 1.2.1.1.1. Oscillation of Insulin Secretion

Insulin is secreted continuously in a pulsatile oscillation manner. This oscillation arises from the feedback cycle between the pancreas and liver [40] and recurs every 5 minutes [41,42]. The rapid insulin oscillation is disturbed in obesity and diabetes [40], causing a reduction in the rhythm amplitude [43]. After meal ingestion, continuous enteral nutrition, or intravenous glucose infusion, an ultradian oscillation of insulin secretion is stimulated [40,42]. This oscillation has a range of 50-135 minutes [40-42].

##### 1.2.1.1.2. Factors Affecting Insulin Secretion

Insulin secretion can be regulated by nutrients (Figure 1-3), neurotransmitters, and hormones that can stimulate or inhibit insulin production and release [44].

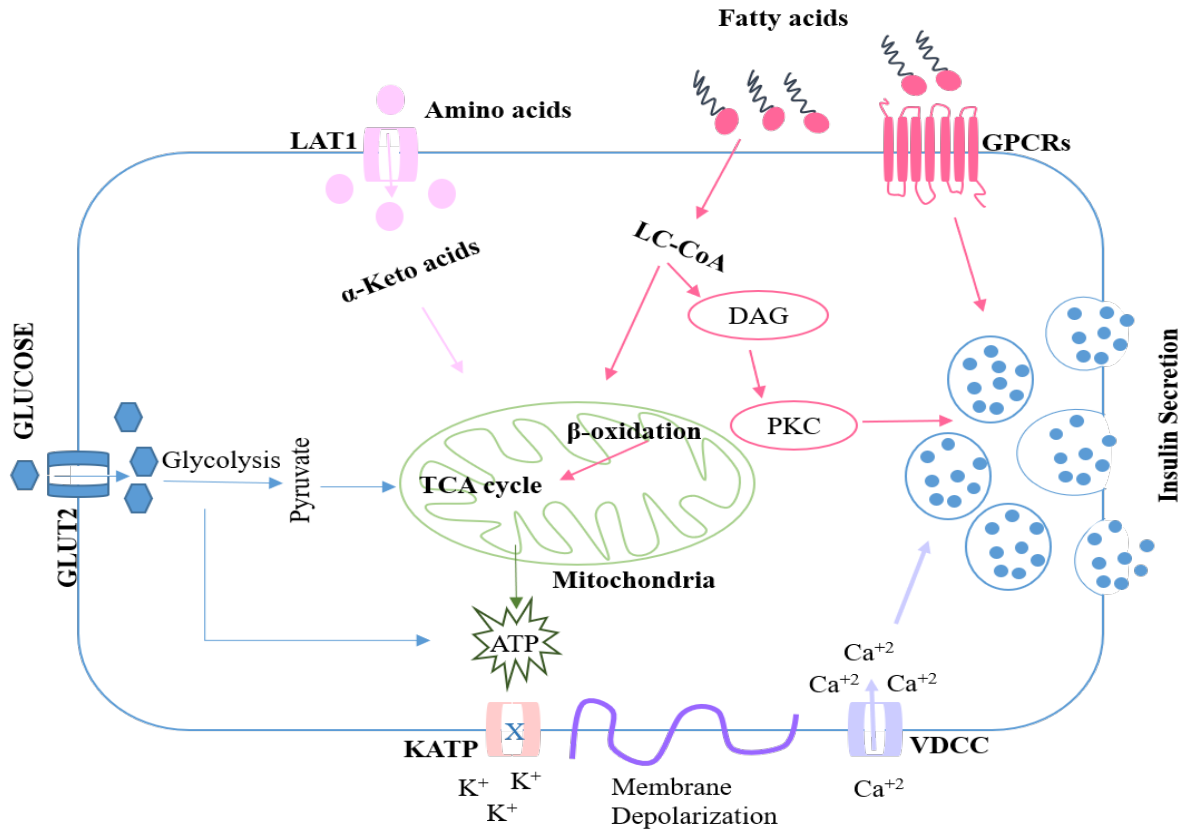



**Figure 1-3. Nutrients such as glucose, amino acids, and fatty acids stimulate insulin secretion in pancreatic β-cells.**
GLUT2: glucose transporter 2; TCA cycle: tricarboxylic acid cycle; ATP: adenosine triphosphate; LAT1: L-amino acid transporter 1; GPDRs: G-protein-coupled receptor; LC-CoA: long chain-CoA; DAG: diacylglycerol; PKC: protein kinase C; KATP: ATP-sensitive potassium channel; $K^+$: potassium ion; VDCC: voltage-dependent calcium channel; $Ca^{+2}$: calcium ion.

*1.2.1.1.2.1. Glucose*

Glucose is a hydrophilic molecule that is too big to pass across the cell membrane and therefore requires specific transporters or carriers [45]. These carriers are proteins known as GLUT (in mammals, also known as solute carrier family (SLC)) with 12 membrane-spanning helices that provide bidirectional glucose transport across the plasma membrane [45,29]. Glucose enters β-cells via facilitated diffusion through GLUT-2 [30]. GLUT-2 is also expressed in the liver, while GLUT-4 is expressed in adipose tissues and skeletal muscle [30]. Insulin plays a crucial role in glucose entry into cells. Binding to its receptor will activate and mobilize GLUT-2, stimulating glucose uptake into the cell [30].

Following glucose transport to the cytosol, glucose is phosphorylated into glucose-6-phosphate that stimulates oxidative and glycolytic metabolism in the mitochondria. Then,

glycolytic-derived pyruvate is converted into acetyl-coenzyme A and oxidized in the Krebs cycle, synthesising ATP. An increase in ATP/AMP ratio activates AMP-protein kinase (AMPK) and closes the ATP-sensitive potassium ($K^+$) channels, reducing $K^+$ efflux and depolarisation of the cell membrane. This process opens the voltage-operated $Ca^{+2}$ channels, increasing cytosolic $Ca^{+2}$ concentration and leading to insulin secretion via granule exocytosis.

Glucose in β-cells stimulates extracellular signal-regulated kinase (ERK) phosphorylation which leads to mTORC1 activation [46], which in turn regulates proinsulin biosynthesis predominantly by stimulation of pre-proinsulin mRNA translation [47]. It has been found that glucose stimulation regulates insulin gene transcription, translation, preRNA splicing, and mRNA stabilization [47].

*1.2.1.1.2.2. Lipids*

Lipids are the main component of cell membrane phospholipids and are stored as triglycerides which can be hydrolysed into fatty acids [48,49]. Fatty acids can cross the cell membrane by diffusion or via cell surface transporters [48,50]. Once inside the cell, fatty acyl-CoA synthetase activates fatty acids by adding a CoA group, generating long-chain acyl-CoA (LC-CoA) [48,50]. The LC-CoA enters the mitochondria, oxidizing it via the β-oxidation pathway producing acetyl-CoA. This acetyl-CoA enters the mitochondrial tricarboxylic acid cycle producing ATP, which affects the $K^+$ efflux causing an increase in $Ca^{+2}$ concentration leading to insulin release. However, fatty acids also exert essential signalling roles in controlling cell responses by activating G protein-coupled receptors at the cell surface, leading to vacuole exocytosis and insulin secretion [48-50].

*1.2.1.1.2.3. Amino acids*

Amino acid movement between cells and intracellular compartments is important in β-cell signalling [51]. Amino acids, especially branched-chain amino acids, regulate insulin secretion, lipolysis, and glucose metabolism [48,50]. They enter the cell via active transporters such as L-amino acid transporter [48]. Amino acids accumulated in the cell are converted into α-keto-acids and enter the mitochondria, going through the tricarboxylic acid cycle producing ATP, which regulates $K^+$-channels in the same way as glucose [51]. Amino acids stimulate protein synthesis via mTOR signalling and promote mitochondrial biogenesis that enhances cellular respiration and energy partitioning, in addition to the activation of AMPK signalling [52].

Arginine is an amino acid that can stimulate insulin and glucagon secretion via membrane receptors [53]. Arginine depolarizes the membrane, activates $Ca^{+2}$ influx, and stimulates insulin or glucagon secretion independent of ATP generation [54,55] and depending on glucose concentration [56].

*1.2.1.1.2.4. Hormones*

Several hormones contribute to the regulation of insulin secretion. Hormones such as glucagon, somatostatin, glucagon-like peptide, and glucose-dependent insulinotropic polypeptide stimulate insulin secretion by binding to their receptors on β-cells, stimulating the mobilization of intracellular calcium ions [35,57].

Adipokines are cell-signalling proteins produced by adipose tissue that stimulate insulin secretion [38]. Leptin and adiponectin both affect insulin secretion. After binding to their receptors on the β-cell, leptin inhibits insulin secretion [58], while adiponectin stimulates insulin secretion [59]. Cortisol, growth hormone, and the catecholamines (epinephrine and norepinephrine) can inhibit insulin action [60-62].

#### 1.2.1.2. Insulin Signalling

Insulin signalling is a biochemical pathway by which insulin exerts its action on insulin-sensitive tissues such as muscle, liver, and adipose tissue, as well as the islet cells. Insulin signalling is initiated when insulin binds to its receptor IR. The IR has two forms, A and B. IR-A functions primarily as a growth-promoting isoform during fetal life, whereas IR-B is the predominant isoform in adults [63]. The insulin signalling cascade is a reversible enzymatic reaction in which each kinase activation by insulin is inhibited by phosphatases [63]. When insulin binds to its receptor, protein kinase phosphorylates the tyrosine molecule on IR and IRS [64]. This phosphorylation translocates mTOR to the lysosome and activates insulin signalling [63,65].

### 1.2.2. Glucagon

Glucagon is a single-chain 29-amino acid peptide hormone released from α-cells. A decrease in the intra-α-cell adenosine triphosphate (ATP)/ adenosine diphosphate (ADP) ratio activates the adenylate cycle, increasing the cyclic adenosine monophosphate (cAMP). This increase stimulates protein kinase A, opening the calcium channels and increasing calcium concentration in α-cells, which triggers glucagon release [55]. It is released into both the systemic

circulation and the hepatic portal vein, having a short half-life of 10-15 minutes due to rapid degradation by proteolysis and enzymatic cleavage within hepatocytes [66].

Glucagon's main physiological role is to stimulate gluconeogenesis and glycogenolysis [67]. It prevents glucose storage as glycogen [68], which protects the body against hypoglycaemia during fasting and exercise. Similarly to insulin, glucagon secretion is primarily regulated by blood glucose concentration, leading to changes in intracellular ATP production [25].

Once released, glucagon binds to the specific glucagon receptor (GCGR) in the liver, the main target organ [66]. This binding activates adenylate cyclase, which catalyses the conversion of ATP to cAMP, leading to the activation of intracellular kinase stimulating glycogenolysis and gluconeogenesis [69]. In addition to glucose, glucagon secretion can be affected by fatty acids and amino acids [67]. Free fatty acids suppress glucagon secretion via a negative feedback system regulating ketogenesis, while amino acids stimulate glucagon secretion [69].

### 1.2.3. Somatostatin

There are two major circulating somatostatin isoforms, consisting of 14 and 28 amino acids, respectively [70]. Somatostatin-14 is formed in gastric, pancreatic, and neural tissue, whereas the intestinal epithelium synthesizes somatostatin-28 [71]. The pancreas accounts for 5% of total body somatostatin [72]. Its secretion from δ-cells can be stimulated by glucose and amino acids such as isoleucine [70]. Somatostatin's main effect is inhibition of insulin and glucagon secretion [73], although it regulates a wide range of physiological processes such as glandular secretion, neurotransmission, smooth muscle contractility, nutrient absorption, and cell division and growth [72,74].

An increase in somatostatin secretion occurs when blood glucose concentration falls, inhibiting insulin secretion and thus preventing insulin hypersecretion and hypoglycaemia [70,72]. An increase in glucose blood concentration can also stimulate somatostatin secretion, inhibiting glucagon secretion, preventing glucose production, glycogen breakdown, and hyperglycaemia [70,72]. Therefore, both an increase and decrease in blood glucose concentrations, can result in δ-cells secreting somatostatin, which binds to somatostatin receptors in β- and α-cells, inhibiting insulin and glucagon secretion [23 75]. This mechanism occurs through paracrine control [71,72], microcirculation through the islet [72], and gap junctions [72]. Somatostatin modulates insulin secretion during stimulation with glucose [71], with the effect on β-cells mediated through gap

junctions (cell-to-cell coupling) [71]. The gap junction connection between β-and δ-cells excites δ-cells (to secrete somatostatin) and β-cells (to secrete insulin) [76] [75]. The activation of β-cells suppresses α-cell activity, and this suppression is mediated through δ-cells [76]. In addition, somatostatin can inhibit glucagon secretion by binding to somatostatin receptors located on α-cells [25]. Somatostatin can also reduce insulin and glucagon secretion by reducing the rate of gene transcription post-transcriptional processing [72].

### 1.2.4. Pancreatic Polypeptide

Pancreatic polypeptide is a hormone consisting of 36 amino acids. Its secretion is stimulated by feeding (chewing). It is considered an anorectic hormone as it reduces energy demand and decreases the amount of food intake [77]. Plasma concentrations of pancreatic polypeptide increase significantly after a meal, particularly meals containing protein and fat, and remain elevated for up to 6 hours [77,78]. This hormone inhibits exocrine pancreatic secretion, insulin secretion, and gastric acid secretion and delays gastric emptying leading to a slow digestive and absorption process, therefore regulating food intake and body weight [77,78]. Pancreatic polypeptide levels negatively correlate with the body mass index (BMI) and body weight [79]. The effect of pancreatic polypeptide is mediated through binding to the G-protein coupled Y receptor superfamily, especially in the hypothalamus [77].

### 1.2.5. Ghrelin

Ghrelin is a 28 amino acid peptide. It is the only known orexigenic (affecting the hypothalamus and opposing leptin) hormone secreted by the islet cells [80]. It stimulates growth hormone secretion [80], influences sleep and eating behaviour, controls energy expenditure through orexin activity, affects gastric motility and acid secretion, and modulates endocrine pancreatic function and glucose metabolism [81]. Circulating ghrelin concentrations rise with fasting and fall after food intake, correlating negatively with insulin concentrations [81]. Ghrelin action starts with binding to the receptor ghrelin-secretagogue receptor type-1a, widely found in various organs such as the pancreas, liver, stomach, adipose tissue, intestine, immune system, and brain [82].

### 1.2.6. Pancreatic Development

Since the focus of this study involves pancreatic function in adulthood after preterm birth, it is necessary to understand pancreatic development during embryogenesis.

The pancreas is a composite organ that develops with an invagination of the distal foregut endoderm into dorsal and ventral buds, which fuse to form the single organ [83,84]. The pancreatic anlage is the earliest stage of development, detected by pancreatic and duodenal homeobox 1 (Pdx1) expressing dorsal and ventral domains in the posterior foregut endoderm [85]. Specification of the pancreas region begins when signals from the mesoderm and ectoderm establish the anterior-posterior pattern of the endoderm, where the anterior endoderm invaginates to form the anterior intestinal portal (AIP), which marks the foregut-midgut boundary [83]. When the anteroposterior patterning of the endoderm starts, endodermal progenitor cells migrate through the primitive streak, contributing to different parts of the gut and expressing different gene signatures [85].

The notochord separates the neural tube and the gut endoderm and signals the expression of Pdx1 in the adjacent pancreatic epithelium [83]. The dorsal aorta displaces the notochord and initiates pancreatic budding. It seems that the dorsal and ventral pancreatic buds display markedly different requirements for mesenchyme-derived fate-inducing signals [85]. Signals from the surrounding mesodermic tissue regulate the expression of transcription factors in the pancreatic epithelium and are responsible for the specification of endocrine versus exocrine tissues [86,87].

Once dorsal and ventral pancreatic buds develop, the buds morph into a rounded bud with a narrow neck connecting the pancreatic bud to the primitive gut tube through a central lumen [88]. The microlumens organized in rosettes later expand and fuse to generate an immature, highly interconnected tubular plexus consisting of stratified epithelial cells, evolving into highly branched ducts [89,90]. The Notch signalling pathway regulates the specification of endocrine cells in the developing pancreatic endoderm, a mechanism also involved in the specification of neurons in the developing neuroectoderm [85,91]. The pancreas is widely innervated by sensory and sympathetic and parasympathetic neurons [92]. In the developing pancreatic epithelium, individual cells or small cell clusters express neurog3$^{+}$, a member of the neurogenin gene family [85]. Neurog3$^{+}$ is expressed only in progenitor cells before islet formation and is undetectable in adult pancreas [93]. The endocrine specification occurs in two major waves, primary and secondary transitions [85]. The primary transition occurs during bud formation, while the secondary transition occurs during trunk plexus [94]. Neurogn3$^{+}$ endocrine precursors generated at the primary transition mainly produce α-cells, while other endocrine cells form during the secondary transition [85]. During embryonic stages, endocrine cells remain

close together with epithelial ducts forming endocrine cord-like structures, which undergo division to form immature islets in late gestation and the neonatal period [95]. In the sheep fetus, insulin has been found to be present in the islets of Langerhans at 40, 65, and 70 days of gestation [96].

### 1.2.7. Pancreatic β-Cell Growth

Pancreatic β-cell growth is a combination of cell replication from existing β-cells and β-cell neogenesis, where new β-cells are formed from pancreatic precursor (stem cells) [97]. Both increases in β-cell number (hyperplasia due to proliferation) and size (hypertrophy) contribute to increased β-cell mass [97]. During late gestation in humans, β-cell mass expansion occurs rapidly [29,98], suggesting fetal life represents a critical time in which a representative number of β-cells develop [98]. However, this number needs to be sufficient to compensate for the increase insulin secretion required with aging due to increased body weight and insulin resistance [98]. The average adult human pancreas has a β-cell mass of about 2% of pancreatic weight, with an approximate weight of the whole pancreas 60-100 g, of which β-cell mass accounts for 1–2 g [29]. There is a linear correlation between β-cell mass and body weight [98], signifying that β-cell mass increases to compensate for changing physiological requirements, such as in obesity or pregnancy. This ability to maintain β-cell mass during adulthood is necessary to maintain glucose homeostasis and prevent diabetes [97].

Although the size of the pancreas can be used to evaluate pancreatic development, this does not necessarily reflect endocrine function as the endocrine pancreas is a small part of the total pancreas. The number and size of the pancreatic islet cells can be used to measure pancreatic development that is more specifically related to pancreatic endocrine function [21].

### 1.2.8. Preterm Birth

Fetal life is a critical period for organ development, in which the appropriate number of β-cells are produced [98]. β-cells are formed during the third trimester and proliferate until about two years of age [99]. Intrauterine or extrauterine environment (preterm birth) can disturb the development of β-cell number [98] and β-cell mass [8], by which cell mass cannot compensate for the body's increased needs [98]. In addition, a dysregulated development of β-cell mass leads to low insulin secretion [8]. For instance, reduced β-cell mass after preterm birth is shown to be associated with reduced insulin secretion in juvenile sheep and reduced insulin mRNA expression in adult sheep [13]. Adults born preterm have low insulin sensitivity [100,101] and are

more susceptible to becoming diabetic due to poor fetal β-cell growth and short gestational age [6].

## 1.2.9. Factors Affecting β-cell Mass

### 1.2.9.1. Fetal Factors

Fetal β-cell mass and insulin secretion can be affected by maternal factors during pregnancy [11]. Maternal undernutrition in the periconceptional period has been shown to reduce fetal insulin secretion [102] and β-cell number [103] in sheep, and β-cell mass in rats [97,98]. In sheep, multiple pregnancies can be a factor, since β-cell mass is higher in single than twin fetuses [104]; this reduction in fetal β-cell mass can affect insulin secretion and sensitivity [104]. Maternal obesity, on the other hand, can induce alteration in fetal pancreatic β-cell numbers and structure [97]. In sheep, maternal obesity can reduce fetal pancreatic growth and β-cell numbers during late gestation [105].

Maternal exposure to corticosteroids may affect fetal growth, and organ maturation [106]. Studies have shown increased maternal concentration of corticoids to be associated with reduced fetal β-cell mass in rat [97] and mice models[107]. However, no such effect was found on β-cell mass in sheep [108]. Repeated doses of maternal glucocorticoid in sheep resulted in alterations in fetal glucose homeostasis [109], and increased basal insulin concentrations in offspring at two years of age [110]. When preterm birth was induced with glucocorticoid, a reduction in β-cell mass was found in adult preterm-born sheep compared to adult spontaneously-born term sheep [13]. However, induction of preterm birth without glucocorticoids resulted in a further reduction in β-cell mass compared with preterm birth induced with exogenous glucocorticoids [111], suggesting that glucocorticoids can partially mitigate the reduction of β-cell mass caused by preterm birth in sheep [111]. In humans, it was reported that preterm-born children exposed to antenatal glucocorticoids had decreased insulin sensitivity compared to term-born children (4-10 years of age) [3]. However, treatment with antenatal corticosteroids to accelerate fetal lung maturation in women at risk of preterm birth reduces the risk of perinatal death and neonatal death [112]. In a 30-year follow-up randomised controlled trial, no differences were found between those exposed to betamethasone or to placebo in body size, blood lipids, blood pressure, plasma cortisol, prevalence of diabetes, or history of cardiovascular disease [113].

β-cell mass can also be affected by factors after birth [11]. Perinatal malnutrition [98] or overfeeding [114] can affect growth leading to obesity, insulin resistance, glucose intolerance, and diabetes [98,114]. Malnutrition can lead to deregulation of fat metabolism resulting in lipotoxicity and insulin resistance due to lipid oversupply [115]. Preterm-born infants receive parenteral nutrition with glucose to maintain adequate glucose blood concentration to prevent hypoglycaemia [116]. However, this may lead to hyperglycaemia due to insulin resistance and/or insulin deficiency [116]. Higher concentrations of amino acids further increase insulin secretion [115], particularly branched-chain amino acids, which can stimulate an increase in pancreatic β-cell mass and function [117]. Glucose stimulates maintenance of β-cell mass by stimulating proliferation and inhibiting apoptosis [47]. Hyperglycaemia can exert its deleterious effects either directly (glucotoxicity) or by unveiling the harmful effects of fatty acids (glucolipotoxicity) [47].

## 1.2.10. β-cell Abnormalities and Malfunction

Changes in β-cell mass, number (hyperplasia), size (hypertrophy), and decline (atrophy) can all affect the production and function of insulin. Impaired glucose homeostasis is associated with decreased β-cell mass, insulin depletion, and reduction in insulin synthesis [29].

### 1.2.10.1. Insulin Resistance

Insulin resistance is a condition in which an insulin response is either suppressed or delayed due to an impaired cellular response to insulin in insulin-dependent tissues despite no impairment in insulin production [45]. Insulin resistance is inversely correlated to insulin sensitivity, which is the ability of insulin to exert its action in stimulating glucose uptake by the tissues and suppressing endogenous glucose production. Insulin resistance reduces insulin sensitivity in insulin-dependent tissues and disables glucose uptake, metabolism, and storage [36].

Pancreatic β-cells adapt to insulin resistance by increasing mass and function by cell hypertrophy [46,97,98] and/or hyperplasia [46,97]. If nutrient excess persists, hyperglycaemia (glucotoxicity) and elevated free fatty acids (glucolipotoxicity) negatively impact β-cell function [98]. This happens by numerous mechanisms, including the generation of reactive oxygen species [37], alterations in metabolic pathways [118], increases in intracellular calcium [118], and the activation of endoplasmic reticulum (ER) stress [118]. These processes adversely affect

β-cells by impairing insulin secretion [50], decreasing insulin gene expression [29], and ultimately causing apoptosis [97].

#### 1.2.10.1.1. Skeletal Muscle

Skeletal muscle provides mobility, supports and protects the skeleton, regulates body temperature, and has a role in glucose homeostasis [119]. It accounts for nearly 40% of body mass and is the largest insulin-sensitive organ, accounting for 75% of whole-body insulin-stimulated glucose uptake [120,121]. Skeletal muscle is composed of muscle fibres, including slow contractile type I fibres that utilize energy by oxidative metabolism and fast contractile type II fibres that use glycolytic metabolism for energy [121].

Insulin resistance in skeletal muscle is mediated by reduced insulin signalling and GLUT-4 translocation [119,122], meaning that insulin-stimulated glucose transport is reduced, leading to elevated postprandial glucose concentration and reduced glucose tolerance [122], causing impaired glucose homeostasis [121]. A decrease in fat oxidation and an elevation in free fatty acids leads to an accumulation of extra lipids in skeletal muscle. This accumulation increases fatty acid metabolism, oxidative stress, and inflammation, suppressing insulin signalling pathways and thereby reducing insulin-stimulated glucose uptake [119,120].

In obese and diabetic humans, GLUT-4 expression in skeletal muscle may be normal; however, an impairment in GLUT-4 translocation and docking or GLUT-4 fusion with the plasma membrane can cause a defect in glucose transport to the cell [36].

#### 1.2.10.1.2. Adipose Tissue (fat)

Adipose tissue predominantly comprises mature white adipocytes, essential insulin-regulated energy stores [38]. Adipocytes store energy in the form of triglycerides [65]. Upon physiological demand, the stored lipid will mobilize as fatty acids and be released into the blood, supplying the peripheral tissues (skeletal muscle and liver) during fasting or by adrenergic stimulation [123]. Insulin regulates adipocyte function since adipocytes are highly insulin-responsive [38]. Adipocytes are recognised as an essential endocrine cell type [38]. Insulin stimulates the storage of adipocyte triglyceride by stimulating glucose transport. Impairment of insulin signalling leads to downregulation in GLUT-4 on adipose tissue and elevated free fatty acids in the blood [36,122].

1.2.10.1.3. Liver

The liver maintains glucose homeostasis by releasing the endogenous glucose via gluconeogenesis when plasma glucose and insulin concentrations are low and glucose uptake and synthesis of glycogen when plasma glucose concentrations are high [98]. Normally, endogenous glucose production is suppressed by insulin. In hepatic insulin resistance, endogenous glucose production remains unsuppressed despite high plasma insulin concentrations, elevating glucose concentrations via enhanced gluconeogenesis and reduced glucose uptake [122]. When the liver takes up the additional circulating plasma free fatty acids, hepatic insulin resistance will develop due to fat accumulation resulting in fatty liver [122,45].

1.2.10.1.4. Mitochondria

The mitochondria in all tissues and cells are the energy-producing organelles [37]. Through glycolysis, mitochondria use glucose to produce energy in the form of ATP by coupling electron transfer from respiratory substrates to oxygen [46]. Reactive oxygen species (ROS) are a group of free radicals and molecules derived from molecular oxygen generated continuously by mitochondria as a by-product of oxidative phosphorylation [124,125]. ROS play an important role in both physiology and pathology of several cell types including β-cells. The cellular capacity of lipid oxidation is regulated by mitochondrial number, activity, and degree of coupling [123]. Dysregulation of mitochondrial function and population can lead to insulin resistance in skeletal muscle [123]. Due to loss in mitochondrial content and/or function, a decrease in mitochondrial oxidative capacity causes insufficient lipid oxidation, increasing excess lipid [123]. An increase in ROS formation and a decrease in in the antioxidant defences leads to oxidative stress [47]. Oxidative stress leads to cellular damage which plays a central role in the development of diabetic complications, insulin resistance and β-cell dysfunction [47]. An increase in oxidative stress triggers defective insulin signalling and leads to loss of skeletal muscle mass [37]. It has been shown that skeletal muscle mitochondria from diabetic patients have lower respiratory chain activity [123].

The impact of preterm birth on mitochondrial function has not been well assessed. However, a study in adults born preterm, found that mitochondrial respiration in adults born preterm consumed more oxygen than adults born at term, suggesting a correlation between preterm birth and impaired mitochondrial function [126].

#### 1.2.10.2. Diabetes

Diabetes is a metabolic disease manifested by elevated blood glucose concentration. There are two major forms of absolute or relative insulin deficiency and malfunction [121]. Type 1 diabetes is an autoimmune disease in which the immune system attacks β-cell and destroys it. Type 2 diabetes is caused by impaired β-cell function or/and decreased β-cell mass, and these two factors lead to low insulin secretion and impaired action. Another contributor to type 2 diabetes involves insulin resistance.

It has been estimated that, in 2014, 422 million adults globally were living with type 2 diabetes and, between 1980 and 2014, the prevalence of diabetes rose from 4.7% to 8.5% in the adult population [127]. It is estimated that diabetes is responsible for 1.5 million deaths per year [127].

##### 1.2.10.2.1. Risk Factors

The prevalence of type 2 diabetes is increasing due to increases in several risk factors. Changing lifestyle by increasing sedentary activities [128-130], unhealthy dietary patterns [128-130], and obesity [128,131] are key risk factors for developing type 2 diabetes. There are also genetic and metabolic factors related to ethnicity and family history [130,132]. Birthweight, early neonatal and childhood nutrition can affect metabolism and increase susceptibility to developing type 2 diabetes in adulthood [132-135]. Despite constant efforts, the number of patients suffering from type 2 diabetes, or its related chronic complications continues to rise.

##### 1.2.10.2.2. Complications

Diabetes is associated with long-term damage, dysfunction, and failure of various organs [60]. The major contributors to diabetes-associated morbidity and mortality are diabetic cardiomyopathy, retinopathy, and nephropathy [118].

## 1.3. Nutrition after Preterm Birth

It has been suggested that the postnatal growth of babies born preterm should be similar to a fetus of the same gestational age growing *in utero* [114]. However, the intra- and extrauterine environments are different [136], and preterm infants will therefore grow differently than fetuses [137]. Being born small, a preterm baby needs postnatal provision of adequate energy, protein, and nutrients for survival, growth, development, and long-term health [16,138], including essential

nutrients such as glucose, lipids, amino acids, and oxygen. [114]. Infant morbidity and mortality following preterm birth can be reduced through various postnatal interventions [1], among which nutrition is the key [139]. With inappropriate nutrition, those who survive are at increased risk of lifetime disability and poor quality of life [135].

Breast milk is the optimal nutrition [114,140]; however, the structural and functional immaturity of the gut, in addition to immature coordination of suckling, swallowing and breathing, often prevents full enteral feeding immediately after preterm birth [16,135]. Preterm-born babies frequently receive nutritional supplementation from birth to maintain an 'adequate' growth rate [114]. Parenteral nutrition (intravenous fluids) is used after birth until enteral feeding is established [16]. The most widely used intravenous fluids contain a high concentration of glucose [16,114,141], which may cause hyperglycaemia in preterms due to the inability to respond to a high glucose load [16,114]. This inability can be associated with low insulin secretion and/or low fat and muscle volume, all of which may impair glucose uptake [16]. Hyperglycaemia in preterm-born babies is associated with an increase in mortality and morbidity [141].

Many studies have demonstrated the necessity of including amino acids in the nutritional supplementation [114,135,142]. Amino acids are important for growth and development, especially for preterm neonates [135,142]. For example, in a randomised control trial, supplementation of preterm infants with amino acids during the first-two postnatal days resulted in better neurodevelopment outcomes in boys at two years of age [143]. In addition, preterm infants who received higher amino acid intakes in the first 52 hours of life had increased whole-body protein gain without evidence of protein toxicity [144]. Another study has shown that intravenous amino acid administration to preterm infants can increase head circumference [145]. Furthermore, since amino acids can stimulate insulin secretion, supplementing preterm infants with amino acids might promote insulin production, enhancing glucose metabolism [114].

## 1.4. Branched-Chain Amino Acids (BCAA)

The branched-chain amino acids leucine, isoleucine, and valine are three of nine essential amino acids with hydrophobic side chains that cannot be synthesized *de novo* in animals and humans and therefore must be acquired from the diet [146]. BCAAs can be utilized in the digestive system (lumen of the gut) by bacteria, and based on the 24-hour disappearance rates of amino acids, leucine has a high disappearance rate while isoleucine and valine have a medium disappearance rate [147]. BCAAs from dietary sources are absorbed from the intestines,

bypassing the liver, and are transported to the peripheral tissues [148]. Dairy products (milk, proteins), meat, fish, eggs, different beans, nuts, and whole-grain products are rich sources of BCAAs [149]. Leucine, isoleucine, and valine account for 15 to 25% of the total protein intake in these products [150,151].

Nutritional supplementation with BCAAs does not stimulate gluconeogenesis or increase glomerular filtration rate in the way other amino acids, such as alanine or a high protein diet have been reported to do [152]. BCAAs are essential for nitrogen synthesis [152] and are precursors for other non-essential amino acids such as alanine and glutamine [150,152]. BCAAs are the only amino acids not degraded in the liver [150,153]. However, the liver can oxidize BCAAs after their conversion to oxo-ketoacids in muscle and other target tissues [151]. Their catabolism is primarily by peripheral tissues, particularly muscle (skeletal) and adipose tissue [150,152,153].

It has been found that BCAAs function as signalling molecules that directly influence feeding behaviour, cell growth and differentiation [154], gene expression [155], energy production [150], and protein and neurotransmitter synthesis [152,156]. It has been suggested that the response to individual BCAAs and their effects might differ from the combination of the three [157]. BCAAs influence body composition by triggering muscle protein synthesis and growth via the mTOR signalling pathway [151,153] and increase diet-induced energy expenditure in skeletal muscle by increasing mitochondrial energy production [151].

All steps of the BCAAs catabolic pathway are located in mitochondria. The common metabolic pathway of the BCAAs starts with transamination, then oxidative decarboxylation, and finally with dehydration [152,158]. BCAAs represent about 50% of skeletal-muscle amino acid uptake [146]. This uptake of the BCAAs by the muscle tissues increases their intracellular concentration, which stimulates the transamination of BCAAs, a reversible reaction process by which an amino nitrogen transfer from BCAAs to pyruvate occurs, producing alanine [150,158]. The transamination reaction is initiated with α-ketoglutarate to form glutamate and branched-chain keto acids by branched-chain aminotransferase [148,159]. Then an irreversible oxidative decarboxylation reaction by the branched-chain ketoacid dehydrogenase complex produces acyl-CoA derivative that will initiate the final step, dehydration, in this catabolism. The end products of leucine are acetoacetate and acetyl coenzyme A, which are ketogenic; valine yields succinyl coenzyme A which is glucogenic, whereas the dehydrogenation products of isoleucine are acetyl coenzyme A and succinyl coenzyme A, therefore ketogenic and glucogenic products

[148,152,158]. The molecular events of intracellular BCAA uptake and BCAA oxidation are mainly regulated by AMPK activating mTOR that promotes protein synthesis, reduces protein degradation and increases cell growth [160,161]. BCAA also increases protein synthesis in the muscle through the Phosphoinositide-3-OH kinase/protein kinase (PI3K/Akt) pathway [161].

Amino acids represent key nutrients during intrauterine life [162]. It has been found that there is a large uptake of amino acids by the pregnant uterus and, similarly, a large uptake of amino acids by the fetus [162]. Enhanced metabolism of the BCAA to their corresponding keto acids was found in both human [163] and sheep [164] placentas with high BCAA transferase activity.

It appears that leucine is the most important BCAA in the process of protein synthesis, with leucine alone being almost equally as efficient as all three BCAAs together in stimulating protein synthesis in skeletal muscle [165]. An increase in leucine concentration is recognized via the insulin signalling pathway, which stimulates muscle protein synthesis [150].

### 1.4.1. Leucine

In comparison to other amino acids, leucine exhibits the highest insulinogenic index [153]. The stimulatory effect of leucine and isoleucine on insulin secretion is more effective in young people than in adults [152]. Leucine is taken up from the maternal circulation into the placenta and transferred to the fetus [162]. In fetal sheep [166,167] and humans (maternal and fetal) [168], leucine infusion stimulates insulin secretion. Leucine can impact β-cell metabolism and function [167,166] by stimulating pancreatic islet growth, development, and insulin secretion during fetal life [167,166].

Leucine effect influences glucose homeostasis and protein turnover [150]. As a metabolic fuel and allosteric activator of glutamate dehydrogenase, leucine stimulates insulin secretion by pancreatic β-cells through increased mitochondrial metabolism by oxidative decarboxylation and/or allosteric activation of glutamate dehydrogenase [153,169]. Glutamine is consumed highly in pancreatic islets and utilized for nucleic acid synthesis and protein synthesis, as well as being a mitochondrial metabolic substrate to promote insulin secretion [33]. Leucine is metabolized in β-cells completely by the mitochondria, initially by its transamination to α-ketoisocaproic acid and finally by oxidative decarboxylation of α-ketoisocaproic acid to acetyl-CoA; both acetyl-CoA and α-ketoglutarate are necessary as Krebs cycle substrates to activate the β-cell mitochondria [34]. This mitochondrial metabolism of

leucine with glucose in β-cells exerts a role in gene expression, protein translation ion channel activity, insulin secretory granule movement, and exocytosis [169].

It has been suggested that leucine stimulates protein synthesis in skeletal muscle through both insulin-dependent and independent pathways. Insulin stimulates leucine uptake and leucine simultaneously promotes insulin secretion *in vivo*. Together, leucine and insulin synergistically stimulate muscle protein synthesis [170]. The insulin-dependent mechanism is associated with the mTOR pathway, while the insulin-independent pathway is unknown [34,165,170].

Leucine activates mTOR, which is involved in ribosome biogenesis [153], protein synthesis, cell growth, and metabolism [155]. Leucine with insulin stimulates TOR complex 1 (TORC1) signalling in skeletal muscle promoting mRNAs translation; TORC1 is the nutrient-responsive mediator of cell mass and size regulation [170]. In the presence of amino acids, glucose can activate mTORC1 in rodent and human islets [153]. For example, in isolated rat islets, continuous exposure to glucose was associated with fully activated mTOR activity, thus supporting a crucial role of mTOR in β-cell proliferation [171].

Leucine not only affects insulin and glucose homeostasis, but it can activate TORC1 to suppress additional food intake by promoting leptin synthesis in adipose tissue that affects immediate and long-term adequate nutrition [170]. As leucine can activate the production of amino acids, it can also stimulate valine and isoleucine catabolism [156].

### 1.4.2. Isoleucine

Isoleucine is an isomer of leucine. It is a ketogenic and glucogenic branched-chain amino acid. Isoleucine has a possible role in regulating adiposity through lipid mobilisation from tissues without affecting food intake [172]. It also regulates glucose homeostasis [172], lowering glucose concentration by stimulating skeletal muscle glucose uptake *in vivo* [173] and increasing glucose oxidation within the whole body without an elevation in plasma insulin [174 175 176]. Furthermore, it can enhance protein expression of GLUT1 and GLUT4 *in vitro* [173,174]. Since it plays an essential role in the protein metabolism pathway, it affects growth parameters and lysosomal enzymes [177] and facilitates body energy production [178].

### 1.4.3. Valine

Valine is a glucogenic amino acid metabolized to propionyl-CoA, which is converted to succinyl-CoA, which enters the Krebs cycle [179-181]. It can modulate glucose metabolism by stimulating the hypothalamus [179]. In an *in vitro* experiment, valine (800 nmol/mL) improved the dendritic cell function in cirrhotic patients, suggesting that valine has an immunological function and may have a role in nutritional therapy, especially for patients with cirrhosis [182]. It has been found that valine and insulin have independent pathways, by which valine concentration has no association with HOMA-insulin resistance [183]. However, in the presence of leucine, isoleucine, obesity, and diabetes, this pathway changes. The mechanism through which valine functions is still unknown.

### 1.4.4. BCAA and Fetal Growth

Nutrition is vital for the fetus's early growth and development. Amino acids can cross the placenta to the fetus, where they are used for fetal growth [184,185]. Compared with healthy fetuses those with intrauterine growth restriction have lower plasma concentrations of amino acids [186] and BCAA in the umbilical artery and vein [187]. Insufficient amino acid concentration during pregnancy can affect fetal growth, contributing to the pathogenesis of islet dysfunction in fetuses [186]. Decreased fetal amino acid supply causes a reduction in fetal insulin concentration and secretion, β-cell mass, islet size, and vascularity [97,98,103,107,186]. BCAA are essential elements in fetal development [147]. BCAA activate the mTOR signalling pathway controlling β-cell differentiation during fetal life [155] and stimulating protein synthesis [147,155]. Amino acids, especially BCAA, are essential for glucose and insulin activation of mTOR in β-cells [169]. So, with nutrient signalling, insulin, insulin-like growth factor-1, and other growth hormones such as growth hormone and parathyroid hormone exert a role in regulating the mTOR pathway. This shows that mTOR incorporates signals from diverse stimuli such as mitogens and nutrients to regulate cell metabolism, growth, and proliferation [171].

### 1.4.5. BCAA Supplementation

Amino acids, such as BCAA, influence the secretion of hormones that regulates anabolic pathways and tissue and lipid deposition during early growth [188]. Accumulating studies indicate that protein-rich diets containing BCAA improve muscle protein synthesis and body composition [151], especially important in preterm babies. Beyond the neonatal period, there are positive effects described on glucose metabolism [167] and muscle growth of high BCAA intake, particularly with leucine [189]. In adult humans, BCAA supplementation at the highest intake is associated with a decrease in the risk of diabetes [190].

### 1.4.6. Potential Adverse Effects of BCAA Supplementation

There are also some potential adverse effects of BCAA supplementation. An increase in plasma leucine, isoleucine and valine was detected among overweight/obese individuals compared to normal-weight individuals [183]. High leucine concentrations were positively associated with high protein intake, elevated fat storage, and increased weight gain [191]. A low protein diet (6% protein) supplemented with 3% leucine can cause growth retardation and decrease relative liver weight in male Sprague–Dawley rats [192].

A positive correlation has been found between BCAA concentration and body mass index (BMI), which associates obesity with an increase in BCAA concentration in children and adolescents [193]. A study in human adults (obese and lean) fed a high-fat diet supplemented with BCAA found that an increase in plasma BCAA concentration correlated with insulin sensitivity and body weight associated with obesity-related insulin resistance, meaning that BCAA levels were high in obese subjects only [194]. An increase in plasma BCAA concentration was associated with diabetes [195] and obesity due to the dysregulated BCAA catabolism that resulted in the elevated plasma BCAA concentrations [157]. However, findings among studies are inconsistent, with one study suggesting that leucine represses β-cell development in rat embryonic pancreatic cell culture by activating the mTOR signalling pathway [155] and, controversially, others findings that leucine had no significant effect on plasma insulin concentration [21,196].

## 1.5. Summary

In humans, preterm birth occurs before 37 weeks of pregnancy, a time when many vital organs are structurally and functionally immature [16]. The early transition to extrauterine life can therefore alter organ development. One of the organs affected by preterm birth is the pancreas [11]. The pancreatic islets secrete insulin from β-cells and glucagon from α-cells, among other hormones. Insulin and glucagon are potent regulators of carbohydrate metabolism and therefore of growth. Their interaction is the primary determinant of gluconeogenic and glycogenolytic flux in the body [30]. Preterm birth in humans is associated with an increased risk of impaired glucose homeostasis, including hypo- and hyperglycaemia in the neonatal period [17,18,197], decreased insulin secretion [13], increased insulin resistance [100,198], and an increased risk of developing type 2 diabetes in adulthood [5,199,200]. These studies suggest the association of preterm birth with impaired pancreatic development. Nutritional supplements are often given to preterm babies to promote growth and development. BCAA leucine, isoleucine, and valine, are key nutrients for pancreatic islet development. Targeted supplementation with BCAA after preterm birth may therefore improve pancreatic function long-term.

## 1.6. Thesis Hypothesis

The hypothesis for this thesis is that postnatal nutritional supplementation with BCAA will ameliorate the reduction in adult β cell mass that has been observed following preterm birth. In addition, we hypothesise that the amino acids, and not an increase in calories, will restore β-cell mass and islet function.

## 1.7. Thesis Aims

Specifically, the aims of this thesis were:

1) To investigate the short- and long-term effects on growth and milk intake of postnatal BCAA supplementation for 15 days after birth in preterm-born lambs (chapter 3).

2) To investigate the effect of BCAA supplementation on endocrine pancreas function, including insulin and glucagon secretion and insulin sensitivity, at one year of age in preterm-born lambs (chapter 4).

3) To investigate, at one year of age, the effect of BCAA supplementation on β-cell, α-cell, δ-cell, and islet number and mass and the ratio of proliferation-to-apoptosis in β-cells and α-cells (chapter 5).

## 1.8. Why Sheep?

There are several reasons for performing this study on sheep rather than other animal models such as rodents. Similar to humans, they have natural singleton-bearing pregnancies [201], and a more extended gestational period (150±3 days) [202] compared to small animals (25±3 days) [201,202]. The offspring are of a similar size to human infants at birth. The gestational timing of the growth and maturation of major organs such as the lungs, brain, kidney, pancreas, and heart is similar to that of humans', such that the stage of organ development in relation to birth is also similar [203], although the pre-birth maturation window is compressed in sheep. In both humans and sheep, pancreatic development occurs before birth, while in rodents, it occurs in the early postnatal period [96,108,203,204]. In humans, insulin and glucagon are first detected in the fetal pancreas on day-70 and 42, respectively (term = 280 days), while in sheep, they are both detected on day-40 (term =150 days) [202]. In sheep, preterm labour and birth can be induced using glucocorticoids [108,201], and with appropriate postnatal care, preterm lambs can survive into adulthood.

# Chapter 2. Research Design and Methods

## 2.1. Ethical Approval

The study was approved by the Animal Ethics Committee of the University of Auckland and registered under the identifier 001873. All experiments were conducted in accordance with the Animal Welfare Act (New Zealand (NZ), 1999), Ministry of Primary Industries Codes of Animal Welfare, National Animal Ethics Advisory Committee guidelines, Institutional standard operating procedures (SOP) and Ngapouri Research Farm SOP705 (appended).

## 2.2. Research Design

### 2.1.1. Subject

Preterm-born lambs (137 days' gestational age) and term-born lambs (148 days' gestational age).

### 2.1.2. Design

All experiments were conducted under modified ARRIVE guidelines [205]. Singleton-bearing ewes of known gestational age were randomised to term or preterm delivery. Preterm (137 days' gestational age) and term-born (148 days' gestational age) lambs were used in this experiment, generated during two consecutive lambing periods from June 2017 to November 2018. At birth, lambs were randomised into four groups stratified by sex. Group 1 (Term/control), group 2 (Preterm/BCAA), which received an oral nutritional supplement containing branched-chain amino acids (BCAA); group 3 (Preterm/Maltodextrin), that received an oral nutritional supplement containing Maltodextrin, and group 4 (Preterm/control/Water). Control groups received an equivalent volume of water. Each group included from five to seven animals of each sex.

A total sample size of 80 ewes was chosen for the pre-delivery group. A random block size (computer-generated by a study statistician) was modified to account for unexpected losses in the preterm groups, of 4 or 8 ewes was used with a ratio of 3:1 preterm: term delivery. After male and female preterm lamb delivery, a random block size of 3 or 6 was used with a ratio of 1:1:1 per preterm group and sex (the allocation was done by sequentially numbered opaque envelopes). The loss of preterm animals in 2017 was greater than expected due to failed

induction of labour, preterm lambs' death, and lactation failure; hence the preterm/term ratio was changed to 9:1 in 2018. The number of seven animals per group was calculated to provide no less than 90% power at a 5% level of significance to detect a 50% increase in pancreatic β cell mass in preterm lambs [from mean (SEM) of 1.1 (0.3) to 1.65 (0.3) g; term β cell mass 3.1 (0.3) g], based on previous data [13].

Lambs were given the nutritional supplements for a period of 15-days whilst continuing to suckle from their mothers. At birth there is a shift from *in utero* amino acid maternal supply to mix nutrients (amino acids and glucose) absorption through feeding. β-cells need to adapt to this shift in the nutritional environment and this occurs within the first 15-days after birth [206]. At the end of the supplementation period, lambs were returned to pasture with their mothers, then brought indoors for monthly blood samples and growth measurements. Lambs from all groups were weaned at three months of age. At 12 months of age (adulthood), lambs were acclimatised to indoor conditions and underwent a hyperglycaemic clamp to determine maximal insulin secretory response and sensitivity. After ten days, lambs were euthanised and organs harvested for further analysis (Figure 2-1).

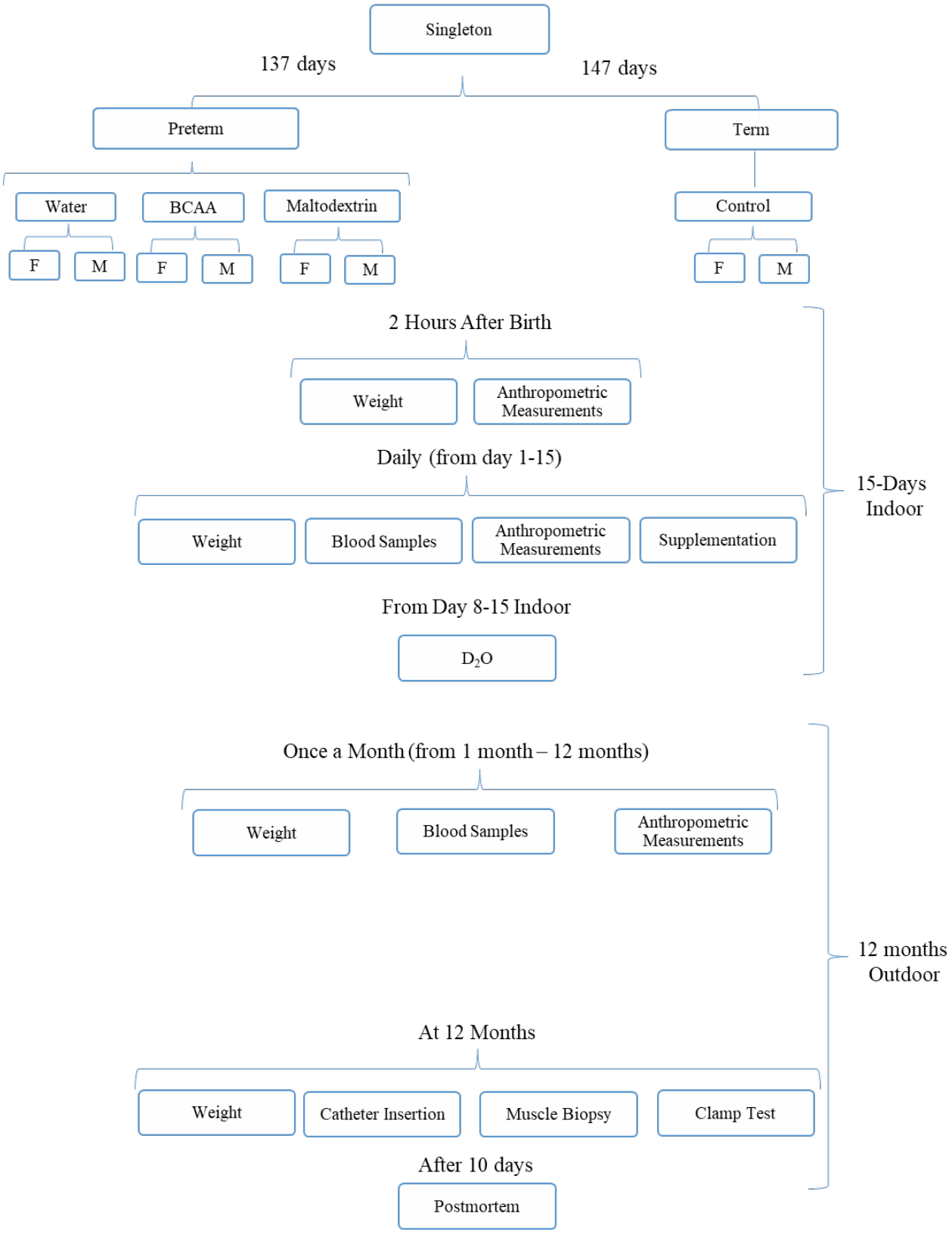



**Figure 2-1. Experimental design from birth until 12 months of age.**

F: female; M: male; $D_2O$: Deuterium Oxide

## 2.3. Animal Care

All animal husbandry and experimental procedures were performed within the framework of Ngapouri Research Farm Laboratory SOP705. This SOP covers selection criteria, health and welfare management, ethical standards, acclimatisation to indoor housing and concentrate feeds, grazing and restricted medicines use.

### 2.3.1. Breeding

Four to five-year-old Romney ewes were managed at Ngapouri Research Farm, Reporoa, NZ. Ewes were bred outdoors. Oestrus was synchronised with intravaginal controlled internal drug release devices (CIDRs, Zoetis, Auckland, NZ) containing 0.3 g progesterone. After 14 days CIDRs were removed, then two days after the removal, ewes were placed into a breeding paddock with Poll-Dorset rams for two days. Gestational age was calculated from the day the rams were introduced. Shorter daylight hours stimulate the breeding season, and the ovulation rate is at a maximum around the shortest day in autumn. The induction of ovulation occurred between June and February. For groups mated outside the natural breeding season, ewes were treated with intramuscular gonadotropin (2.5 mg pregnecol injection, serum gonadotropin, Vetoquinol, Queensland, Australia (AU)) to induce ovulation. Ultrasound scanning was carried out at 40 and 70 days after mating to distinguish between pregnant and non-pregnant (dry) ewes and confirm singleton pregnancy. Ewes with multiple pregnancies were excluded from this study. Following paddock-based feed, ewes were acclimatised to indoor conditions with a complete pellet feed (Uni C, Dunstan Nutrition Ltd, Hamilton, NZ) and 12-hour-controlled photoperiods for two weeks before lambing. The ewes were placed in group pens for the first ten days, then shifted to clean individual pens with open mesh sides for continuous visual and auditory contact with other ewes. Individual feeding aligned with recommended nutritional standards for pregnant or lactating animals was provided daily with free access to water. All ewes were checked daily; any problems with welfare or acclimatisation were identified and managed according to SOP705.

### 2.3.2. Induction of Labour

Acclimatised singleton-bearing ewes with known gestation were randomised to their experimental group (preterm/ term birth). Using a previously published model of dexamethasone-induced preterm birth by our group [13,207-209], labour was induced in all animals

with two injections of intramuscular steroid (dexamethasone sodium phosphate; DEXA 0.2, Phoenix pharm, Whakatane, NZ), approximately 12 hours apart, at a dose of 0.25 mg/kg as per Institutional Drug Administration Orders (IDAOs). Induction of labour commenced on day 135 of gestation to induce preterm birth at 137-138 days or on day 145 to induce term birth at 147-148 days. From the evening of day 137, pregnant ewes induced to give birth preterm were under observation for signs of labour and monitored closely until the lamb was born. Assistance with the delivery was provided when necessary.

### 2.3.3. Lamb Care

When lambs were born (term and preterm), time was given for ewe-lamb bonding to occur, allowing the ewe to lick and smell the lamb. Handling the lamb was postponed until after the bonding period, during which intervention only occurred if required for ewe or lamb welfare. The first breeding group lambed in May and the last in November, a period which included the coldest months of the year with overnight temperatures often around 0$^{o}$C. The farm feedlot is not a controlled temperature unit, and the pens have an open mesh floor to maintain pen hygiene. To reduce thermal stress, lambs were dried using a towel, had a woollen jacket put on, and were cared for in a pen with their mother. Weight was recorded. The temperature was checked to check if the lamb looked unwell. If a lamb was hypothermic, it was kept in a box, in its ewe's pen, containing a hot water bottle wrapped in a towel, and covered with a foil hypothermia blanket.

One 60 mL feed of artificial colostrum (newborn pure colostrum, Halen Health, Christchurch, NZ) with a concentration of 0.1 g/mL warm water was given in the first 6 hours after birth for both term and preterm lambs. The lambs were allowed to feed *ad libitum* from the ewe. Expressed ewe milk was given if lambs were unable to feed spontaneously, and this care was extended to three to five days depending on how long it took the lamb to feed independently. A welfare monitoring sheet for each lamb was utilised for recording expressed milk, body weights, and any feeding assistance required. Two ear tags were placed for lamb identification. If a lamb died or had to be euthanised, a diagnostic post-mortem was performed, and organs were examined. The lambs remained in the feedlot with their ewes for 15-days until they had completed the intervention period.

## 2.4. Methods

### 2.4.1. Nutritional Supplementation

At birth, lambs were randomised into four groups stratified by sex (Term, BCAA, Maltodextrin and Water). Lambs were given the nutritional supplements for a period of 15-days whilst continuing to suckle from their mothers. All supplements, including water, were administered daily in two divided doses via a syringe for 15-days from birth. The control (Term) received water in different volumes starting with 20 mL for the first three days, then 30 mL until the 7$^{th}$ day, and 40 mL until the 14$^{th}$ day. The three preterm experimental groups were either given BCAA (Ajinomoto, Japan, imported by Dunstan Nutrition Ltd, Hamilton, NZ), Maltodextrin (Interchem, Auckland, NZ), or water. BCAA supplementation contained leucine, isoleucine, and valine in a ratio of 2:1:1, suspended in water in similar volumes to control. The Maltodextrin was dissolved with an equivalent volume of water (Table 2-1). Both supplements (BCAA & Maltodextrin) were isocaloric.

**Table 2-1. Nutritional supplementation. The amount of water, BCAA, and Maltodextrin doses prepared and used for the first 15-days after birth.**

| Days | Body weight (Kg) | Milk intake (mL.day$^{-1}$) | BCAA (g.day$^{-1}$) | Maltodextrin (g.day$^{-1}$) | Water (mL) | Dose/day |
|---|---|---|---|---|---|---|
| 1-3 | 6 | 600 | 5.70 | 9.60 | 20 | 2 |
| 4-7 | 8 | 960 | 9.12 | 15.36 | 30 | 2 |
| 8-14 | 10 | 1500 | 14.25 | 24.00 | 40 | 2 |

#### 2.4.1.1. Calculation of Nutritional Supplementation

The composition of ewe milk is different between term and preterm birth (Table 2-2) [210]. The estimated term ewe milk protein was 6.0 g/100 mL, and the whey protein isolate supplement was 3.8 g protein/100 mL estimated milk intake. The whey protein isolate comprised 25% BCAA, equivalent to 950 mg BCAA/100 mL of milk. This would be further broken into the targeted leucine, isoleucine, and valine in a ratio of 2:1:1. Each gram of BCAA yields 4 Kcal, while Maltodextrin (the carbohydrate calorie equivalent) powder contains 94% of carbohydrates and each gram of carbohydrate yields 4 Kcal. Supplements were estimated

based on birth weight and milk intake. For days 1-3 of birth, the estimated milk intake was 100 mL.Kg$^{-1}$.day$^{-1}$, for days 4-8, was 120 mL.Kg$^{-1}$.day$^{-1}$ and 150 mL.Kg$^{-1}$.day$^{-1}$ for days 8-14. To estimate the doses, we used the following equation:

$$\text{Dose} = \frac{\text{Milk intake (mL.day}^{-1}) \times \text{BCAA (0.95 g)}}{100} \quad \text{OR} \quad \frac{\text{Milk intake (mL.day}^{-1}) \times \text{Maltodextrin (1.6 g)}}{100}$$

**Table 2-2. Composition of ewe milk.**

| | Term ewe milk/100 mL | Preterm ewe milk /100 mL |
|---|---|---|
| **Protein (g)** | 6.0 | 5.2 |
| **Fat (g)** | 7.0 | 9.2 |
| **Carbohydrate (g)** | 5.3 | 4.3 |
| **Energy (Kcal)** | 108 | 121 |

## 2.4.2. Morphometric Measurements

Morphometric (linear) assessments (including body weight) were taken from term and preterm born lambs within 12 hours of birth on day one, then on days 4, 8, 11, 15, 30, and once monthly thereafter. After being weighed, the lamb was placed in an adjusted cradle with limbs hanging free. The morphometric measurements were taken with a measuring tape (Figure 2-2).

| | | |
|---|---|---|
| Crown-rump length | : CRL | The length from the mid-orbital peak (top of the head) to the *ischiatic tuber* (the junction of the spine and tail) |
| Chest Girth | : CHEST | The chest circumference at the peak of the spinous processes (withers), posterior to the forelimb (shoulder and elbow) |
| Abdominal Girth | : ABDO | The distance around the abdomen at the largest portion between the last rib and the pelvic limb |
| Hock-to-toe length | : HT | The length from the top of the hock joint (*calcaneal tuber*) to the end of the hoof (toe, *distal phalanx*) |
| Hind limb length | : HL | The length from the top of the hip joint (*greater trochanter*) down to the tibiofemoral joint, to the hock and end of the hoof |
| Biparietal diameter | : BPD | The width posterior to the orbital sockets (eyes) |

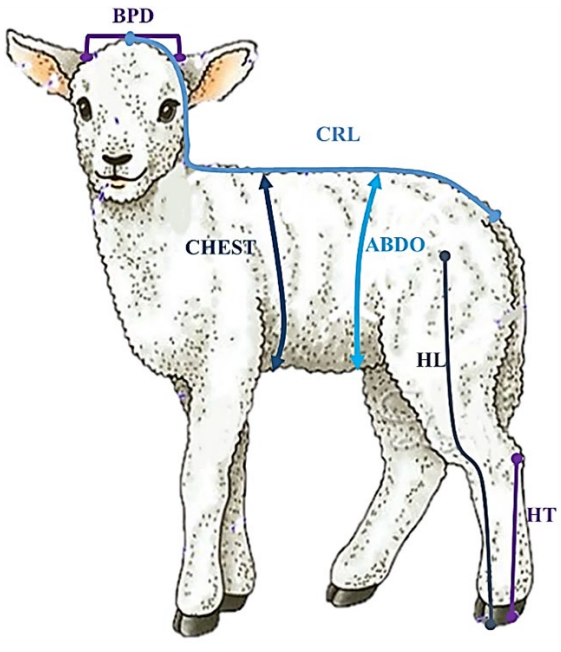



**Figure 2-2. Morphometric measurements.**
From day one after birth until 12 months of age, morphometric measurements were taken by measuring tape or calliper. BPD: Biparietal diameter, CRL: Crown-rump length, CHEST: Chest girth, ABDO: abdominal girth, HL: Hind limb length, and HT: Hock-to-toe length.

## 2.4.3. Growth Velocity

Growth velocity (GV) measures weight gain over a period of interest, represented by grams per kilogram per day. It is derived from the exponential relationship between the initial weight (W1) and the weight at the required time point (Wn) over time (D), representing the day of life. A factor of 1000 was used to correct units to $g.Kg^{-1}.day^{-1}$ [211].

$$GV\ (g.Kg^{-1}.day^{-1}) = [1000 \times \ln (Wn / W1)] / (Dn-D1)$$

Growth velocity of linear measures (CRL, CHEST, ABDO, HL, HT, and BPD) was calculated in the same manner.

## 2.4.4. Blood Samples

Heparinised blood samples were collected using vacutainers (Greiner-bio-one, Kremsmünster, Austria) simultaneously with morphometric measurements during days 1, 4, 8, 11, 15, 30, and then once monthly. Blood samples were taken by jugular venepuncture. Blood samples were kept on ice, then centrifuged at 3780 rpm for 10 minutes at 4°C. Plasma samples were collected and stored at -80 °C for further analysis.

## 2.4.5. Deuterium Oxide Test ($D_2O$)

Lamb milk intake was assessed by $D_2O$ (Sigma-Aldrich, Auckland, NZ) dilution to investigate whether intake was affected by the supplement intervention. $D_2O$ is a stable isotope of water distributed in total body water; therefore, it measures total body water. Water intake at one week of age is a result of milk intake from the mother. Therefore, milk consumption can be estimated by measuring water's total body turnover using $D_2O$ dilution [212].

On day eight after birth, lambs were weighed, and baseline blood samples were collected. A 60% $D_2O$ solution was prepared by adding 0.9 g of sodium chloride (NaCl), 40 mL MilliQ water, and 60 mL of $D_2O$. Before injection, the solution was filtered (0.2 µm in-line Luer-lock filter, Sigma-Aldrich, Auckland, NZ). Based on the lamb weight, a dose of 0.5 $mL.kg^{-1}$ $D_2O$ solution was prepared for each lamb, and the syringe containing the solution was weighed (S1). $D_2O$ solution was injected into the lamb's jugular vein, and the time was recorded. The empty syringe (S2) was weighed to calculate gravimetrically the actual amount of $D_2O$ administered. Blood samples were collected at 2- and 6-hours post-injection and on the morning of days 2, 3, 4, 5, and 8 following the injection. Blood was centrifuged, and plasma was collected and stored at -80°C for further analysis.

### 2.4.5.1. Analysis

$D_2O$ concentration was determined by nuclear magnetic resonance spectroscopy. The fluid intake was calculated using the $D_2O$ disappearance curve generated for each lamb. The actual amount of $D_2O$ (mg) administered was calculated as follows:

$$D_2O \text{ (mg) dose} = (S2\text{-}S1) \times 0.06^{\#}$$

S2 is the final syringe weight, S1 is the start syringe weight, and # is the amount of $D_2O$ in the solution. The total milk intake was calculated from the disappearance of $D_2O$ in the plasma samples over time by the formula:

$$\text{Milk intake } (mL.hr^{-1}) = (D_2O \text{ dose} \times \text{exponential decay of } D_2O \text{ (K)}) / (D_2O \text{ at time 0})$$

The milk intake was then divided by weight across the testing period to estimate volume intake per body weight ($mL.hr^{-1}.Kg^{-1}$).

## 2.4.6. Plasma Amino Acid Concentration

To investigate the effect of oral supplementation (BCCA or Maltodextrin) on plasma amino acid concentration, the amino acid concentration were measured on days 1, 8, 11, and 15 after birth. $D_2O$ samples at day 8 (2 and 6 hrs after $D_2O$ injection; BCAA supplement given between baseline and 2 hour blood samples) were used to measure amino acid plasma concentration post BCAA supplementation. Amino acid concentrations (valine, leucine, isoleucine, methionine, phenylalanine, lysine, histidine, threonine, taurine, serine, glycine, arginine, tyrosine, ornithine, hydroxyproline, proline, aspartate, citrulline, asparagine, glutamine, and glutamate) were measured by ultra-performance liquid chromatography (UPLC) [213] following acid-tungstate precipitation. Briefly, 160 µL of 0.04 M Sulphuric acid (containing 15 µM L-Nor-Valine) was aliquoted on ice. Then 20 µL of the sample or quality control samples (QC) were added, mixed, and hold on ice for a few minutes. To this, 20 µL of 10% Sodium Tungstate was added, mixed instantly, and hold on ice for 3 minutes. All samples were immediately mixed before they were centrifuged at 4°C in an Eppendorf centrifuge at14000 rpm for 10 minutes. Pre column fluorescent derivatisation or "tagging" was achieved by adding 70 µL of Borate buffer (0.2 M) (1.24 g boric acid in 100 mL pH 8.8, adjusted with fresh 5 M NaOH) into a small glass tube, to which 10 µL of the supernatant (sample or QC) or standard mix was added. Then 10 µL of AccQ-tag reagent (2.8 $mg.mL^{-1}$ dry acetonitrile, sonicated to dissolve) was added and mixed instantly. This was transferred to a UPLC vial, capped, and heated at 55°C for 10 minutes. Vials were removed from heat and placed in the correct order in the carousel for injection by the UPLC. The free amino acids in plasma samples were measured using the software Chromeleon7. The samples were analysed if the quality control samples coefficient of variance was ≤ 10%.

## 2.4.7. Hyperglycaemic Clamp

Hyperglycaemic clamps (HGC) were performed as described previously [13,209,214] with minor modifications at 12 months of age to assess insulin sensitivity (IS) and secretion [214]. Sheep were acclimatised to indoor conditions a day prior to testing.

### 2.4.7.1. Catheter Insertion

A day before the clamp test, catheters were inserted into both jugular veins [13]. One catheter was used as an infusion line and the other as a sampling line. The neck was sprayed with

sodium chloride 0.9% (Baxter healthcare, Toongabbie, AU), and wool was shaved above the jugular veins on both sides, exposing the skin, while the sheep was restrained. One mL of local anaesthetic (Xylocaine 2%, AstraZeneca, North Ryde NSW, AU) was injected subcutaneously over the jugular vein according to IDAO. A small cut was made with the scalpel blade in the anaesthetised area. A 12-gauge needle was inserted through the cut in the skin and into the jugular vein. A polyvinyl catheter (single lumen, tube, ID 1.0 mm, OD 2.0 mm, SteriHealth, Victoria, AU) flushed with heparinised saline (0.9% saline, 10 $U.mL^{-1}$ sodium heparin) was inserted into the needle until the marked point 15 cm from the end. The needle was removed, and a stopcock was connected to the distal end of the catheter. The catheter was fixed to the skin by masking tape and superglue, and both connectors were kept in a plastic bag and tied with tick cotton lace tape to the wool on the back of the neck/shoulder. The catheters were secured with a tubular bandage (Surgifix, BSN Medical, Victoria, AU) covering the neck.

#### 2.4.7.2. Muscle Biopsies

Over the area of the *biceps femoris*, wool was sprayed with sodium chloride 0.9% and shaved. One mL of local anaesthetic (Xylocaine 2%, AstraZeneca, North Ryde NSW, AU) was injected subcutaneously over the muscle. A small cut was made with a scalpel blade, and tissues were dissected to reach the muscle plane. Muscle samples were collected. One sample was kept in RNA-later (Invitrogen, Thermo Fisher, Auckland, NZ) overnight at 4°C, then stored at -80°C until analysed. A second sample was kept in a tube in a dried-ice box and stored at -80°C until analysed.

#### 2.4.7.3. Arginine Preparation

A 100 $mg.mL^{-1}$ Arginine (L-arginine hydrochloride 98%, Thermo Fisher, Auckland, NZ) solution was prepared with a 0.9% saline solution. The pH was adjusted to 8.0 with 2N hydrochloric acid. The solution was filtered through a syringe filter (0.2 µm Supor Membrane, Pall Acrodisc, Sigma-Aldrich, Auckland, NZ) prior to the HGC and kept on ice until required.

#### 2.4.7.4. Hyperglycaemic Clamp Test

Sheep were fasted overnight (12-16 hours) with access to water. Catheters were flushed with heparinised saline. Blood samples were collected for glucose and insulin concentration measurements at different time points throughout the test, and 5 mL blood samples were needed for plasma collection. Baseline blood samples of 5 mL were collected at -20, -10, and 0 minutes

and additional 0.5 mL blood samples were collected at -15 and -5 minutes. After the collection, blood samples were immediately analysed for whole blood glucose concentration using a YSI 2300 glucose analyser (Yellow Springs Instruments, Ohio, USA). At 0 minutes, a bolus infusion of 25% glucose (Baxter healthcare, Toongabbie, AU) was started with an infusion rate calculated using a fixed equation according to body weight and surface area, using a computer algorithm [13], to increase glucose baseline concentration to 10 $mmol.L^{-1}$ (priming dose). This increase was maintained at a steady-state for the clamp duration (165 minutes) by collecting blood samples every 5 minutes, measuring the glucose concentration, and adjusting the infusion rate using the computer algorithm. At 135 minutes, a bolus infusion of arginine was given to stimulate maximal insulin secretion. Blood samples for glucose and insulin concentration measurements were collected at 5-minute intervals for a period of 30 minutes after arginine infusion. At 165 minutes, the infusion was stopped, and blood sampling ceased. Throughout the clamp, catheters were flushed with heparinised saline following each blood collection. Blood samples were transferred into 5 mL tubes on ice, centrifuged, and plasma was collected and stored at -80°C for further analysis. The catheters were removed after the clamp study, and sheep were kept for at least 2 hours for observation and then returned to the paddock.

#### 2.4.7.5. Hyperglycaemic Clamp Data Analysis

Hyperglycaemic clamp samples were analysed if the blood glucose concentration coefficient of variance was ≤ 10% during the steady-state insulin response (60-120 minutes) [13]. The mean plasma insulin and blood glucose concentrations at baseline were calculated. The first phase insulin response (FIR) was calculated from the plasma insulin concentration during the first 20 minutes of the clamp test. The steady-state insulin response (SIR) was calculated from the insulin concentration during the test's last hour (60-120 minutes). Arginine challenge (AC) was calculated from the insulin concentration immediately after arginine infusion (140-165 minutes). The area under the FIR curve, SIR, and AC curve were calculated using the trapezoid rule. Insulin sensitivity (IS) was calculated from the formula [13]:

$$IS\ (mmol.L^{-1}.Kg^{-1}.min^{-1}.\mu g^{-1}) = \frac{\text{mean glucose infused during the steady-state } (mmol.Kg^{-1}.min^{-1})}{\text{mean plasma insulin concentration during the steady-state } (\mu g.L^{-1})}$$

## 2.4.8. Post-mortem

Ten days after the clamp study, sheep were brought indoors. Bodyweight was measured, and the sheep were euthanised by intravenous pentobarbitone overdose (Pentobarb 300, Provet, Auckland, NZ) injection. Organs were identified, removed, weighed, and selected tissues were preserved. Tissues were collected in formalin (chemical care and storage, Auckland, NZ) and/or cryomold with OCT (optimal cutting temperature, Tissue-Tek, Prosci Tech, Kirwan, AU) and/or snap-frozen in liquid nitrogen (Table 2-3).

**Table 2-3. Tissue collection and sample preparations after sheep post-mortem at 12 months of age; OCT: optimal cutting temperature.**

| Organ | | Tissue preparation |
|---|---|---|
| Pancreas | | 1. Formalin<br>2. Microdissection (OCT)<br>3. Snap freeze |
| Omental fat | | 1. Snap freeze |
| Muscle | | 1. Snap freeze |
| Liver | | 1. Snap freeze |
| Kidney | | 1. Formalin<br>2. Snap freeze |
| Gut | **Duodenum** | 1. RNA-*later*™ (Scraping & contents)<br>2. Formalin (flat & tube) |
| | **Jejunum** | 1. RNA-*later*™ (Scraping & contents)<br>2. Formalin (flat & tube) |
| | **Colon** | 1. RNA-*later*™ (Scraping & contents)<br>2. Formalin (flat & tube) |
| | **Ileum** | 1. RNA-*later*™ (Scraping & contents)<br>2. Formalin (flat & tube) |
| Stomach | | 1. Formalin |

Samples were kept in formalin for 24 hours at room temperature and then changed to fresh formalin for 48 hours at room temperature. Seventy-two hours after collection, samples were stored in 70% ethanol (Thermo Fisher, Auckland, NZ) at 4°C until processing. Cryomold samples with OCT were kept directly in cooled iso-pentane (Thermo Fisher, Auckland, NZ) in an icebox with dry ice. Samples were then wrapped in aluminium foil and parafilm and stored

at -80ºC until processing. The snap-frozen samples were wrapped in aluminium foil and parafilm and stored at -80ºC until processing.

The pancreas was sectioned along the head-tail axis. One part was immediately snap-frozen in liquid nitrogen for molecular analysis. The second part was placed in formalin for immunohistochemistry analysis. Small pieces from different areas (head, body, and tail) were collected and placed in cryomolds, then covered with OCT for microdissection immunohistochemistry analysis.

One cubic centimetre sections of the right lobe of the liver, muscle (left side above the rump and *ischiatic tuber*, *fasciae latae*), and omental fat were collected for molecular analysis. Two approximately one $cm^3$ cross-sections containing both medulla and cortex were collected from the right kidney, one section for molecular analysis, and the second for immunohistochemistry. Fat thickness above the rump and the $7^{th}$ rib was measured.

The weights of the left kidney, adrenal (left and right), perirenal fat (left and right), spleen, lung, heart, brain, thymus (neck and chest), thyroid, cerebellum, and brain stem were recorded. Empty carcass weight was recorded.

## 2.4.9. Enzyme-Linked Immunosorbent Assay (ELISA)

### 2.4.9.1. Insulin Measurement

Plasma samples collected from the hyperglycaemic clamp were used to measure the insulin concentration using an ELISA kit (cat # 10-1202-01, Mercodia, Uppsala, Sweden) following the manufacturer's instructions. The enzyme conjugate solution and buffer solution were prepared according to the table provided in the manual. A 25 µL of the calibrators and samples were pipetted into the wells, then 100 µL of the enzyme conjugate was added to each well. The microplate was incubated on a plate shaker (700-900 rpm) for 2 hours at room temperature, then washed with 700 µL wash buffer 1 × solution per well using an automatic plate washer. After the final wash, the plate was inverted, discharging the remaining washing buffer and tapped firmly against absorbent paper to remove excess liquid. Then, 200 µL of the substrate was added to each well, and the microplate was incubated for 15 minutes at room temperature. A 50 µL of stop solution was added to each well, and the microplate was incubated for 5 minutes on the plate shaker. The microplate's optical density was read at 450 nm by a plate reader (BioTek Instruments, Vermont, USA).

Using the calibrators, a cubic spline regression curve of absorbance versus concentration was plotted, and the plasma insulin concentration was extrapolated from the standard curve (Figure 2-3). The intra- and inter-assay coefficients of variation were < 10%.

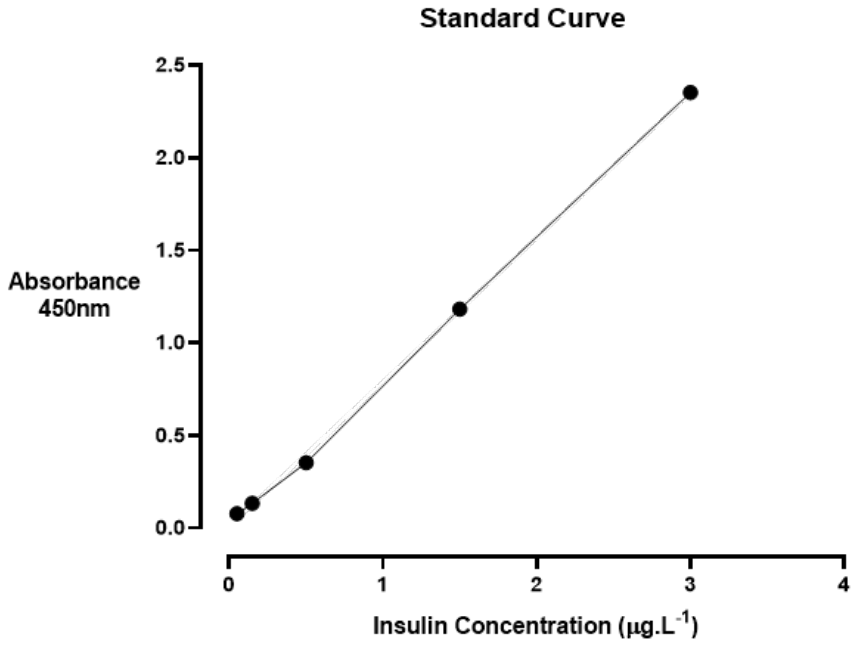



**Figure 2-3. A cubic spline regression standard insulin concentration curve was used to analyse plasma samples collected at the hyperglycaemic clamp.**

### 2.4.9.2. Glucagon Measurement

Plasma samples collected from the hyperglycaemic clamp were used to measure the glucagon concentration using a commercially available ELISA kit (DuoSet Glucagon, cat # DY1249, R&D Systems, Minneapolis, USA). This kit was previously used to measure glucagon in both mice [215] and pigs [216]. No citations were found of this kit having been used to measure ovine glucagon. Therefore, as far as we could determine, this is the first-time glucagon concentration in sheep plasma has been measured using this kit. Validation tests were conducted according to the manufacturer's instructions. This validation included dilution, spiking, and evaluation of intra- and inter-assay coefficients of variation for high and low glucagon control samples. Since glucagon circulates in the low picomolar concentration range [217], we had low or no colour development in the initial trials. Troubleshooting steps were taken to overcome the low colour development:

1. Blocking the plate with reagent diluent was increased from 1 to 2 hours.

2. The volume of the sample was increased from 100 µL to 150 µL.

3. The incubation time of the sample was intensified from two hours to overnight incubation at room temperature on a shaker (300 rpm).

4. The detection antibody working concentration was doubled from 50 $ng.mL^{-1}$ to 100 $ng.mL^{-1}$.

5. The substrate solution incubation time increased to 30 minutes.

The working concentrations of the glucagon capture antibody, glucagon detection antibody, glucagon standard, and streptavidin-HRP solutions were prepared according to the certificate of analysis provided by the manufacturer. The washing buffer, reagent diluent, and substrate solutions were prepared according to the information provided in the manual. The plate was coated with 100 µL per well of the glucagon capture antibody, sealed, and incubated overnight at room temperature on a plate shaker (300 rpm). Wells were aspirated and washed with 300 µL of 1 × washing buffer solution using an automatic plate washer, and the process was repeated five times. After the final wash, the plate was inverted, discharging the remaining washing buffer and tapping firmly against absorbent paper to remove excess liquid. Then, the plate was blocked by adding 300 µL of the reagent diluent to each well and incubated for 2 hours at room temperature on a plate shaker (300 rpm). The aspiration/wash was repeated, then 150 µL of the sample and 100 µL of the standard were added per well, and the plate was sealed and incubated overnight at room temperature on a plate shaker (300 rpm). The aspiration/wash was repeated, and 100 µL of the glucagon detection antibody was added to each well. The plate was sealed and incubated for 2 hours at room temperature on a plate shaker (300 rpm). The aspiration/wash was repeated, followed by the addition of 100 µL streptavidin-HRP to each well and incubated for 20 minutes at room temperature on a plate shaker (300 rpm). The aspiration/wash was repeated, and 100 µL of the substrate solution was added per well. The plate was incubated for 30 minutes at room temperature in the dark. A 50 µL of the stop solution was added to each well. The plate's optical density was read at 450 nm by a plate reader (BioTek Instruments, Vermont, USA).

The duplicate readings of each standard, control, and sample were averaged. The computer software created a standard curve using the four-parameter logistic (4-PL) curve-fit (Figure 2-4). The concentration of plasma glucagon was extrapolated from the standard curve. The intra- and inter-assay coefficients of variance were ≤ 10%.

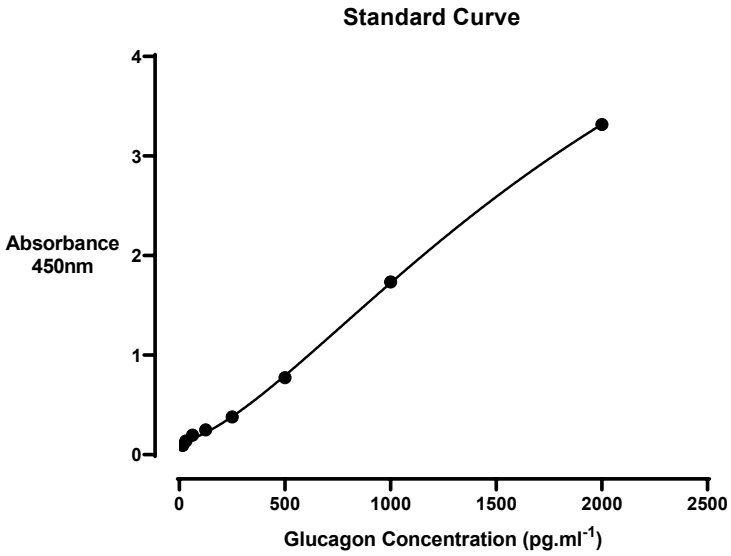



**Figure 2-4. Four parameter logistic (4-PL) standard curve fit was used to calculate the plasma glucagon concentration collected from the hyperglycaemic clamp.**

## 2.4.10. Immunohistochemistry

### 2.4.10.1. Sample Preparation

The 54 pancreata recovered from this study were stored in 70% ethanol. Each pancreas was oriented longitudinally and trimmed using a sharp scalpel. A 2-3 mm deep slices of the pancreas were taken, representing a section of the head, body, and tail regions, and each slice was placed in lidded embedding histological cassettes (TechnoPlas, Adelaide, AU). The cassettes were immersed with 70% ethanol to ensure that the tissues did not dry out, and two cassettes were made per animal and stored in 70% ethanol until embedded in paraffin.

#### 2.4.10.2. Tissue Processing

The tissue sections were dehydrated with increasing ethanol concentration using an automated tissue processing machine (Leica APS 300S, Leica Biosystems, Heidelberger, Germany), followed by a clearing process with chloroform (Thermo Fisher, Auckland, NZ), and then infiltrated with paraffin wax (ParaPlas, Leica Biosystems, Heidelberger, Germany) (Table 2-4).

**Table 2-4. Tissue dehydration and wax infiltration process.**

| Chemicals | Incubation (hour) | Cycles |
|---|---|---|
| 70% Ethanol | 1 | 1 |
| 80% Ethanol | 1 | 1 |
| 95% Ethanol | 1 | 2 |
| 100% Ethanol | 1 | 3 |
| Chloroform | 1 | 2 |
| Paraffin wax | 1 | 3 |

#### 2.4.10.3. Paraffin Embedding

Paraffin blocks were formed using a mould and melted paraffin wax. The tissue sections were embedded into paraffin wax blocks using Leica Microsystem Embedder (Leica Biosystems, Heidelberger, Germany). A plastic mould was kept at the heated paraffin embedding station and filled with melted paraffin wax. Tissue sections (head, body, and tail) were placed into the melted wax with heated forceps. The tissue sections were kept as close as possible without overlapping, away from the edges, and positioned at the bottom of the mould rather than floating. When the tissues were in the appropriate position in the wax, the mould was transferred onto the embedding centre's cooling plate. The tissues were pressed firmly onto the bottom of the mould using the labelled cassette from which the tissues had been taken. Once the wax had solidified, the mould was removed and stored at room temperature until microtomy.

#### 2.4.10.4. Microtomy

One hundred slides were generated per pancreas. The paraffin block was loaded into the microtome (Leica RM 2135, Leica Biosystems, Heidelberger, Germany) and trimmed until the tissue area was exposed. The block was placed in a water bath (50$^{o}$C) for 1-2 seconds to soften the tissues and the infiltrated wax to have a flat and even surface, then incubated on ice for one

hour for a smooth cut. The block was then reloaded into the microtome, and sections were cut at a 5 µm thickness using an S35 fine microtome blade (Feather Safety Razor Co, Osaka, Japan), creating a ribbon. The ribbon was transferred to the water bath using a forceps and a painting brush to flatten the section and remove any wrinkles. Sections were then transferred to the prelabelled glass slides (Superfrost Plus Microscope Slides, LabServ, Auckland, NZ), and the slides were kept in a rack and incubated at 37ºC to dry out and then stored at room temperature until required.

#### 2.4.10.5. Immunofluorescence Multiple Labelling

Four tissue sections at 100 µm intervals were multi-labelled for insulin, glucagon, and somatostatin. The protocol was performed across multiple rounds due to the number of animals, and each round included one section per animal. This was to overcome any day-to-day variation in the process.

##### 2.4.10.5.1. Deparaffinization and Antigen Retrieval

The process started with melting the wax by incubating the slides at 60ºC for one hour. Slides were then deparaffinized in xylene, rehydrated in different ethanol concentrations, followed by washing in 1 × phosphate buffer saline (PBS) (Table 2-5). Slides were then transferred to a slide chamber filled with 1 × sodium citrate buffer (0.05% Tween-20, pH 6) and placed into a pressure cocker (2100-Retriever, Prestige Medical, Lancashire, UK) filled with milli-Q water and incubated at 121ºC for 20 minutes for antigen retrieval. Once the cycle was completed, the slides were cooled to room temperature in the sodium citrate buffer, washed with 1 × PBS (3 × 5 minutes) and soaked with 1 × PBS-Tween-20. Excess PBS was removed by blotting the edge of the slide and around the tissue section with KimWipes (Kimtech, Auckland, NZ). A line was drawn, with a hydrophobic pen (ImmEdge pen, Vector laboratories, California, USA), around the tissue area on each slide to minimise the surface area and control the amount of antibody used.

**Table 2-5. Deparaffinization and tissue rehydration process.**

| Chemicals | Incubation (minutes) | Cycles |
|---|---|---|
| Xylene | 5 | 2 |
| 100% Ethanol | 2 | 2 |
| 90% Ethanol | 2 | 2 |
| 70% Ethanol | 2 | 2 |
| 50% Ethanol | 2 | 2 |
| 1 × PBS | 5 | 2 |

2.4.10.5.2. Blocking

Slides were transferred to a humidity chamber, and 200 µL of goat serum blocking buffer was pipetted to each slide (10% goat serum (cat # 16210-064, Gibco, Auckland, NZ) in 1 × PBS) to cover the tissue section. Slides were then incubated in the humidity chamber at room temperature for one hour.

2.4.10.5.3. Primary and Secondary Antibody Application

Primary and secondary antibodies specific to ovine proteins insulin, glucagon, and somatostatin could not be sourced. Therefore, the antibodies used in our study were from different species. These antibodies went through method development to validate their use in ovine pancreas tissue.

*2.4.10.5.3.1. Insulin, Glucagon, and Somatostatin antibodies*

Eighteen trials were performed to confirm the suitable antibodies (primary and secondary), dilution, and incubation time. The trial started with using the primary antibodies guinea pig anti-insulin, mouse anti-glucagon, and rabbit anti-somatostatin and the secondary antibodies goat anti-guinea pig Alexa Fluor 647, goat anti-mouse IRDye 800CW, and goat anti-rabbit Alexa Fluor 594. In the beginning, a somatostatin signal was seen in the 647-channel, meaning a cross-reaction between guinea pig and rabbit. This reactivity was confirmed when a signal was captured for slide staining with primary rabbit anti-somatostatin antibody and goat anti-guinea pig secondary antibody Alexa Fluor 647. Antibodies were incubated at different times to avoid this problem. The problem persisted, and somatostatin was still seen at the 647-channel with a weaker signal. It was found that goat anti-guinea pig secondary antibody Alexa Fluor 647 combines with goat anti-rabbit Alexa Fluor 594 even if incubated at different times (the secondary antibodies cross-react). Therefore, a switch was made between 594 and 800

channels and the host of the anti-rabbit secondary antibody. A goat anti-mouse Alexa Fluor 594 and donkey anti-rabbit IRDye 800CW antibodies were used, but a similar problem was found between goat anti-guinea pig secondary antibody Alexa Fluor 647 and goat anti-mouse Alexa Fluor 594. We found that Alexa Fluor 647 reacted with all Alexa Fluor 594 secondary antibodies. A goat anti-mouse (IgG1) Alexa Fluor 555 was sourced, and no cross-reaction was found between the antibodies. Table 2-6 summarises the problems and solutions to the primary and secondary antibody applications.

**Table 2-6. Primary and secondary antibodies troubleshooting and method development.**

| Problem | Solution |
|---|---|
| Cross-reaction between anti-guinea pig Alexa Fluor 647 and goat rabbit anti-somatostatin | Three days of staining, starting with insulin primary and secondary, then the rest of the antibodies on the second and third day (changing incubation time) |
| Cross-reaction between anti-guinea pig Alexa Fluor 647and goat anti-rabbit Alexa Fluor 594 | Donkey anti-rabbit IRDye 800CW (changing the host of the antibody and fluorophore wavelength) |
| Cross-reaction between anti-guinea pig Alexa Fluor 647and goat anti-mouse Alexa Fluor 594 | Goat anti-mouse (IgG1) Alexa Fluor 555 (changing to a subtype of IgG) |

Due to the cross-reactivity between antibodies, antibodies were applied at different incubation times. The immunohistochemical staining was performed over three days. Excess blocking buffer was removed, so the antibody would not be diluted when added to the slide. Hundred and fifty µL of insulin primary antibody diluted in 2% bovine serum albumin (BSA) in 1 × PBS with sodium azide was applied to each slide on day one (Table 2-7). Slides were incubated in a humidity chamber for one hour at room temperature, then overnight at 4$^{o}$C.

On day two, slides were kept at room temperature in a humidity chamber to equilibrate. Slides were washed with 1 × PBS (3 × 5 minutes) and soaked with 1 × PBS-Tween-20. Excess buffer was removed, and slides were returned to the humidity chamber. A 150 µL of goat anti-guinea pig Alexa Fluor 647 prepared in 1 × PBS (Table 2-7) was applied to each slide and incubated for one hour at room temperature. Slides then were washed with 1 × PBS-Tween-

20 (3 × 5 minutes) and soaked with 1 × PBS in the darkroom. Excess buffer was removed, and slides were returned to the humidity chamber. A 150 µL aliquot of glucagon and somatostatin primary antibodies diluted in 2% BSA in 1 × PBS with sodium azide (Table 2-7) was applied to each slide. Slides were incubated in a humidity chamber for one hour at room temperature, then overnight at 4°C.

On day three, slides were kept at room temperature in a humidity chamber to equilibrate. Slides were washed with 1 × PBS (3 × 5 minutes) and soaked with 1 × PBS-Tween-20 in the darkroom. Excess buffer was removed, and slides were kept back in the humidity chamber. A 150 µL aliquot of goat anti-mouse (IgG1) Alexa Fluor 555 and donkey anti-rabbit IRDye 800CW prepared in 1 × PBS (Table 2-7) was applied to each slide and incubated for one hour at room temperature. Slides then were washed with 1 × PBS-Tween-20 (3 × 5 minutes) and soaked with 1 × PBS in the dark. Excess buffer was removed, and slides were mounted with three drops of ProLong Gold antifade mounting medium with 4', 6-diamidino-2-phenylindole (DAPI, to stain the nucleus) (cat # P36941, Invitrogen, Massachusetts, USA), and a coverslip (Thermo Fisher Scientific, Massachusetts, USA) was applied. Slides were kept at room temperature for 48 hours in the dark before being imaged. All slides were imaged within six days of staining completion and were protected from light when a secondary antibody was applied. A flowchart summarizing primary and secondary antibodies application is shown in Figure 2-5.

**Table 2-7. Primary and secondary antibodies used for insulin, glucagon, and somatostatin.**

| Target/Protein | Host | Clonality | Isotype | Dilution | Supplier | Catalogue # |
|---|---|---|---|---|---|---|
| Insulin | Guinea pig | Polyclonal | IgG | 1:150 | ABCAM | 7842 |
| Glucagon | Mouse | Monoclonal | IgG1 | 1:200 | ABCAM | 10988 |
| Somatostatin | Rabbit | Polyclonal | IgG | 1:300 | ABCAM | 108456 |
| Anti-guinea pig Alex Flour 647 | Goat | Polyclonal | IgG | 1:400 | Invitrogen | A-21450 |
| Anti-mouse Alex Flour 555 | Goat | Polyclonal | IgG1 | 1:300 | Invitrogen | A-21127 |
| Anti-rabbit IRDye 800CW | Donkey | Polyclonal | IgG (H+L) | 1:400 | LI-COR | 926-32213 |

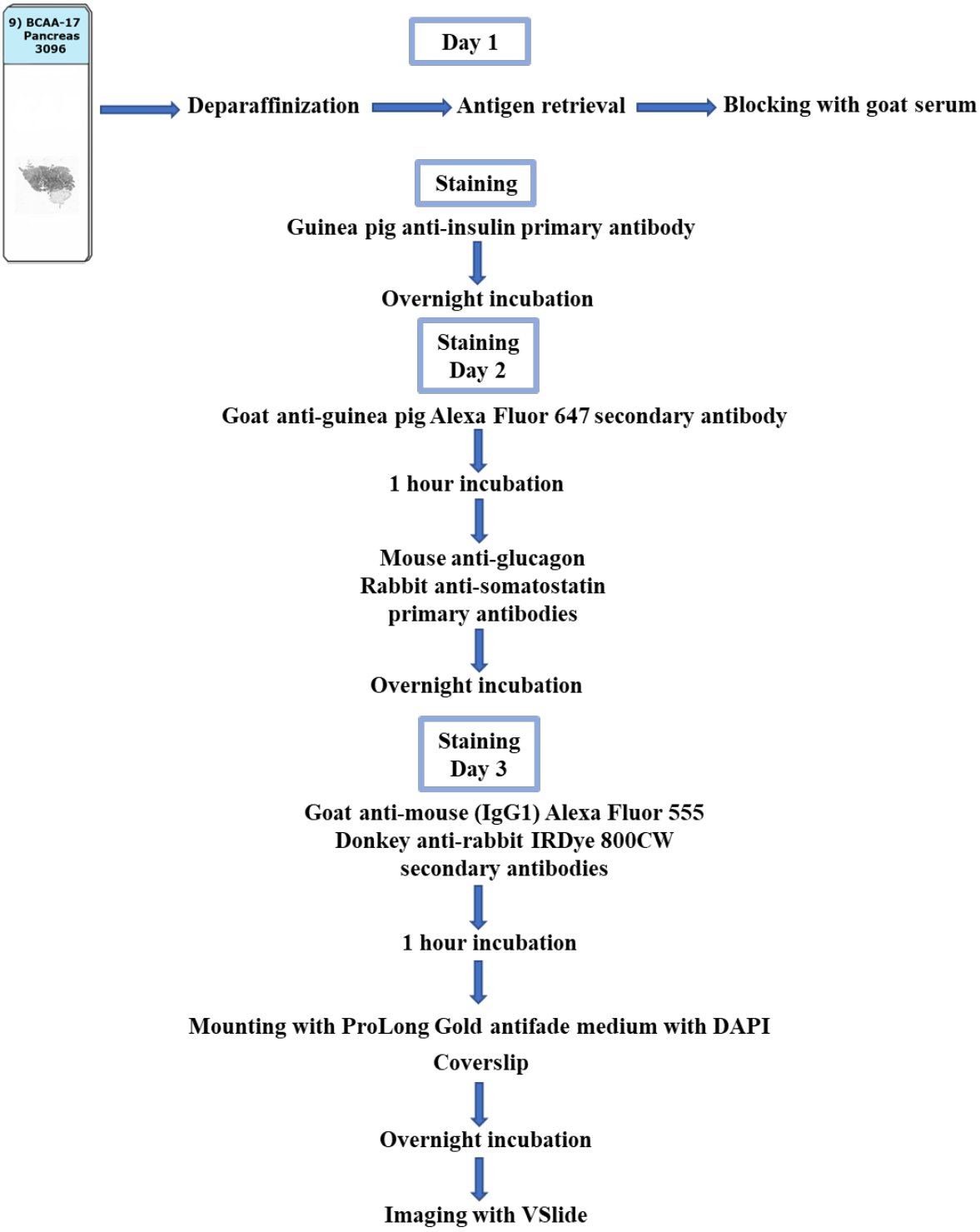



**Figure 2-5. Application of primary and secondary antibodies for insulin, glucagon, and somatostatin.**

*2.4.10.5.3.2. Insulin, Glucagon, Ki67, and Active-Caspase-3 Antibodies*

Ten different trials were performed to confirm the suitable antibodies (primary and secondary), dilution, and incubation time. To overcome the challenges in the previous antibody staining, mouse anti-insulin primary antibody conjugated with Alexa Fluor 647, mouse anti-glucagon primary antibody with specific isotype ($IgG_{2A}$) that can be only labelled with anti-mouse $IgG_{2A}$ secondary antibody, and mouse anti-Ki67 primary antibody with specific isotype (IgG1) were used. Non-specific labelling was observed by rabbit anti-caspase-3 primary antibody (cat # AF835, R&D Systems, Minneapolis, USA) in pancreatic α-cells, which was confirmed with fourteen trials with different primary and secondary antibodies along with the information cited by the Cell Signaling company in the specificity/sensitivity of cleaved caspase-3 that non-specific labelling may be observed in pancreatic α-cells. A monoclonal cleaved active-caspase-3 antibody (cat # 9664, Cell Signaling, Massachusetts, USA) was used to overcome the non-specific binding. A Tyramide SupperBoost kit (Thermo Fisher Scientific, Massachusetts, USA) was used to amplify the active-caspase-3 signal (Table 2-8).

The immunohistochemical staining was performed over three days. Excess blocking buffer was removed so that the antibody would not be diluted when added to the slide. A 150 µL aliquot of mouse anti-glucagon, mouse anti-Ki65, and rabbit anti-caspase-3 primary antibodies diluted in 2% BSA in 1 × PBS with sodium azide was applied to each slide, and slides were incubated in a humidity chamber for one hour at room temperature then overnight at 4°C.

On day two, slides were kept at room temperature in a humidity chamber to equilibrate. Slides then were washed with 1 × PBS (3 × 5 minutes) and soaked with 1 × PBS-Tween-20. Excess buffer was removed, and slides were returned to the humidity chamber. A 150 µL aliquot of the poly-HRP conjugated anti-rabbit secondary antibody was added to all the slides, and slides were incubated in a humidity chamber for one hour at room temperature. Slides then were washed with 1 × PBS (3 × 5 minutes) and soaked with 1 × PBS-Tween-20. Excess buffer was removed, and slides were placed into the humidity chamber. A 100 µL aliquot of Tyramide working solution (following the manual instructions: Alexa Flour 488 in DMSO added to 100 × hydrogen peroxide and 1 × reagent buffer) was applied to each slide for 10 minutes, followed by 110 µL of reaction stop reagent for one minute. Slides then were washed with 1 × PBS (3 × 5 minutes) and soaked with 1 × PBS-Tween-20. Excess buffer was removed, and slides were placed into the humidity chamber.

**Table 2-8. Primary and secondary antibodies used for insulin, glucagon, Ki67 and active-caspase-3.**

| Target/Protein | Host | Clonality | Isotype | Dilution | Supplier | Catalogue # | Notes |
|---|---|---|---|---|---|---|---|
| Insulin | Mouse | Monoclonal | IgG1 Kappa | 1:250 | Novusbio | NBP2-346611AF647 | Conjugated with Alexa Flour 647 |
| Glucagon | Mouse | Monoclonal | $IgG_{2A}$ | 1:200 | R&D | MAB1249 | Clone #181402 |
| Active-Caspase-3 | Rabbit | Monoclonal | IgG | 1:200 | Cell Signaling | 9664 | Cleaved ASP175 |
| Ki67 | Mouse | Monoclonal | IgG1 | 1:100 | Dako | M7240 | Clone MIB-1 |
| Anti-mouse Alex Flour 555 | Goat | Polyclonal | IgG2a | 1:500 | Invitrogen | A-21137 | |
| Anti-rabbit Alexa Flour 488 | Goat | Polyclonal | IgG | | Invitrogen | B40943 | Tyramide SupperBoost Kit |
| Anti-mouse IRDye 800CW | Goat | Polyclonal | IgG1 | 1:500 | LI-COR | 926-32350 | |

A 150 µL aliquot of goat anti-mouse Alexa Fluor 555 and goat anti-mouse IRDye 800CW prepared in 1 × PBS was applied to each slide and incubated for one hour at room temperature. Next, slides were washed with 1 × PBS-Tween-20 (3 × 5 minutes) and soaked with 1 × PBS in the darkroom. Excess buffer was removed, and slides were returned to the humidity chamber. A 150 µL aliquot of mouse anti-insulin primary antibody conjugated with Alexa Flour 647 diluted in 2% BSA in 1 × PBS with sodium azide was applied to each slide. Slides were then incubated in a humidity chamber for one hour at room temperature and then overnight at 4ºC.

On day three, slides were kept at room temperature in a humidity chamber to equilibrate, then were washed with 1 × PBS-Tween-20 (3 × 5 minutes) and soaked with 1 × PBS in the dark. Excess buffer was removed, and slides were stained with DAPI. After 30 minutes of incubation, slides were washed with 1 × PBS and mounted with three drops of ProLong Gold antifade mounting medium (cat # P36934, Invitrogen, Massachusetts, USA) and a coverslip (Thermo Fisher Scientific, Massachusetts, USA) was applied. Slides were kept at room temperature overnight in the dark. Slides then were placed in a box protected from light, and stored at 4ºC before being imaged. One hour before slides were imaged, the slides' box was kept at room temperature. All slides were imaged within six days of staining completion. A flowchart summarizing primary and secondary antibodies application is shown in (Figure 2-6).

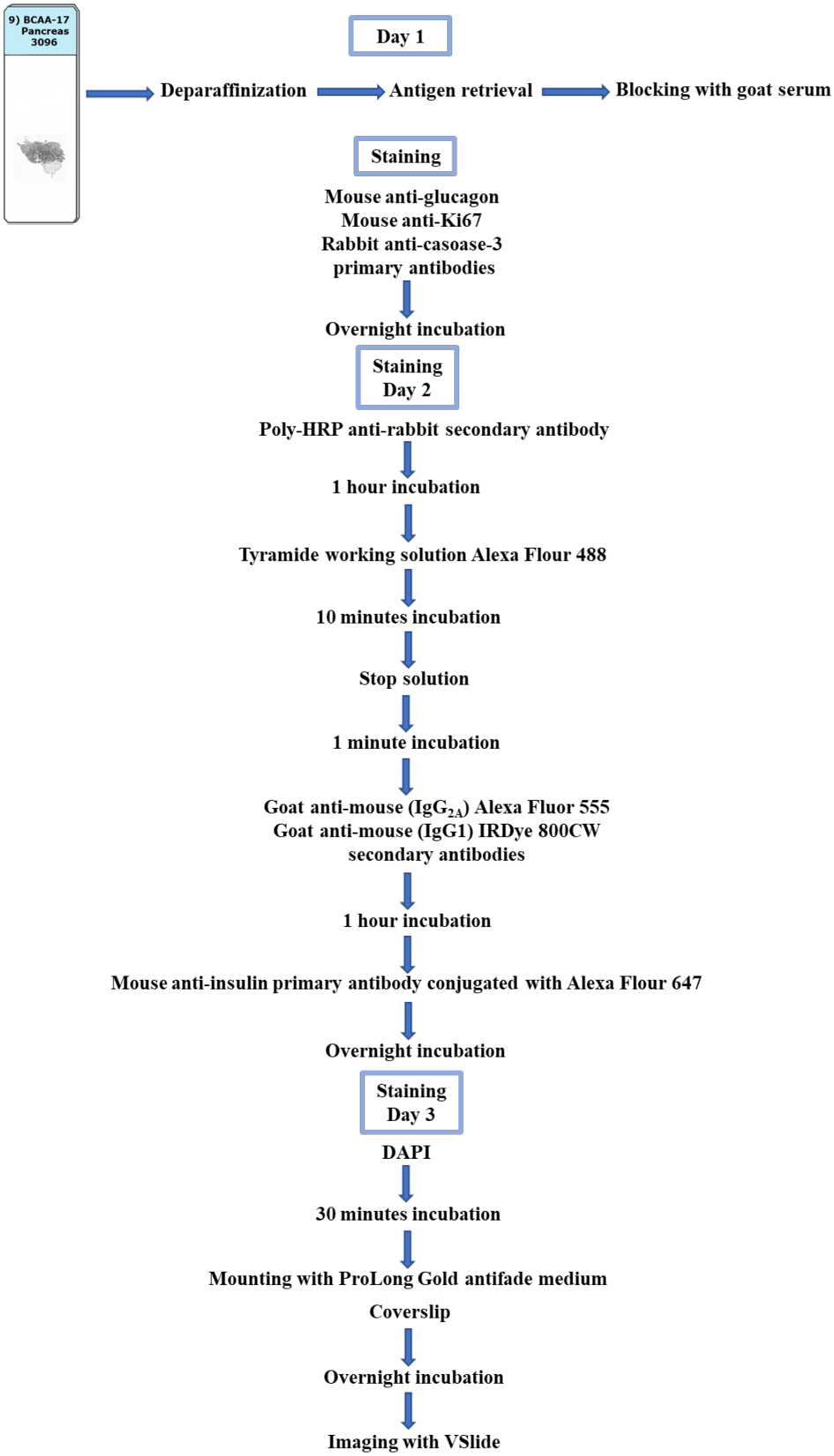



**Figure 2-6. Application of primary and secondary antibodies for insulin, glucagon, Ki67, and active-caspase-3.**

#### 2.4.10.6. Imaging

The antibodies were visualised using a VSlide slide scanner system1.1.128 (MetaSystems, Altlussheim, Germany) consisting of a microscope and a camera. The microscope Axio Imager Z2 (Carl Zeiss Microscopy, Jena, Germany) was equipped with a motorised five-position scanning stage (Marzhauser, Wetzlar, Germany), an automated 80 slide feeder (MetaSystems, Altlussheim, Germany), and fluorescence filter sets with fluorescence light source (Solid-state Light Source Colibri 7, Carl Zeiss Microscopy, Jena, Germany). The camera CoolCube 4m TEC monochrome sCMOS (MetaSystems, Altlussheim, Germany) had a resolution of 4096 × 300 pixels (12 MPixel), 30 frames per second read-out speed, and thermo-electric cooling. Metafer 5 MetaCyte 3.16.150 acquisition software was used to program light sources, filter sets, and exposure times for each assay, and VSlide 1.1.128 software for stitching raw images into one virtual slide image.

##### 2.4.10.6.1. Insulin, Glucagon, and Somatostatin

To acquire the images, five single band filters were used out of seven (DAPI, Fluorescein Isothiocyanate (FITC), Tetramethylrhodamine (TRITC), Cyanine 5 (Cy5), and Cyanine 7 (Cy7)) (Table 2-9). Slides were cleaned with 70% ethanol and KimWipes before loading to the SlideFeeder. One slide was loaded and scanned each time. Every slide was scanned at 10 X microscope magnification with 10 CCD camera gain to identify regions on each slide positive for DAPI (nucleus), insulin, glucagon, and somatostatin using different channels and integration times per second. Starting with DAPI, by choosing the channel, a LiveImage SetUp was open to set the right integration time. This step was repeated for all the required channels. A classifier (Figure 2-7) was constructed to determine the microscope's imaging settings. The classifier contained five channels, four for the antibodies needed and the fifth (FITCI) for background subtraction. The integration time differed between each slide with ±0.01 seconds for all filters.

Slides were set up in the MetaCyte fluorescence program (MetaSystems, Altlussheim, Germany), in which they were labelled by project name, antibody stained, magnification, animal number, and slide number (e.g., BCAA2017_SH_DpInGlSo_SgF10x_3096_slide060). The prepared classifier was selected and used in conjunction with the Slide SetUp command. A manual scanning search was used to avoid scanning areas that did not contain any tissues. The image was focused at 2.5 × magnification with an integration time of 0.400 seconds in the

search section. A preview scan was completed, and then rectangular regions of interest representing 35-40 fields of view were chosen. Once the section plane map had been generated, the selected areas were imaged according to the classifier. After the scanning, two files were generated per slide, a raw data file, and a stitched file. Raw images for each field of view, one image per filter (colour channel), were generated in a TIF format, black and weight (five images per field of view named and stored as B (Dapi, nuclei), C (somatostatin), G (FITC), M (glucagon) and Y (insulin)). One stitched virtual image was generated per slide in visual studio community content installer (vsi) format. Stitched images were viewed with MetaViewer software (version 2.0.133).

**Table 2-9. The Single-band filter sets are used to acquire the images.**

| Filter set | LED Used | Emission |
|---|---|---|
| #01 DAPI | 385 | 447/60nm |
| #03 FITC | 475 | 527/20nm |
| #04 TRITC | 555 | 580/23nm |
| #06 Cy5 | 630 | 676/29nm |
| #07 Cy7 | 735 | 809/81nm |

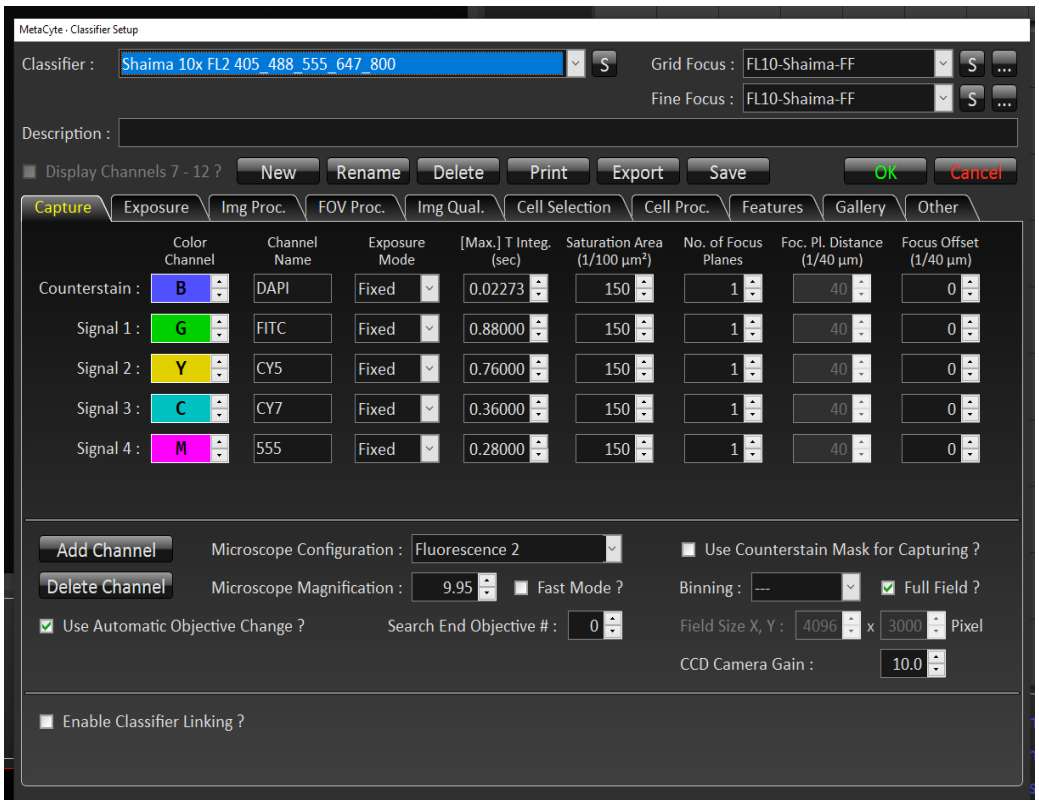



**Figure 2-7. Classifier used to determine the microscope's imaging settings.**

2.4.10.6.2. Insulin, Glucagon, Ki67 and Active-Caspase-3

Slides were imaged as the above section (2.1.10.6.1) by using six single band filters (DAPI, A430, FITC, TRITC, Cy5, and Cy7) to acquire the images. A classifier with six channels was used. Raw images for each field of view were generated in a TIF format (six images per field of view named and stored as B (Dapi, nuclei), C (Ki67), G (caspase), M (glucagon), O (A430) and Y (insulin)). One stitched virtual image was generated per slide in visual studio community content installer (vsi) format.

**2.4.10.7. Image Analysis**

The image analysis was performed on raw images from each field of view. The analysis was supported by a macro written in ImageJ Macro programming language and performed using FIJI (Fiji is Just ImageJ version 1.53f51, Maryland, USA) (Appendix I). All image analysis was performed by the same person who was blinded to treatment allocation.

2.4.10.7.1. Insulin Glucagon and somatostatin

The analysis command started with combining all the five raw images generated from each filter (B, C, G, M, and Y) into one image per field of view, then defining each colour image starting with DAPI as blue (image B), somatostatin as red (image C), FITC as white (image G), glucagon as green (image M), and insulin as yellow (image Y). The channels were then merged. The exact tissue area was measured after scanning the field of view area since some fields of view contained no tissue area (Figure 2-8-A). The FITC background was subtracted from all the filters (to remove the background noise since the pancreas is an auto-fluorescent organ) **(**Figure 2-8-B). This was followed by counting the number of cells stained with DAPI (Figure 2-8-C). The insulin area was measured, followed by counting β-cell numbers (cells stained with DAPI in the insulin area), then adding insulin area to the β-cell area, representing a positive insulin area used to measure β-cell mass. In detail, images containing insulin staining (image Y) were opened, a threshold appropriate for the entire slide run was applied, and areas positive for insulin were measured, then the cells stained with DAPI in the insulin-positive areas (β-cells) were counted. Insulin-positive areas and β-cells were selected and drawn onto each field of view image and saved for quality control (Figure 2-8-D). The exact process was done for glucagon and α-cells (Figure 2-8-E), somatostatin, and δ-cells (Figure 2-8-F). To

measure islet size and number, positive insulin area, positive glucagon area, and positive somatostatin area were combined (Figure 2-8-G).

2.4.10.7.2. Insulin, Glucagon, Ki67 and Active-Caspase-3

The analysis command is as described previously. The difference is the addition of one more filter (B, C, G, M, O, and Y). Images containing insulin staining (image Y) were opened, a threshold appropriate for the entire slide run was applied, and areas positive for insulin were measured. The cells stained with DAPI in the insulin-positive areas (β-cells) were counted. Cells stained with active-caspase-3 or/and Ki67 in the insulin-positive area were counted. The exact process was done for glucagon and α-cells.

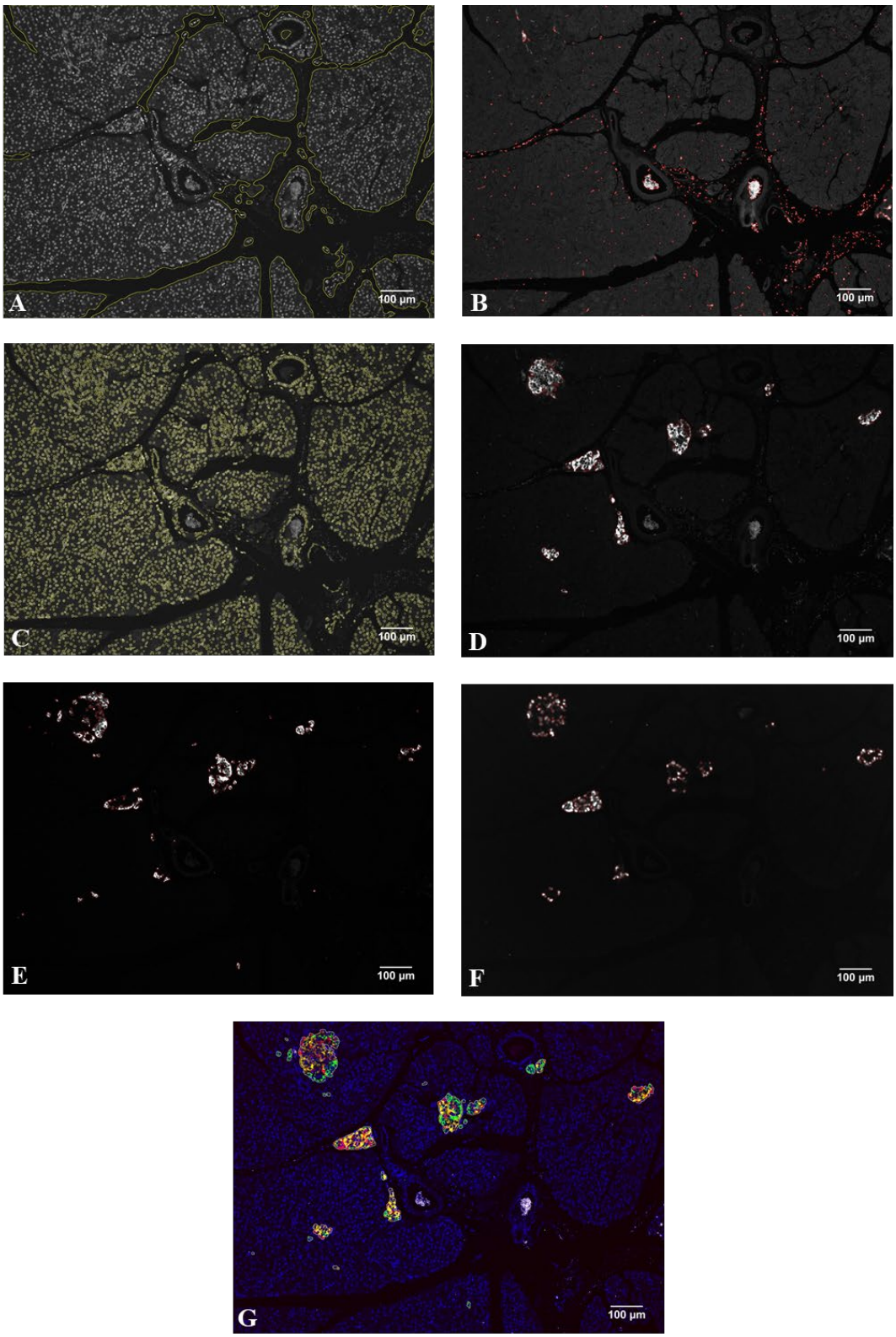



**Figure 2-8. Representative images from the process of image analysis.**
(A) Tissue area measured. (B) FITC image used as a background subtraction. (C) Cell count by DAPI. (D) Insulin area and β-cell area. (E) Glucagon area and α-cell area. (F) Somatostatin area and δ-cell area. (G) All channels combined showing the FITC, DAPI, and Islets.

2.4.10.7.3. Image Quality Control

Several steps of quality control were applied to all images. Before the analysis, every individual field of view (43,200 images (5 channels × 40 fields of view × 4 slides × 54 animals)) was checked manually for artefacts such as folding in the sections, out-of-focus areas, and air bubbles. Any field of view with artefacts was excluded from the analysis. After the analysis, 164,160 images (19 output images × 40 fields of view × 4 slides × 54 animals) were produced from ImageJ and were manually checked to ensure that the results generated from the macro represent the staining from each field of view. All analysed images were compared with the original field of view images to ensure that the analysis was correct, and that the results produced were an accurate representation. Images with edge effects (non-specific differential staining around the edge of the tissue that produces inaccurate data read-out or false positive) were either re-analysed with different thresholds or excluded from the study.

#### 2.4.10.8. Endocrine Cell Mass Calculation

β-cells, α-cells, and δ-cells were marked and calculated as areas positive with insulin, glucagon, and somatostatin staining, respectively. The β-cell area is an area of both positive insulin area and β-cell number. β-Cell mass was calculated by the formula [13]:

$$\beta\text{-cell mass (mg)} = \frac{\beta\text{-cell area per field of view } (\mu m^2) \times \text{pancreas weight (mg)}}{\text{Tissue area per field of view } (\mu m^2)}$$

The α-cell mass and δ-cell mass were measured from a similar equation, using glucagon positive area and α-cell, somatostatin positive area, and δ-cell. The total islet cell area was calculated as a sum of the β-cell, α-cell, and δ-cell area per field of view.

### 2.4.11. Statistical Analysis

Statistical analysis was performed using JMP (version 15.2.1, SAS Institute Inc., North Carolina, USA) and GraphPad Prism (version 8.2.1, GraphPad Software, Inc., California, USA). Data were analysed by two-way analysis of variance (ANOVA) to determine the effects of sex and treatments on each parameter. Significant differences identified by ANOVA were followed by post hoc analysis using either the student t-test for paired analysis or Tukey's test for multiple comparisons. Data are presented for both sexes together when no difference was

found between the sexes. The data were presented and analysed independently if there were significant differences between the sexes.

Longitudinal changes over time were examined using multivariate analysis of variance (MANOVA) for the analysis of repeated measures data. Pearson's correlation was used to examine bivariate relationships, and the correlation coefficient (r) was calculated. The area under the curve (AUC) was calculated using the trapezoidal rule. $P \leq 0.05$ was considered statistically significant. Results are reported as mean ± SEM.

# Chapter 3. Effect of Branched-Chain Amino Acid Supplementation on Plasma Amino Acid Concentrations and Growth of Preterm-Born Lambs

Growth encompasses an increase in body length and mass and functional organ development and maturation. Fetal and postnatal nutrition is a key determinant of health; growth is one indicator of sufficient nutrition. Poor fetal and neonatal growth is associated with organ development and related physiological pathways, contributing to poor metabolic outcomes in adulthood [218-221].

Growth faltering is common after preterm birth [141,222]. Nutritional supplements are often given to preterm babies to promote growth [223]. The pattern of postnatal growth may not be equivalent for all measures; for example, weight gain is not always directly associated with an equivalent increase in linear growth [224]. Body measurements alone do not necessarily reflect body composition; for example, weight gain may be due to an excessive increase in adipose tissue rather than an increase in lean and bone mass. Furthermore, it is unclear what constitutes optimal postnatal growth after preterm birth and if the way to achieve it is different for males and females. While it is important to prevent postnatal growth failure, excess weight gain from birth to age two years is a predictor of later excess weight, obesity, and adiposity [219], and rapid early neonatal weight gain in the first two weeks after birth is associated with insulin resistance in later life [221,225]. Children born preterm have historically received a high carbohydrate neonatal diet, including intravenous dextrose solution; this has been associated with greater weight gain in childhood than in term-born children [226]. However, these nutritional supplements were beneficial in improving preterm babies' survival [135].

Also important, although more challenging to assess than physical growth, are the consequences of preterm birth on organ structure and function in key metabolic organs such as the pancreas. Adults born preterm are more susceptible to metabolic disease, and many studies associate preterm birth with the risk of pancreatic impairment in later life [11,13,100]. Poor weight gain between preterm birth and term equivalent age has been associated with poorer neurodevelopmental outcomes [227]. These outcomes are likely contributed to by organ immaturity at birth, postnatal nutritional exposure, and early growth patterns. To improve growth and development, nutritional supplements are often given to enhance macro- and micronutrient intake and accelerate growth [223], which may have long-term metabolic effects [228]

including an increased risk of later childhood obesity [226]. Recent meta-analyses of the effects on growth and development of postnatal nutritional supplements in humans and animals have found mixed results, but the overall quality of evidence is poor [223,229]. Many studies have demonstrated the necessity of including amino acids in nutritional supplementation [114,135,142]. Amino acids are important for growth and development, especially for neonates [135,142]. It is unknown whether it is possible to enhance long-term pancreatic function using postnatal nutritional interventions specifically targeting pancreatic islet development rather than the more general approach of caloric supplementation for growth promotion.

BCAA have an indirect effect on muscle growth, being involved in growth regulation rather than increasing muscle protein synthesis *per se* [230], but are key nutrients for the development of the endocrine pancreas, particularly β-cells. It might therefore be possible to use BCAA supplements to mitigate the adverse effects of preterm birth on pancreatic development without concurrent alterations in growth patterns. A previous study by our group in preterm lambs investigated the tolerability, absorption, and effect on growth of oral BCAA supplements given during the first two weeks after birth and found no difference in growth to 28 days compared with control groups (unpublished data).

We hypothesised that BCAA supplements given to preterm lambs after birth would enhance endocrine pancreatic development and therefore function long-term, without resulting in excessive early weight gain and independent of effects on growth.

## 3.1. Methods

### 3.1.1. Experimental Design

The animals were generated as outlined in Chapter 2 (methods section 2.3). Preterm lambs were randomized into three groups according to their oral supplementation: BCAA, iso-caloric carbohydrate supplement Maltodextrin (to control any effect on the pancreatic function due to increased energy intake), or water. The Term group received water. Lambs were given the nutritional supplements daily in two divided doses for a period of 15-days after birth while continuing to suckle from their ewe. At the end of the supplementation period, lambs were returned to pasture with their ewes (methods section 2.4.1). The assessment of nutritional status involved the measurement of weight and linear body dimensions (morphometry). These included crown-rump length (CRL), hind limb length (HL), hock-to-toe length (HT), biparietal diameter (BPD), and chest and abdominal circumference. The measures were recorded for all

experimental animals from birth through 12 months of age (methods section 2.4.2). Intravenous deuterium oxide ($D_2O$) dilution assessment of milk intake was conducted in the second week after birth (methods section 2.4.5). Growth velocity for weight and all linear measurements was calculated for days 1-15 and monthly thereafter (methods section 2.4.3). Amino acid plasma concentrations were measured in blood samples taken in the early morning, prior to the morning dose of the supplement being given and approximately 16 hours after the previous dose, on days 1, 8, 11, and 15 after birth using ultra-performance liquid chromatography (UPLC) tandem mass spectrometry following acid-tungstate precipitation. $D_2O$ samples on day 8 (2 and 6 hrs post-dose) were used to measure amino acid plasma concentration post-BCAA supplementation. The samples were analysed if the quality control samples coefficient of variance was ≤ 10% (methods section 2.4.6). Lambs were euthanised at 12 months of age, and select organs like the pancreas were harvested for further analysis (methods section 2.4.8).

### 3.1.2. Statistical Analysis

Data were analysed by two-way ANOVA (independent variables were sex and treatment, in addition to the interaction between the variables) followed by a post hoc test, either the student t-test for paired analysis or Tukey's test for multiple comparisons. Pearson's correlation was used to examine bivariate relationships and the correlation coefficient (r) was calculated. Longitudinal changes over time were analysed using repeated measures MANOVA. $P < 0.05$ was considered statistically significant. Results are reported as mean ± SEM.

## 3.2. Results

### 3.2.1. Animal Numbers and Groups

Fifty-eight lambs were included in this study, and 54 lambs completed the research for 12 months (Figure 3-1). Forty-five ewes were randomly allocated to Preterm delivery at 137 days of gestation. Preterm lambs were randomly divided into three different groups according to their oral supplementation (water - control (n = 16: M = 9, F = 7), BCAA (n = 14: M = 7, F = 7), or Maltodextrin (n = 15: M = 7, F = 8)). Three male lambs died during the experimental period, all from the water group (two after day 60 and one after day 90). Therefore, forty-two Preterm lambs completed the study (Figure 3-1).

Thirteen ewes were randomly allocated to Term delivery at 147 days of gestation. All Term lambs received water (n = 13: M = 6, F = 7). One male lamb died after day 60; therefore, twelve Term lambs completed the study (Figure 3-1).

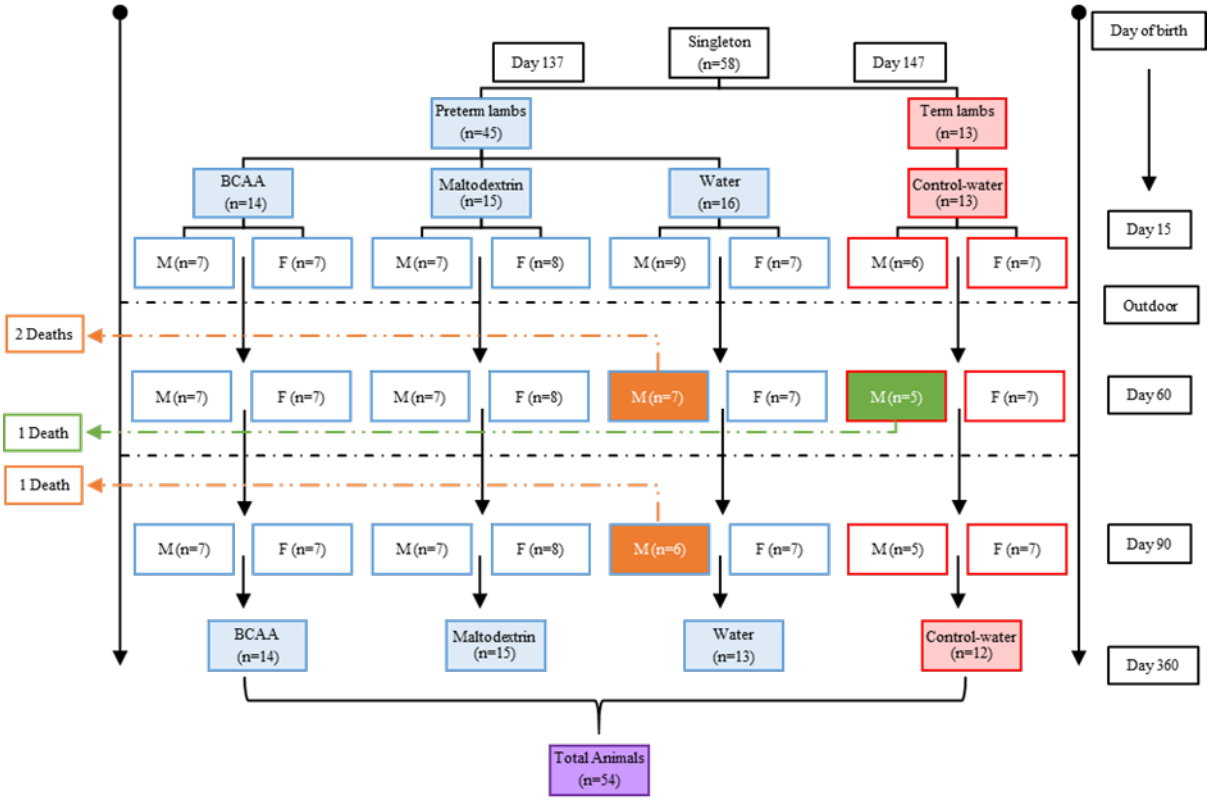



**Figure 3-1. Description of experimental groups and design.**
Labour was induced with dexamethasone in singleton-bearing ewes. Two intramuscular injections of dexamethasone were received 12-hours apart on day-135 and day-136 of gestation to induce labour at day-137 (Preterm birth) or on day-145 and day-146 to induce labour at day-147 (Term birth). The Preterm group was divided into three different groups according to their oral supplementation (BCAA, Maltodextrin, or Water). F: female, M: male.

## 3.2.2. Plasma Amino Acid Concentration

### 3.2.2.1. Day Zero

Plasma concentrations of many amino acids at day zero were different between Preterm and Term lambs. In general, where there were differences, concentrations were higher in Term than Preterm lambs including those of BCAA (Term > Preterm: leucine $P = 0.0006$, isoleucine $P < 0.0001$, and valine $P < 0.0001$). The exceptions were serine and taurine, where plasma concentrations were higher in Preterm lambs (Table 3-1 and Figure 3-2). There was a sex

difference in the amino acid concentrations in both Term and Preterm groups, however, this difference was not consistent for all amino acids.

**Table 3-1. Plasma amino acid concentration at day zero in Preterm and Term lambs.**

| | Preterm n = 45 | | Term n = 13 | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|
| (μg.mL$^{-1}$) | Female | Male | Female | Male | Sex | Birth | Interaction |
| **Ala** | 505±44 | 460±31 | 636±42 | 434±40 | **0.02** | 0.34 | 0.16 |
| **Arg** | 187±17$^{b}$ | 171±13$^{b}$ | 364±36$^{a}$ | 193±19$^{b}$ | **0.0002** | **0.0001** | **0.001** |
| **Asn** | 95±10$^{b}$ | 79±6$^{b}$ | 179±22$^{a}$ | 113±21$^{a,b}$ | **0.005** | **0.0001** | 0.08 |
| **Asp** | 17±7 | 17±2 | 18±3 | 12±4 | 0.35 | 0.46 | 0.99 |
| **Glu** | 151±15 | 170±17 | 132±15 | 134±33 | 0.67 | 0.24 | 0.71 |
| **Gln** | 441±32 | 448±17 | 731±59 | 581±60 | 0.09 | **<0.0001** | 0.06 |
| **Gly** | 464±41 | 452±56 | 623±37 | 459±72 | 0.20 | 0.22 | 0.26 |
| **His** | 124±16 | 102±9 | 153±29 | 151±31 | 0.57 | 0.06 | 0.63 |
| **Hyp** | 65±5$^{a,b}$ | 52±3$^{b}$ | 81±7$^{a}$ | 52±2$^{b}$ | **0.001** | 0.19 | 0.20 |
| **Ile** | 84±7 | 104±7 | 152±11 | 148±36 | 0.55 | **<0.0001** | 0.35 |
| **Leu** | 165±17 | 211±17 | 278±40 | 324±80 | 0.14 | **0.0006** | 0.99 |
| **Lys** | 98±8$^{b}$ | 99±9$^{b}$ | 192±21$^{a}$ | 102±25$^{b}$ | **0.002** | **0.001** | **0.002** |
| **Met** | 35±4$^{b}$ | 27±2$^{b}$ | 69±7$^{a}$ | 37±11$^{b}$ | **0.0005** | **<0.0001** | **0.02** |
| **Phe** | 123±11$^{b}$ | 123±10$^{b}$ | 199±24$^{a}$ | 135±33$^{a,b}$ | 0.07 | **0.01** | 0.07 |
| **Pro** | 1073±129$^{b}$ | 449±62$^{b}$ | 779±214$^{a,b}$ | 428±92$^{b}$ | **0.003** | 0.31 | 0.38 |
| **Ser** | 372±25 | 327±22 | 277±19 | 263±48 | 0.39 | **0.02** | 0.65 |
| **Thr** | 607±47 | 602±60 | 781±95 | 624±65 | 0.31 | 0.22 | 0.34 |
| **Trp** | 66±13 | 61±18 | 98±24 | 25±4 | 0.08 | 0.94 | 0.12 |
| **Tyr** | 166±15$^{b}$ | 171±13$^{b}$ | 245±23$^{a}$ | 181±13$^{a,b}$ | 0.15 | **0.03** | 0.09 |
| **Val** | 375±26$^{b}$ | 366±24$^{b}$ | 750±65$^{a}$ | 524±98$^{b}$ | **0.01** | **<0.0001** | **0.01** |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are shown for the following groups of lambs: Preterm male (n = 23), Preterm female (n = 22), Term male (n = 6), and Term female (n = 7). Ala: alanine; Arg: arginine; Asn: Asparagine; Asp: Aspartic acid; Glu: Glutamic acid; Gln: Glutamine; Gly: Glycine; His: Histidine; Hyp: Hydroxyproline; Ile: Isoleucine; Leu: Leucine; Lys: Lysine; Met: Methionine; Phe: Phenylalanine; Pro: Proline; Ser: Serine; Thr: Threonine; Trp: Tryptophan; Tyr: Tyrosine; Val: Valine. Data are mean ± SEM.

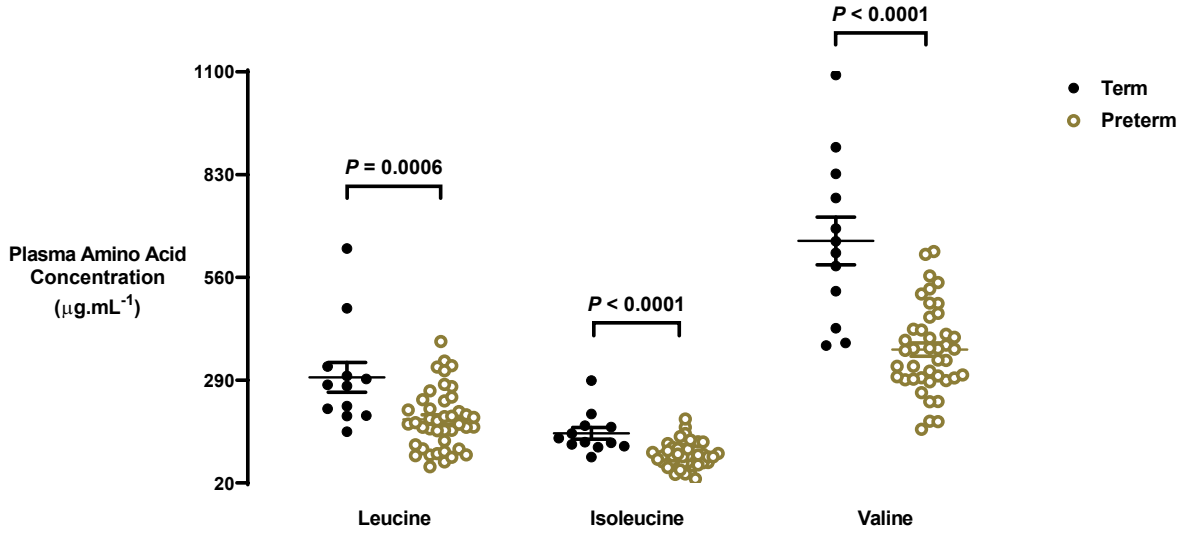



**Figure 3-2. Plasma BCAA concentration at day zero.**
The Term group (n = 13) had higher leucine, isoleucine, and valine concentration compared to the Preterm groups (all groups combined, n = 45). Data are mean ± SEM. *P*-values for differences between groups by ANOVA.

### 3.2.2.2. Plasma BCAA Concentration During Supplementation

Plasma concentrations of BCAA changed differently among groups over time (leucine (Time $P < 0.0001$, Time × Treatment $P < 0.002$), isoleucine (Time $P < 0.0001$, Time × Treatment $P < 0.04$), and valine (Time $P < 0.0001$, Time × Treatment $P < 0.002$). No differences were found between the sexes (Table 3-2).

There was no difference in plasma concentrations of BCAA between sexes; therefore, the results are reported with both sexes combined.

On day four, plasma concentrations of leucine and valine were higher in the Term group than those in all Preterm groups, and the concentration of isoleucine was higher in Term than in Maltodextrin and Water groups (Table 3-2).

On day eight, plasma leucine concentration was not different among groups. Plasma isoleucine concentration was higher in the Term and BCAA groups than in the Maltodextrin group (P = 0.002, on post hoc analysis), and valine concentration was higher in the Term group than in the Maltodextrin group (P = 0.008, on post hoc analysis). Two and six hours post BCAA supplementation, isoleucine, leucine, and valine were different among groups with the highest concentrations in the BCAA group (Table 3-3 and Figure 3-3).

**Table 3-2. The BCAA (isoleucine, leucine, and valine) plasma concentrations during the supplemented period.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=9) | (F=7) | (M=6) | Sex | Treatment | Interaction |
| **Leucine (µg.mL$^{-1}$)** | | | | | | | | | | | |
| Day 4 | 316±50 $^{a,b}$ | 312±56 $^{a,b}$ | 324±33 $^{a,b}$ | 246±39 $^{b}$ | 334±47 $^{a,b}$ | 238±31 $^{b}$ | 388±35 $^{a,b}$ | 502±49 $^{a}$ | 0.61 | **0.002** | 0.09 |
| Day 8 | 253±25 | 248±21 | 172±28 | 215±19 | 193±36 | 237±35 | 201±32 | 225±38 | 0.21 | 0.25 | 0.82 |
| Day 11 | 237±24$^{a,b}$ | 276±28$^{a}$ | 163±18 $^{b}$ | 222±15$^{a,b}$ | 200±31$^{a,b}$ | 240±20$^{a,b}$ | 179±10$^{a,b}$ | 245±25$^{a,b}$ | **0.002** | **0.03** | 0.91 |
| Day 15 | 233±42 | 291±34 | 215±35 | 214±16 | 215±32 | 209±26 | 200±22 | 251±23 | 0.25 | 0.33 | 0.62 |
| **Isoleucine (µg.mL$^{-1}$)** | | | | | | | | | | | |
| Day 4 | 177±12 $^{a,b}$ | 163±26 $^{a,b}$ | 174±17 $^{a,b}$ | 134±20 $^{b}$ | 174±19 $^{a,b}$ | 129±15 $^{b}$ | 197±19 $^{a,b}$ | 248±27 $^{a}$ | 0.40 | **0.003** | 0.08 |
| Day 8 | 153±10 $^{a,b}$ | 143±12 $^{a,b}$ | 110±16 $^{b}$ | 120±9 $^{b}$ | 138±12 $^{a,b}$ | 131±19 $^{a,b}$ | 182±11 $^{a}$ | 114±17 $^{b}$ | 0.05 | 0.05 | **0.04** |
| Day 11 | 159±10$^{a,b}$ | 140±12 $^{a,b}$ | 115±14$^{b}$ | 120±8 $^{b}$ | 127±6$^{a,b}$ | 134±11 $^{a,b}$ | 178±18 $^{a}$ | 129±13 $^{a,b}$ | 0.11 | **0.01** | 0.10 |
| Day 15 | 157±15$^{a}$ | 155±10$^{a}$ | 96±10$^{b}$ | 121±9 $^{a,b}$ | 139±8$^{a,b}$ | 116±14 $^{a,b}$ | 171±17 $^{a}$ | 132±11 $^{a,b}$ | 0.27 | **0.0006** | 0.06 |
| **Valine (µg.mL$^{-1}$)** | | | | | | | | | | | |
| Day 4 | 617±104 $^{b,c}$ | 561±106 $^{b,c}$ | 601±59 $^{b,c}$ | 460±76 $^{c}$ | 605±69 $^{b,c}$ | 480±35 $^{c}$ | 849±50 $^{a,b}$ | 1062±102 $^{a}$ | 0.63 | **<0.0001** | 0.13 |
| Day 8 | 543±43$^{a,b}$ | 487±32$^{a,b}$ | 377±46 $^{b}$ | 415±29 $^{b}$ | 466±51$^{a,b}$ | 446±62 $^{b}$ | 672±65 $^{a}$ | 453±63$^{a,b}$ | 0.07 | **0.009** | 0.08 |
| Day 11 | 549±75$^{a,b}$ | 544±52$^{a,b}$ | 418±29 $^{b}$ | 425±18$^{a,b}$ | 507±48$^{a,b}$ | 510±51$^{a,b}$ | 623±45$^{a}$ | 472±33 $^{a,b}$ | 0.28 | **0.02** | 0.32 |
| Day 15 | 482±65$^{a,b}$ | 535±64$^{a,b}$ | 482±42$^{a,b}$ | 427±35$^{a,b}$ | 489±48$^{a,b}$ | 399±56 $^{b}$ | 655±58 $^{a}$ | 493±55$^{a,b}$ | 0.10 | 0.09 | 0.27 |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. F: female; M: male

**Table 3-3. The BCAA (isoleucine, leucine, and valine) plasma concentrations two and six hours post supplementation.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=9) | (F=7) | (M=6) | Sex | Treatment | Interaction |
| **Isoleucine (µg.mL$^{-1}$)** | | | | | | | | | | | |
| Day 8 | | | | | | | | | | | |
| 2 hrs | 285±37$^{a}$ | 285±30$^{a}$ | 104±6$^{b}$ | 96±7$^{b}$ | 109±13$^{b}$ | 114±11$^{b}$ | 128±14$^{b}$ | 100±21$^{b}$ | 0.58 | **<0.0001** | 0.88 |
| 6 hrs | 142±22$^{a,b}$ | 162±20$^{a}$ | 102±5$^{b}$ | 96±6$^{b}$ | 138±12$^{a,b}$ | 108±7$^{a,b}$ | 138±10$^{a,b}$ | 109±17$^{a,b}$ | 0.24 | **0.002** | 0.21 |
| **Leucine (µg.mL$^{-1}$)** | | | | | | | | | | | |
| Day 8 | | | | | | | | | | | |
| 2 hrs | 632±86$^{a}$ | 589±74$^{a}$ | 206±13$^{b}$ | 207±21$^{b}$ | 204±21$^{b}$ | 214±17$^{b}$ | 258±28$^{b}$ | 195±36$^{b}$ | 0.47 | **<0.0001** | 0.84 |
| 6 hrs | 350±63$^{a,b}$ | 361±41$^{a}$ | 198±10$^{c}$ | 187±14$^{c}$ | 296±35$^{a,b,c}$ | 201±13$^{b,c}$ | 263±20$^{a,b,c}$ | 211±33$^{a,b,c}$ | 0.13 | **<0.0001** | 0.40 |
| **Valine (µg.mL$^{-1}$)** | | | | | | | | | | | |
| Day 8 | | | | | | | | | | | |
| 2 hrs | 852±94$^{a}$ | 767±55$^{a}$ | 366±24$^{b}$ | 357±23$^{b}$ | 383±50$^{b}$ | 386±33$^{b}$ | 503±58$^{b}$ | 408±63$^{b}$ | 0.23 | **<0.0001** | 0.73 |
| 6 hrs | 570±96$^{a,b}$ | 617±63$^{a}$ | 352±21$^{b}$ | 346±25$^{b}$ | 471±49$^{a,b}$ | 369±36$^{b}$ | 483±40$^{a,b}$ | 414±61$^{a,b}$ | 0.40 | **0.0003** | 0.51 |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. F: female; M: male

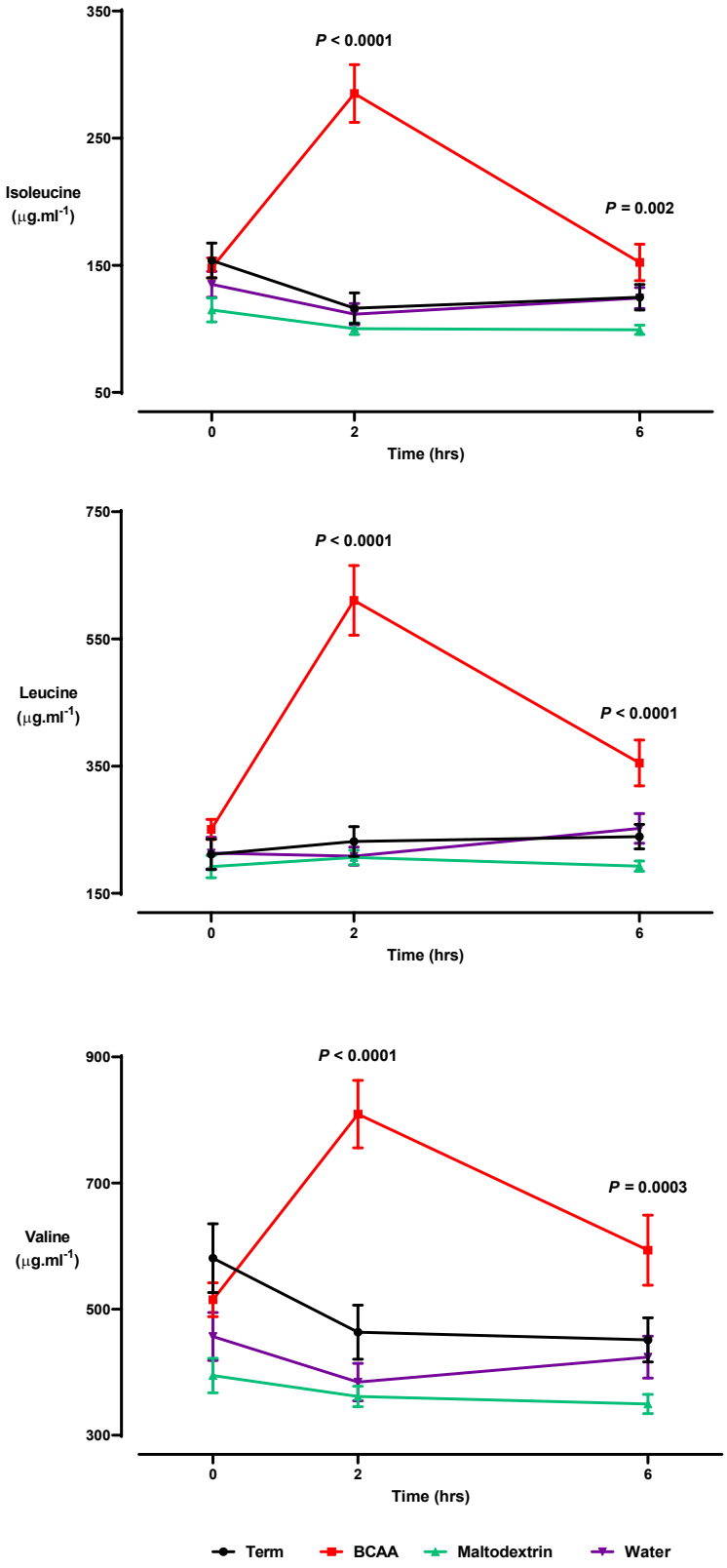



**Figure 3-3. The BCAA (isoleucine, leucine, and valine) plasma concentrations two and six hours post supplementation.**
Data are mean ± SEM. *P*-values for differences between groups by ANOVA.

On day 11, plasma leucine concentration was higher in the BCAA group than in the Maltodextrin group ($P = 0.02$, post hoc analysis). Isoleucine concentration was higher in the Term and BCAA groups than in the Maltodextrin group ($P = 0.01$, on post hoc analysis). Valine concentration was higher in the BCAA group than in the Maltodextrin group ($P = 0.04$, post hoc analysis).

On day 15, there was no difference in plasma leucine and valine concentrations among groups, but isoleucine concentrations were higher in Term and BCAA groups than the Maltodextrin group ($P = 0.005$ and $P = 0.001$, respectively, on post hoc analysis).

Plasma concentrations of the essential amino acids tryptophan, threonine, and histidine were higher in the Term group on day four compared to all Preterm groups ($P < 0.0001$). Plasma concentration of lysine was higher in the Term group than in the BCAA group on day 15 ($P = 0.02$) (Figure 3-4).

For non-essential amino acids, plasma concentrations of arginine, asparagine, glycine, serine, tryptophan, and proline were higher in the Term group compared to some or all of the Preterm groups, particularly on day 4 (Figure 3-5).

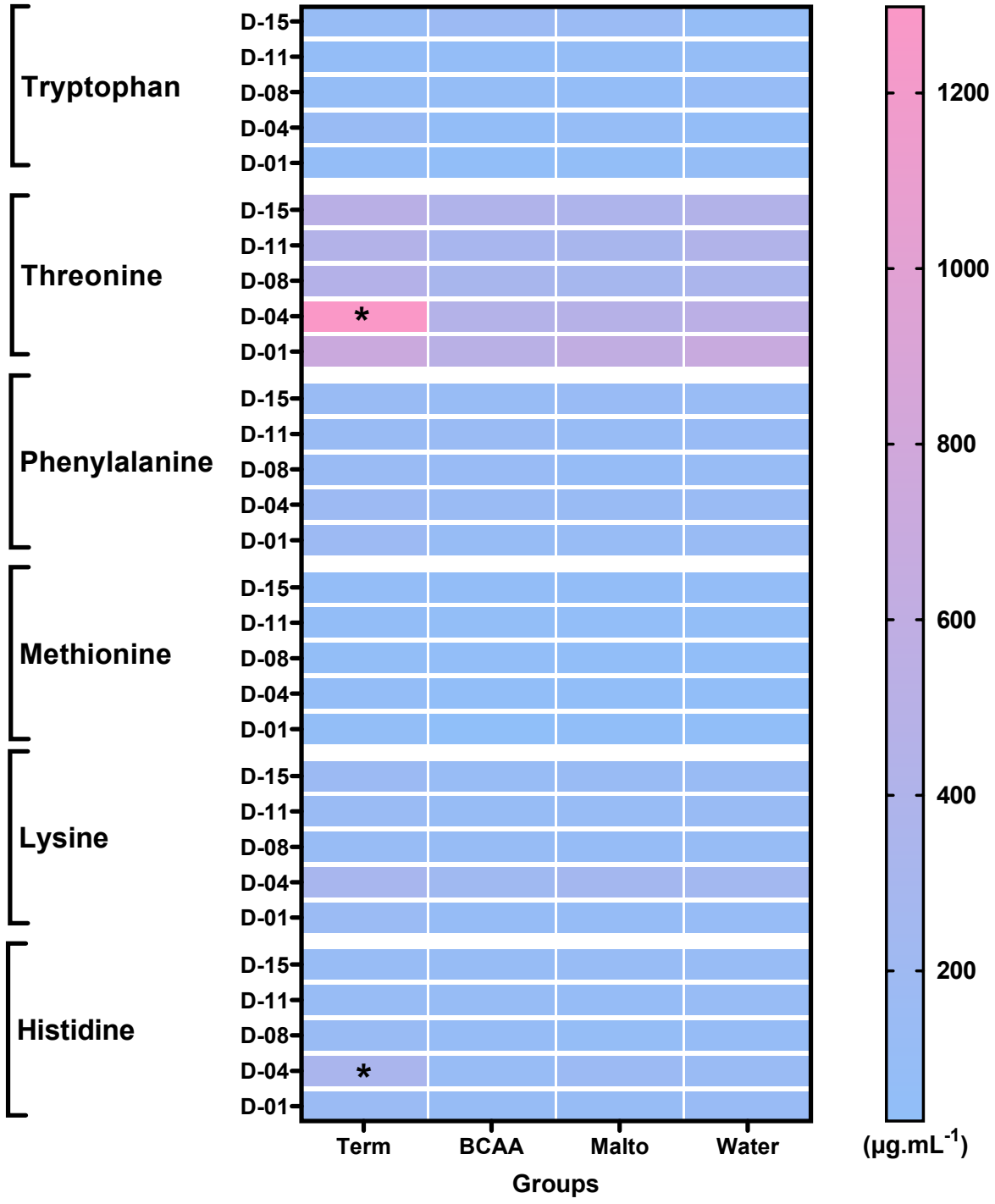



**Figure 3-4. The plasma concentrations of essential amino during the supplemented 15 days.**

Data are mean ± SEM. The blue colour indicates a lower concentration, while the pink colour indicates a higher concentration. * *P* values for differences between experimental Term and all Preterm groups by ANOVA ($P < 0.0001$) Malto: Maltodextrin.

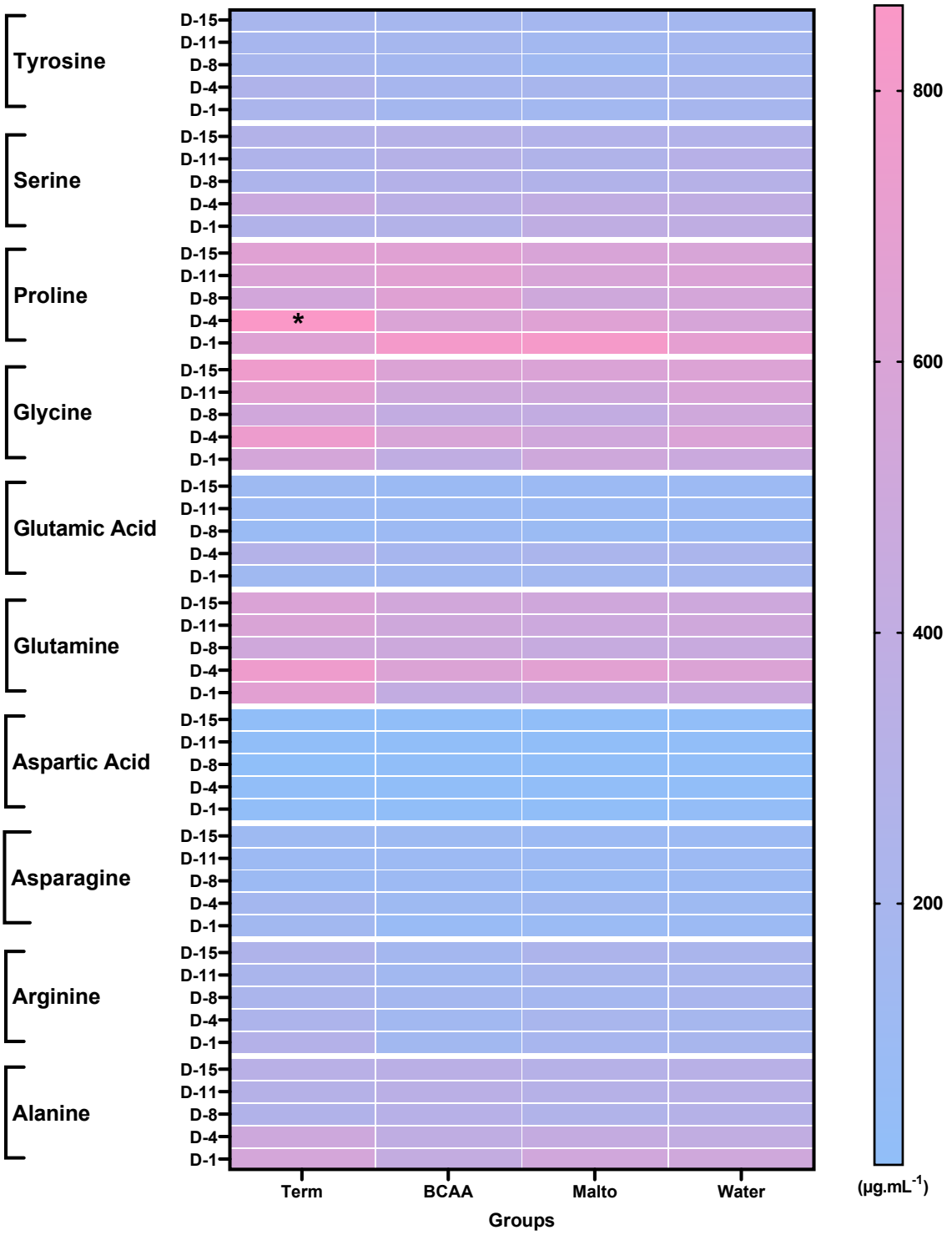



**Figure 3-5. The plasma concentrations of non-essential amino during the supplemented 15 days.**

Data are mean ± SEM. The blue colour indicates a lower concentration, while the pink colour indicates a higher concentration. * *P*-values for differences between experimental Term and all Preterm groups by ANOVA ($P = 0.0003$) Malto: Maltodextrin.

## 3.2.3. Growth Parameters

### 3.2.3.1. Day zero

Weight and linear measurements at day zero were greater in the Term group than in the Preterm groups in both males and females ($P < 0.05$) (Table 3-4). Term males had greater BPD than Term females ($P < 0.05$). There was no difference in birth weight or linear measurements among the Preterm groups, but bodyweight and BPD were greater in males than females ($P < 0.05$).

### 3.2.3.2. Day 1-15: Morphometry, Growth Velocity, and Milk Intake

From day 4 to day 15, weight and linear measures increased similarly over time in all four groups (Table 3-5 and Table 3-6). On day 15, the Term group had the greatest weight and linear measurements overall compared with all Preterm groups. In males, all growth parameters except for abdominal girth were greater than those of females, with the greatest difference between sexes being in leg length measurements (HL and HT) and BPD (Table 3-5 and Table 3-6).

Growth velocity for all growth parameters from day 1-15 was similar in all groups and both sexes (Table 3-7). The growth pattern was different in males in the Maltodextrin group, who had the lowest growth velocity on days 1-4 (Figure 3-6).

Regardless of their supplementation, the Term and Preterm groups had similar milk intake (Figure 3-7). There was a positive correlation between the milk intake and growth velocity in all Preterm groups, but not in the Term group (Figure 3-8).

**Table 3-4. Bodyweight and morphometric data were collected from Preterm and Term lambs at day zero.**

| | Body weight (kg) | | CRL (cm) | | ABDOM (cm) | | CHEST (cm) | | HL (cm) | | HT (cm) | | BPD (cm) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Preterm** | **Term** | **Preterm** | **Term** | **Preterm** | **Term** | **Preterm** | **Term** | **Preterm** | **Term** | **Preterm** | **Term** | **Preterm** | **Term** |
| **All** | 5.0±0.1 | 6.0±0.2[a] | 48±0.5 | 52±1[a] | 36±0.5 | 40±0.7[a] | 40±0.3 | 41±0.6[a] | 37±0.2 | 40±1[a] | 20±0.1 | 21±0.3[a] | 6.1±0 | 6.5±0.1[a] |
| **Male** | 5.0±0.1[b] | 6.5±0.3[a] | 48±1.0 | 54±2[a] | 36±1.0 | 40±1.0[a] | 40±0.4 | 42±1.0 | 38±0.3 | 41±1[a] | 20±0.2 | 22±0.5[a] | 6.2±0[b] | 6.7±0.1[a,b] |
| **Female** | 4.5±0.1 | 6.1±0.2[a] | 47±1.0 | 51±1[a] | 37±1.0 | 40±1.0[a] | 39±0.5 | 40±0.4 | 37±0.4 | 39±1[a] | 19±0.2 | 21±0.4 | 6±0.1 | 6.3±0.1[a] |

Preterm lambs n = 45 (22 females and 23 males), Term lambs n = 13 (7 females and 6 males).
Abbreviations: CRL: crown-rump length, ABDOM: abdominal girth, CHEST: chest girth, HL: hind limb length, HT: hock-to-toe length, BPD: biparietal diameter.
Data are shown for the following groups of lambs: Preterm male (n = 23), Preterm female (n = 22), Term male (n = 6), and Term female (n = 7). Data are mean ± SEM.
[a] $P < 0.05$ for Term vs Preterm.
[b] $P < 0.05$ for male vs female.

**Table 3-5. Morphometric measurements including weight, crown-rump length, abdominal girth, and chest girth for the four groups from day four until day 15.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=9) | (F=7) | (M=6) | Sex | Treatment | Interaction |
| **Weight (Kg)** | | | | | | | | | | | |
| Day 4 | 4.9±0.3$^{b}$ | 5.4±0.2$^{b}$ | 5.7±0.2$^{b}$ | 5.7±0.1$^{b}$ | 5.1±0.3$^{b}$ | 5.7±0.2$^{b}$ | 7.3±0.2$^{a}$ | 7.6±0.4$^{a}$ | **0.03** | **0.001** | 0.71 |
| Day 8 | 6.2±0.4$^{b}$ | 6.9±0.2$^{b}$ | 7.1±0.2$^{b}$ | 7.1±0.2$^{b}$ | 6.4±0.4$^{b}$ | 7.0±0.2$^{b}$ | 8.5±0.2$^{a}$ | 8.9±0.4$^{a}$ | **0.03** | **0.001** | 0.67 |
| Day 11 | 7.2±0.5$^{c}$ | 7.9±0.2$^{c}$ | 8.1±0.3$^{c}$ | 8.3±0.2$^{b,c}$ | 7.4±0.5$^{c}$ | 8.1±0.3$^{c}$ | 9.7±0.3$^{a,b}$ | 10.2±0.5$^{a}$ | 0.05 | **0.001** | 0.86 |
| Day 15 | 8.6±0.6$^{c}$ | 9.3±0.2$^{b,c}$ | 9.4±0.3$^{b.c}$ | 9.5±0.3$^{b,c}$ | 8.7±0.6$^{c}$ | 9.5±0.4$^{b,c}$ | 10.9±0.2$^{a,b}$ | 11.7±0.6$^{a}$ | **0.03** | **0.001** | 0.81 |
| **CRL (cm)** | | | | | | | | | | | |
| Day 4 | 50±1$^{b}$ | 51±2$^{a,b}$ | 52±1$^{a,b}$ | 52±1$^{a,b}$ | 49±2$^{b}$ | 52±1$^{a,b}$ | 54±1$^{a,b}$ | 56±2$^{a}$ | **0.03** | **0.001** | 0.84 |
| Day 8 | 53±1$^{a}$ | 55±1$^{a,b}$ | 55±1$^{a,b}$ | 56±1$^{a,b}$ | 53±2$^{a}$ | 56±1$^{a,b}$ | 57±1$^{a,b}$ | 60±2$^{a}$ | **0.02** | **0.01** | 0.78 |
| Day 11 | 57±1$^{b}$ | 58±1$^{b}$ | 58±1$^{b}$ | 59±1$^{b}$ | 56±1$^{b}$ | 59±1$^{b}$ | 60±1$^{a,b}$ | 64±1$^{a}$ | **0.01** | **0.001** | 0.30 |
| Day 15 | 61±1$^{b}$ | 61±1$^{b}$ | 6±1$^{b}$ | 61±1$^{a,b}$ | 59±2$^{b}$ | 62±1$^{a,b}$ | 63±1$^{a,b}$ | 66±1$^{a}$ | **0.03** | **0.01** | 0.31 |
| **ABDOM (cm)** | | | | | | | | | | | |
| Day 4 | 40±2$^{a,b}$ | 40±1$^{a,b}$ | 41±1$^{a,b}$ | 40±1$^{a,b}$ | 39±1$^{b}$ | 40±1$^{b}$ | 44±2$^{a,b}$ | 43±1$^{a}$ | 0.44 | **0.01** | 0.79 |
| Day 8 | 44±1$^{b,c}$ | 43±1$^{c}$ | 45±1$^{b,c}$ | 44±1$^{b,c}$ | 43±1$^{c}$ | 44±1$^{c}$ | 47±1$^{a,b}$ | 49±1$^{a}$ | 0.43 | **0.001** | 0.43 |
| Day 11 | 45±1$^{b}$ | 46±1$^{b}$ | 47±1$^{a,b}$ | 47±1$^{a,b}$ | 45±1$^{b}$ | 47±1$^{a,b}$ | 51±1$^{a}$ | 51±1$^{a}$ | 0.50 | **0.001** | 0.48 |
| Day 15 | 48±2$^{b}$ | 50±1$^{a,b}$ | 50±2$^{a,b}$ | 49±1$^{a,b}$ | 48±1$^{a,b}$ | 49±1$^{a,b}$ | 54±1$^{a}$ | 53±1$^{a,b}$ | 0.57 | **0.01** | 0.69 |
| **CHEST (cm)** | | | | | | | | | | | |
| Day 4 | 40±1$^{b}$ | 42±1$^{a,b}$ | 42±1$^{a,b}$ | 42±1$^{a,b}$ | 40±1$^{b}$ | 42±1$^{a,b}$ | 43±1$^{a,b}$ | 44±1$^{a}$ | 0.06 | **0.01** | 0.39 |
| Day 8 | 43±1$^{b}$ | 44±1$^{a,b}$ | 44±1$^{a,b}$ | 45±1$^{a,b}$ | 43±1$^{b}$ | 44±1$^{a,b}$ | 46±1$^{a,b}$ | 48±1$^{a}$ | **0.01** | **0.001** | 0.75 |
| Day 11 | 45±1$^{b}$ | 45±0$^{a,b}$ | 46±1$^{a,b}$ | 47±1$^{a,b}$ | 44±1$^{b}$ | 46±1$^{a,b}$ | 48±1$^{a,b}$ | 49±1$^{a}$ | 0.06 | **0.001** | 0.96 |
| Day 15 | 47±1$^{b,c}$ | 49±1$^{a,b,c}$ | 48±1$^{a,b,c}$ | 48±1$^{a,b,c}$ | 46±1$^{c}$ | 48±1$^{a,b,c}$ | 50±1$^{a,b}$ | 51±1$^{a}$ | **0.04** | **0.001** | 0.72 |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. CRL: crown-rump length; ABDOM: abdominal girth, CHEST: chest girth F: female; M: male.

**Table 3-6. Morphometric measurements including hind limb length, hock-to-toe length, and PBD for the treatment groups and controls from day four until day 15.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=9) | (F=7) | (M=6) | Sex | Treatment | Interaction |
| **HL (cm)** | | | | | | | | | | | |
| Day 4 | $38\pm1^{c}$ | $39\pm0^{b,c}$ | $40\pm0^{a,b,c}$ | $40\pm1^{b,c}$ | $39\pm1^{b,c}$ | $40\pm0^{b,c}$ | $42\pm1^{a,b}$ | $43\pm1^{a}$ | 0.14 | **0.001** | 0.47 |
| Day 8 | $39\pm1^{c}$ | $41\pm1^{b,c}$ | $41\pm0^{b,c}$ | $42\pm1^{b,c}$ | $40\pm1^{c}$ | $41\pm0^{b,c}$ | $43\pm0^{a,b}$ | $44\pm1^{a}$ | **0.02** | **0.001** | 0.53 |
| Day 11 | $40\pm1^{d}$ | $42\pm1^{b,c,d}$ | $42\pm0^{b,c,d}$ | $43\pm0^{b,c}$ | $42\pm1^{c,d}$ | $42\pm0^{b,c}$ | $44\pm0^{a,b}$ | $45\pm0^{a}$ | **0.01** | **0.001** | 0.32 |
| Day 15 | $42\pm1^{c}$ | $44\pm0^{a,b,c}$ | $44\pm0^{b,c}$ | $44\pm1^{a,b,c}$ | $43\pm1^{b,c}$ | $44\pm0^{a,b}$ | $45\pm0^{a,b}$ | $46\pm1^{a}$ | **0.01** | **0.001** | 0.41 |
| **HT (cm)** | | | | | | | | | | | |
| Day 4 | $20\pm0^{c}$ | $21\pm1^{b,c}$ | $20\pm0^{b,c}$ | $21\pm0^{a,b,c}$ | $20\pm0^{b,c}$ | $21\pm0^{b,c}$ | $21\pm0^{a,b}$ | $23\pm0^{a}$ | **0.001** | **0.001** | 0.85 |
| Day 8 | $20\pm0^{c}$ | $22\pm1^{b,c}$ | $21\pm0^{b,c}$ | $22\pm0^{a,b,c}$ | $21\pm1^{b,c}$ | $22\pm0^{b,c}$ | $22\pm0^{a,b}$ | $23\pm1^{a}$ | **0.001** | **0.001** | 0.75 |
| Day 11 | $21\pm0^{c}$ | $22\pm0^{b,c}$ | $22\pm0^{b,c}$ | $22\pm0^{a,b,c}$ | $22\pm1^{b,c}$ | $22\pm0^{b,c}$ | $23\pm0^{a,b}$ | $24\pm0^{a}$ | **0.001** | **0.001** | 0.58 |
| Day 15 | $22\pm0^{c}$ | $23\pm0^{b,c}$ | $22\pm0^{b,c}$ | $23\pm0^{a,b,c}$ | $22\pm1^{b,c}$ | $23\pm0^{a,b,c}$ | $23\pm0^{a,b}$ | $25\pm0^{a}$ | **0.01** | **0.001** | 0.88 |
| **BPD (cm)** | | | | | | | | | | | |
| Day 4 | $6.2\pm0.1^{c}$ | $6.2\pm0.1^{c}$ | $6.3\pm0.0^{b,c}$ | $6.4\pm0.1^{b,c}$ | $6.1\pm0.1^{c}$ | $6.3\pm0.1^{b,c}$ | $6.5\pm0.1^{a,b}$ | $6.8\pm0.1^{a}$ | **0.01** | **0.001** | 0.57 |
| Day 8 | $6.4\pm0.1^{c}$ | $6.5\pm0.0^{b,c}$ | $6.5\pm0.0^{b,c}$ | $6.5\pm0.1^{b,c}$ | $6.4\pm0.1^{c}$ | $6.5\pm0.1^{b,c}$ | $6.7\pm0.1^{a,b}$ | $6.9\pm0.0^{a}$ | **0.01** | **0.001** | 0.44 |
| Day 11 | $6.5\pm0.1^{b,c}$ | $6.6\pm0.0^{b,c}$ | $6.7\pm0.0^{b,c}$ | $6.6\pm0.1^{b,c}$ | $6.5\pm0.1^{c}$ | $6.7\pm0.0^{b,c}$ | $6.8\pm0.1^{a,b}$ | $7.0\pm0.0^{a}$ | **0.01** | **0.001** | 0.15 |
| Day 15 | $6.7\pm0.1^{b}$ | $6.8\pm0.1^{b}$ | $6.8\pm0.0^{b}$ | $6.9\pm0.0^{a,b}$ | $6.7\pm0.1^{b}$ | $6.9\pm0.1^{a,b}$ | $7.0\pm0.1^{a,b}$ | $7.2\pm0.1^{a}$ | **0.01** | **0.001** | 0.66 |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. CRL: crown-rump length, HL: hind limb length, HT: hock to toe length, BPD: biparietal diameter; F: female; M: male

**Table 3-7. Growth velocity for weight and linear measures from 1-15 days.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=9) | (F=7) | (M=6) | Sex | Treatment | Interaction |
| **GV Weight ($g.Kg^{-1}.day^{-1}$)** | | | | | | | | | | | |
| From 1-15 | 21±1 | 21±1 | 21±1 | 19±1 | 21±1 | 20±1 | 18±1 | 18±2 | 0.28 | 0.07 | 0.49 |
| **GV CRL ($mm.cm^{-1}.day^{-1}$)** | | | | | | | | | | | |
| From 1-15 | 8±0 | 8±1 | 8±0 | 6±0 | 7±1 | 8±1 | 7±1 | 6±0 | 0.50 | 0.08 | 0.40 |
| **GV ABDO ($mm.cm^{-1}.day^{-1}$)** | | | | | | | | | | | |
| From 1-15 | 9±1 | 10±1 | 9±1 | 10±1 | 10±1 | 10±1 | 9±0 | 9±1 | 0.41 | 0.74 | 0.94 |
| **GV CHEST ($mm.cm^{-1}.day^{-1}$)** | | | | | | | | | | | |
| From 1-15 | 21±1 | 21±1 | 21±1 | 19±1 | 21±1 | 20±1 | 18±1 | 18±2 | 0.28 | 0.07 | 0.49 |
| **GV HL ($mm.cm^{-1}.day^{-1}$)** | | | | | | | | | | | |
| From 1-15 | 4±0 | 5±1 | 4±0 | 5±0 | 5±1 | 5±0 | 4±0 | 4±1 | 0.60 | 0.21 | 0.13 |
| **GV HT ($mm.cm^{-1}.day^{-1}$)** | | | | | | | | | | | |
| From 1-15 | 3±0 | 4±1 | 4±0 | 4±1 | 5±0 | 5±0 | 4±0 | 4±0 | 0.94 | 0.19 | 0.58 |
| **GV BPD ($mm.cm^{-1}.day^{-1}$)** | | | | | | | | | | | |
| From 1-15 | 3±0 | 3±0 | 4±0 | 3±0 | 4±1 | 3±0 | 3±0 | 2±0 | 0.12 | 0.25 | 0.19 |

Data are mean ± SEM. GV: growth velocity; GV weight; CRL: crown-rump length; ABDOM: abdominal girth; CHEST: chest girth; HL: hind limb length; HT: hock to toe length; BPD: biparietal diameter; F: female; M: male.

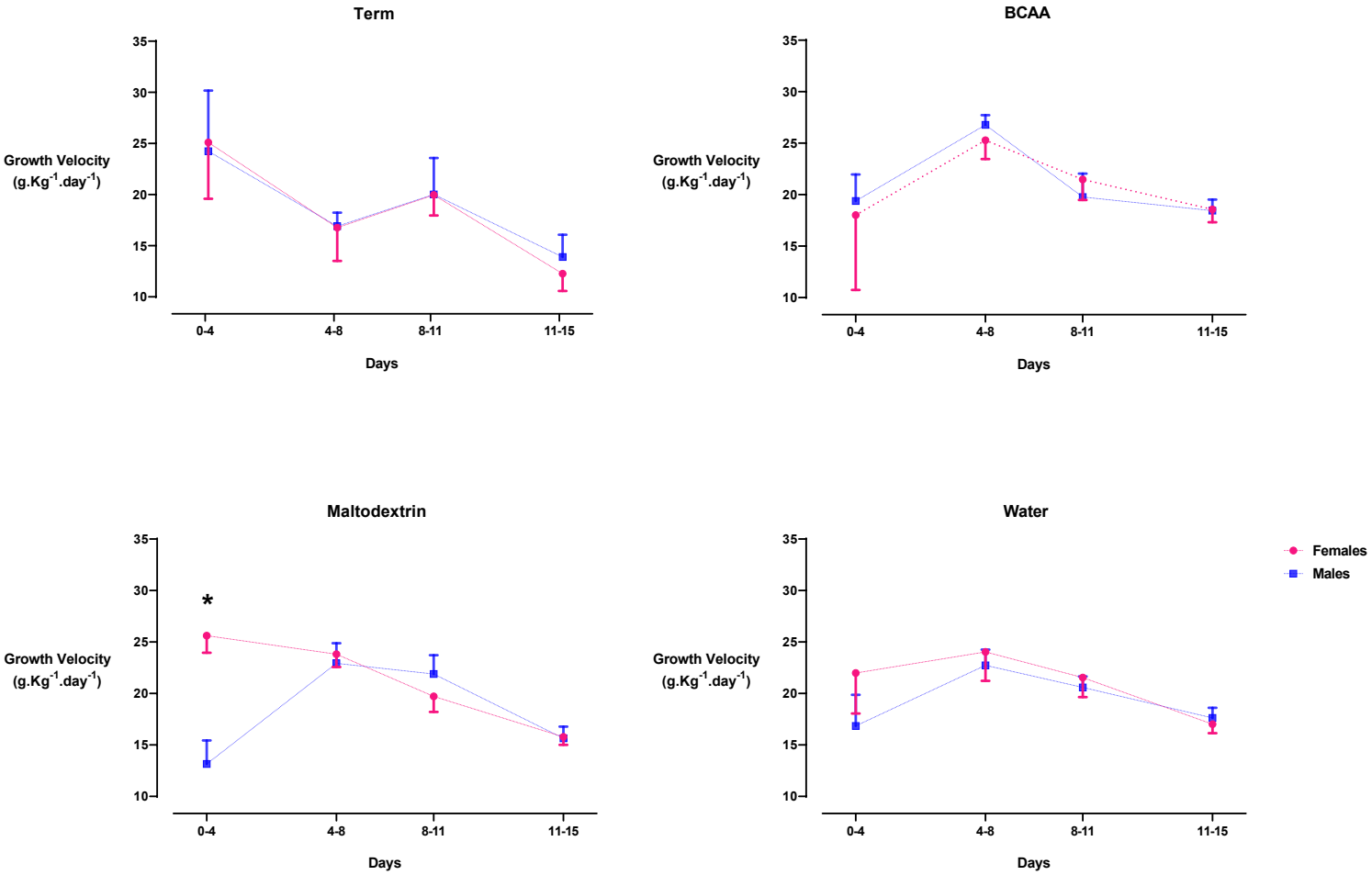



**Figure 3-6. The growth velocity for weight in the four different groups.**
There was no significant difference in growth velocity for weight among the group. There was a sex difference in the Maltodextrin group; the females had higher growth velocity on day 4 (* $P < 0.05$). Data are mean ± SEM.

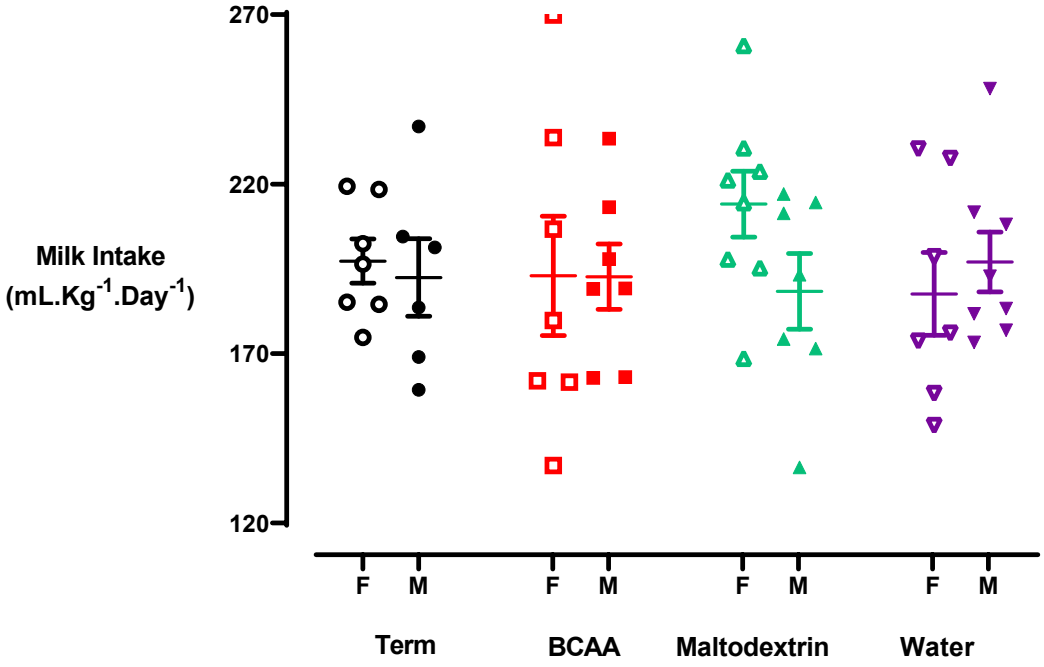



**Figure 3-7. The milk intake in the four different groups.**
Data are mean ± SEM.

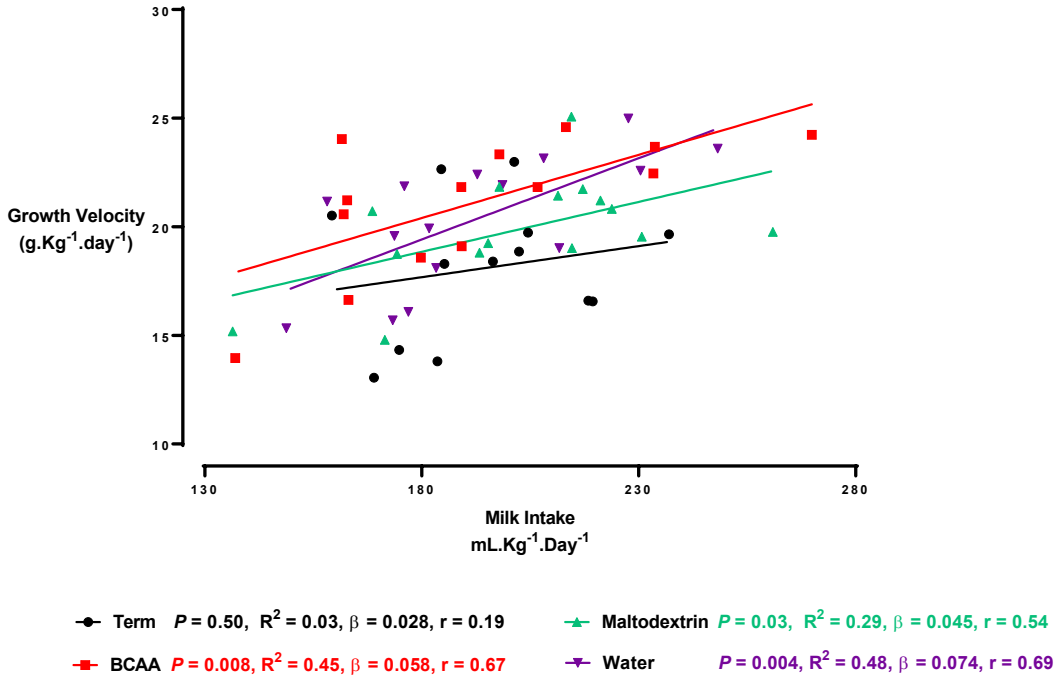



**Figure 3-8. Relationship between growth velocity and milk intake by treatment group.**
Pearson's correlation was used to examine bivariate relationships and calculate the correlation coefficient (r) r and β-coefficient.

#### 3.2.3.3. Day 30-120 (pre-puberty): Measurement and Growth Velocity

By day 120, earlier differences in growth parameters between Term and all Preterm treatment groups were no longer present. Males tended to be heavier ($P = 0.06$) and had greater limb length (HL and HT) and BPD than females (Table 3-8 and Table 3-9).

There was a sex × treatment interaction on day 120 in abdominal girth in the supplemented groups, with BCAA males having greater abdominal girth than BCAA females and males in the Maltodextrin group having lesser abdominal girth than Maltodextrin females. There was no difference in abdominal girth between sexes in the Term and Preterm water groups (Table 3-8).

There was a trend towards a sex × treatment interaction in BPD at day 120 ($P = 0.05$), with the greatest difference in BPD between sexes in the Preterm water group (males > females) and no difference between sexes in the Maltodextrin group (Table 3-9).

Growth velocity for weight was different among groups, with the BCAA group having a higher growth velocity than the Term group from 15-30 days ($P = 0.01$), 30-60 days ($P = 0.005$), 60-90 days ($P = 0.01$), and 90-120 days ($P = 0.03$). The CRL growth velocity was not different among the group; however, there was a sex difference from 30-60 days (females > males, $P = 0.04$). HL growth velocity differed among groups, especially between BCAA and Term groups from 30-60 days (BCAA > Term $P = 0.03$). The BPD growth velocity differed among groups, being greater in the Water group than in the Term group from 60-120 days ($P = 0.03$) (Table 3-10 and Table 3-11).

**Table 3-8. Morphometric measurements including weight, crown-rump length, abdominal girth, and chest girth for the treatment groups and controls from day 30 until day 120.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=9) | (F=7) | (M=6) | Sex | Treatment | Interaction |
| **Weight (Kg)** | | | | | | | | | | | |
| Day 30 | 13±1[b] | 14±0[a,b] | 14±1[a,b] | 14±1[a,b] | 13±1[a,b] | 14±1[a,b] | 16±0[a,b] | 16±1[a] | 0.22 | **0.001** | 0.68 |
| Day 60 | 21±1[b] | 23±1[a,b] | 22±1[a,b] | 22±1[a,b] | 22±2[a,b] | 24±0[a,b] | 26±1[a,b] | 27±2[a] | 0.07 | **0.006** | 0.93 |
| Day 90 | 23±2 | 32±1 | 31±2 | 30±1 | 29±3 | 34±1 | 33±1 | 36±2 | 0.06 | 0.14 | 0.54 |
| Day 120 | 33±2 | 41±2 | 38±3 | 37±2 | 36±4 | 42±3 | 41±1 | 42±3 | 0.06 | 0.27 | 0.22 |
| **CRL (cm)** | | | | | | | | | | | |
| Day 30 | 69±2[a,b] | 71±1[a,b] | 70±1[a,b] | 70±1[a,b] | 67±2[b] | 71±1[a,b] | 69±1[a,b] | 75±2[a] | **0.004** | 0.31 | 0.13 |
| Day 60 | 81±1 | 81±2 | 80±3 | 80±2 | 78±3 | 82±2 | 84±1 | 85±2 | 0.47 | 0.10 | 0.65 |
| Day 90 | 88±2 | 90±2 | 88±2 | 87±2 | 86±4 | 90±2 | 91±1 | 90±2 | 0.48 | 0.62 | 0.61 |
| Day 120 | 94±2 | 95±1 | 96±2 | 94±1 | 93±4 | 97±2 | 95±1 | 97±2 | 0.26 | 0.85 | 0.56 |
| **ABDOM (cm)** | | | | | | | | | | | |
| Day 30 | 54±2 | 58±1 | 58±2 | 57±1 | 57±2 | 60±3 | 60±1 | 59±2 | 0.50 | 0.18 | 0.44 |
| Day 60 | 68±2[b] | 71±2[a,b] | 71±1[a,b] | 73±1[a,b] | 71±2[a,b] | 71±2[a,b] | 77±1[a] | 76±1[a] | 0.26 | **0.001** | 0.67 |
| Day 90 | 78±2 | 83±2 | 82±2 | 82±2 | 82±3 | 81±1 | 85±1 | 87±2 | 0.25 | 0.07 | 0.44 |
| Day 120 | 85±1 | 93±2 | 90±2 | 86±2 | 89±4 | 87±2 | 93±2 | 94±4 | 0.59 | 0.12 | **0.04** |
| **CHEST (cm)** | | | | | | | | | | | |
| Day 30 | 52±1 | 53±1 | 54±1 | 53±1 | 53±1 | 57±2 | 56±1 | 57±2 | 0.19 | 0.11 | 0.39 |
| Day 60 | 64±2 | 65±1 | 65±2 | 65±0 | 64±2 | 66±1 | 70±1 | 69±2 | 0.63 | **0.01** | 0.80 |
| Day 90 | 71±2 | 74±1 | 73±2 | 72±2 | 73±3 | 74±2 | 76±2 | 76±2 | 0.71 | 0.29 | 0.82 |
| Day 120 | 76±2 | 84±3 | 80±2 | 79±2 | 79±4 | 79±3 | 82±1 | 85±4 | 0.19 | 0.44 | 0.27 |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. F: female; M: male; CRL: crown-rump length; ABDOM: abdominal girth; CHEST: chest girth

**Table 3-9. Morphometric measurements including hind limb length, hock-to-toe length, and biparietal diameter for the treatment groups and controls from day 30 until day 120.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=9) | (F=7) | (M=6) | Sex | Treatment | Interaction |
| **HL (cm)** | | | | | | | | | | | |
| Day 30 | 46±1$^{b}$ | 47±1$^{a,b}$ | 48±0$^{a,b}$ | 48±1$^{a,b}$ | 47±1$^{a,b}$ | 47±1$^{a,b}$ | 49±1$^{a,b}$ | 50±1$^{a}$ | 0.09 | **0.003** | 0.83 |
| Day 60 | 52±1 | 54±1 | 53±1 | 54±1 | 52±2 | 54±1 | 53±1 | 56±1 | **0.005** | 0.27 | 0.90 |
| Day 90 | 55±2 | 58±1 | 56±1 | 58±1 | 56±2 | 56±1 | 58±1 | 58±2 | 0.16 | 0.28 | 0.73 |
| Day 120 | 57±1$^{b}$ | 60±1$^{a,b}$ | 60±1$^{a,b}$ | 61±1$^{a,b}$ | 59±2$^{a,b}$ | 61±1$^{a,b}$ | 60±1$^{a,b}$ | 62±1$^{b}$ | **0.005** | 0.23 | 0.72 |
| **HT (cm)** | | | | | | | | | | | |
| Day 30 | 24±1$^{b}$ | 24±0$^{a,b}$ | 24±0$^{a,b}$ | 25±0$^{a,b}$ | 24±1$^{a,b}$ | 25±0$^{a,b}$ | 25±0$^{a,b}$ | 26±0$^{a}$ | **0.003** | **0.01** | 0.90 |
| Day 60 | 26±1 | 27±0 | 26±1 | 27±0 | 26±1 | 27±1 | 27±0 | 28±1 | **0.0008** | 0.26 | 0.96 |
| Day 90 | 27±1 | 29±1 | 28±1 | 29±1 | 28±1 | 29±1 | 27±0 | 29±1 | **0.004** | 0.81 | 0.94 |
| Day 120 | 28±1 | 29±0 | 59±1 | 29±0 | 28±1 | 30±1 | 28±0 | 30±1 | **0.001** | 0.84 | 0.87 |
| **BPD (cm)** | | | | | | | | | | | |
| Day 30 | 7.0±0.1$^{b,c}$ | 7.1±0.1$^{b,c}$ | 7.2±0.1$^{b,c}$ | 7.2±0.1$^{a,b,c}$ | 7.0±0.1$^{c}$ | 7.2±0.1$^{b,c}$ | 7.4±0.1$^{a,b}$ | 7.6±0.1$^{a}$ | **0.02** | **<0.0001** | 0.57 |
| Day 60 | 7.6±0.1$^{b}$ | 7.7±0.1$^{b}$ | 7.7±0.1$^{b}$ | 7.7±0.1$^{b}$ | 7.5±0.2$^{b}$ | 8.0±0.1$^{b}$ | 7.7±0.1$^{b}$ | 8.3±0.1$^{a}$ | **0.0008** | **0.001** | **0.03** |
| Day 90 | 7.8±0.1$^{b}$ | 8.2±0.1$^{a,b}$ | 8.0±0.1$^{a,b}$ | 8.0±0.1$^{a,b}$ | 8.0±0.2$^{b}$ | 8.2±0.1$^{a,b}$ | 8.2±0.1$^{a,b}$ | 8.6±0.1$^{a}$ | **0.008** | **0.02** | 0.39 |
| Day 120 | 8.2±0.1$^{b}$ | 8.6±0.1$^{a,b}$ | 8.3±0.1$^{a,b}$ | 8.3±0.1$^{a,b}$ | 8.1±0.1$^{b}$ | 9.0±0.1$^{a}$ | 8.3±0.1$^{a,b}$ | 8.7±0.2$^{a}$ | **<0.0001** | 0.28 | 0.05 |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. F: female; M: male, HL: hind limb length, HT: hock to toe length, BPD: biparietal diameter;

**Table 3-10. Growth velocity of weight, crown-rump length, abdominal girth, and chest girth for the treatment groups and controls from day 30 until day 120.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=9) | (F=7) | (M=6) | Sex | Treatment | Interaction |
| **GV Weight ($g.Kg^{-1}.day^{-1}$)** | | | | | | | | | | | |
| From 15-30 | 16±1 | 16±1 | 16±0 | 15±0 | 16±1 | 15±1 | 14±1 | 14±1 | 0.12 | **0.02** | 0.48 |
| From 30-60 | 12±0 | 12±0 | 11±0 | 11±0 | 12±1 | 11±0 | 10±0 | 10±0 | 0.31 | **0.01** | 0.61 |
| From 60-90 | 9±0 | 9±0 | 9±0 | 9±0 | 9±0 | 9±0 | 8±0 | 8±0 | 0.74 | **0.02** | 0.56 |
| From 90-120 | 7±0 | 8±0 | 8±0 | 7±0 | 8±0 | 8±0 | 7±0 | 7±0 | 0.83 | **0.03** | 0.28 |
| **GV CRL ($mm.cm^{-1}.day^{-1}$)** | | | | | | | | | | | |
| From 15-30 | 6±0 | 6±0 | 6±0 | 5±0 | 6±0 | 6±0 | 5±0 | 5±1 | 0.59 | 0.08 | 0.25 |
| From 30-60 | 4±0 | 4±0 | 4±0 | 3±0 | 4±0 | 3±1 | 4±0 | 3±0 | **0.04** | 0.16 | 0.58 |
| From 60-90 | 3±0 | 3±0 | 3±0 | 3±0 | 3±0 | 2±0 | 3±0 | 3±0 | 0.10 | 0.22 | 0.48 |
| From 90-120 | $3±0^{a,b}$ | $3±0^{a}$ | $3±0^{a}$ | $2±0^{a,b}$ | $3±0^{a,b}$ | $2±0^{b}$ | $2±0^{a,b}$ | $2±0^{a,b}$ | 0.06 | 0.11 | 0.11 |
| **GV ABDOM ($mm.cm^{-1}.day^{-1}$)** | | | | | | | | | | | |
| From 15-30 | 6±1 | 7±0 | 6±0 | 7±1 | 7±0 | 8±1 | 6±0 | 6±0 | 0.31 | 0.04 | 0.83 |
| From 30-60 | 4±0 | 5±0 | 5±0 | 5±0 | 5±0 | 5±0 | 5±0 | 5±0 | 0.42 | 0.54 | 0.46 |
| From 60-90 | 4±0 | 4±0 | 4±0 | 4±0 | 4±0 | 4±0 | 4±0 | 4±0 | 0.47 | 0.44 | 0.57 |
| From 90-120 | 3±0 | 3±0 | 3±0 | 3±0 | 3±0 | 3±0 | 3±0 | 3±0 | 0.80 | 0.36 | 0.17 |
| **GV CHEST ($mm.cm^{-1}.day^{-1}$)** | | | | | | | | | | | |
| From 15-30 | 4±0 | 4±0 | 4±0 | 4±0 | 5±0 | 5±1 | 5±0 | 4±0 | 0.44 | 0.26 | 0.77 |
| From 30-60 | 4±0 | 3±0 | 3±0 | 3±0 | 4±0 | 4±0 | 4±0 | 4±0 | 0.06 | 0.06 | 0.63 |
| From 60-90 | 3±0 | 3±0 | 3±0 | 3±0 | 3±0 | 3±0 | 3±0 | 3±0 | 0.14 | 0.50 | 0.71 |
| From 90-120 | 2±0 | 3±0 | 2±0 | 2±0 | 3±0 | 2±0 | 3±0 | 3±0 | 0.79 | 0.83 | 0.23 |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. GV: growth velocity; GV weight; CRL: crown-rump length; ABDOM: abdominal girth; CHEST: chest girth; F: female; M: male.

**Table 3-11. Growth velocity of hind limb length, hock-to-toe length, and biparietal diameter for the treatment groups and controls from day 30 until day 120.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=9) | (F=7) | (M=6) | Sex | Treatment | Interaction |
| **GV HL ($mm.cm^{-1}.day^{-1}$)** | | | | | | | | | | | |
| From 15-30 | 3±0 | 4±0 | 3±0 | 3±0 | 4±0 | 3±0 | 3±0 | 3±0 | 0.99 | 0.32 | 0.66 |
| From 30-60 | 3±0 | 3±0 | 2±0 | 3±0 | 3±0 | 3±0 | 2±0 | 2±0 | 0.29 | **0.03** | 0.88 |
| From 60-90 | 2±0 | 2±0 | 2±0 | 2±0 | 2±0 | 2±0 | 2±0 | 2±0 | 0.91 | 0.22 | 0.23 |
| From 90-120 | 2±0 | 2±0 | 2±0 | 2±0 | 2±0 | 2±0 | 2±0 | 2±0 | 0.19 | 0.06 | 0.50 |
| **GV HT ($mm.cm^{-1}.day^{-1}$)** | | | | | | | | | | | |
| From 15-30 | 3±0 | 3±0 | 3±0 | 3±0 | 3±0 | 3±0 | 3±0 | 2±0 | 0.50 | 0.05 | 0.84 |
| From 30-60 | 2±0 | 2±0 | 2±0 | 2±0 | 2±0 | 2±0 | 2±0 | 2±0 | 0.78 | 0.27 | 0.54 |
| From 60-90 | 2±0 | 2±0 | 2±0 | 2±0 | 2±0 | 1±0 | 1±0 | 1±0 | 0.66 | 0.15 | 0.24 |
| From 90-120 | 1±0 | 1±0 | 1±0 | 1±0 | 1±0 | 1±0 | 1±0 | 1±0 | 0.52 | 0.13 | 0.16 |
| **GV BPD ($mm.cm^{-1}.day^{-1}$)** | | | | | | | | | | | |
| From 15-30 | 2.1±0.2 | 2.3±0.2 | 2.5±0.1 | 1.9±0.1 | 2.5±0.5 | 2.0±0.1 | 2.3±0.2 | 1.9±0.2 | 0.05 | 0.92 | 0.20 |
| From 30-60 | 1.5±0.1 | 1.7±0.1 | 1.8±0.1 | 1.4±0.1 | 1.8±0.3 | 1.5±0.0 | 1.5±0.1 | 1.6±0.1 | 0.19 | 0.722 | 0.05 |
| From 60-90 | 1.2±0.1 | 1.4±0.1 | 1.4±0.1 | 1.2±0.0 | 1.4±0.2 | 1.2±0.1 | 1.3±0.1 | 1.2±0.0 | 0.37 | 0.83 | 0.07 |
| From 90-120 | 1.1±0.0 | 1.2±0.1 | 1.2±0.0 | 1.0±0.0 | 1.2±0.1 | 1.1±0.1 | 1.0±0.1 | 0.9±0.1 | 0.81 | **0.03** | **0.04** |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. GV: growth velocity; HL: hind limb length; HT: hock to toe length; BPD: biparietal diameter; F: female; M: male.

### 3.2.3.4. Day 150-360 (young adulthood): Measurements and Growth Velocity

As the lambs matured, there were no differences in morphometry among treatment groups, but the difference between sexes (M > F) in all growth parameters increased over time (Table 3-12-Table 3-15).

Growth velocity for the weight from 120-150 days was different among groups, being greater in the BCAA group than in the Term group ($P = 0.02$). From day 180 to 270, the BCAA and Water groups had greater growth velocity than the Term group ($P < 0.05$). From day 300 to 360, all the Preterm groups had higher growth velocity than the Term group ($P < 0.05$). There was a sex difference in the growth velocity for the weight from 300-330 and 330-360 days, being greater in males than females ($P < 0.001$) (Table 3-16).

The CRL growth velocity was not different among the groups; however, there was a sex difference from 120-150 days (females > males, $P = 0.04$) (Table 3-16).

HL growth velocity differed among groups, especially between BCAA and Term groups from 120-150 and 180-210 days (BCAA > Term $P = 0.03$). From 150-180 days, BCAA and Water groups had greater HL growth velocity than the Term group ($P < 0.05$), while from 210-240 days, BCAA and Maltodextrin were greater than Term ($P < 0.05$). All the Preterm groups had greater HL growth velocity from day 270 to 360 than the Term group ($P = 0.01$). There was a sex difference in the HL growth velocity from 210-240, 240-270, 300-330, and 330-360 days (males > females $P < 0.05$) (Table 3-18).

The BPD growth velocity was different among groups, being greater in BCAA than Term from 270-300 days ($P = 0.03$) and greater than Term in all the Preterm groups from 330-360 days ($P = 0.01$). From day 270-360, males had greater BPD growth velocity than females ($P < 0.01$) (Table 3-19).

The growth velocity of ABDO, CHEST, and HT was similar among groups and sexes (Table 3-17 and Table 3-18).

**Table 3-12. Morphometric measurements including weight and crown-rump length for the treatment groups and controls from day 150 until day 360.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=6) | (F=7) | (M=5) | Sex | Treatment | Interaction |
| **Weight (Kg)** | | | | | | | | | | | |
| Day 150 | 39±2 | 46±2 | 45±3 | 41±3 | 42±4 | 46±4 | 45±2 | 50±3 | 0.13 | 0.47 | 0.30 |
| Day 180 | 41±2 | 50±3 | 48±3 | 45±2 | 46±4 | 53±5 | 47±2 | 55±3 | **0.01** | 0.26 | 0.14 |
| Day 210 | 44±4 | 53±3 | 51±3 | 49±3 | 47±5 | 58±5 | 50±1 | 57±4 | **0.02** | 0.53 | 0.21 |
| Day 240 | 47±4 | 57±3 | 53±3 | 52±3 | 49±4 | 61±5 | 53±2 | 58±4 | **0.01** | 0.61 | 0.25 |
| Day 270 | 51±4 | 58±3 | 55±4 | 53±3 | 49±4 | 62±4 | 53±2 | 62±4 | **0.01** | 0.79 | 0.20 |
| Day 300 | 51±4 | 63±3 | 57±4 | 58±2 | 51±4 | 65±3 | 55±2 | 62±3 | **0.001** | 0.96 | 0.24 |
| Day 330 | $55±4^{b}$ | $71±1^{a}$ | $59±4^{a,b}$ | $66±3^{a,b}$ | $55±4^{b}$ | $71±2^{a,b}$ | $56±2^{b}$ | $67±4^{a}$ | **0.001** | 0.95 | 0.40 |
| Day 360 | $61±2^{b}$ | $77±3^{a}$ | $51±2^{b}$ | $75±4^{a}$ | $59±4^{b}$ | $80±3^{a,b}$ | $59±2^{b}$ | $72±4^{a}$ | **0.001** | 0.69 | 0.65 |
| **CRL (cm)** | | | | | | | | | | | |
| Day 150 | 96±2 | 100±2 | 100±3 | 98±2 | 97±3 | 101±3 | 101±2 | 99±2 | 0.49 | 0.91 | 0.46 |
| Day 180 | 100±2 | 105±2 | 103±2 | 101±2 | 101±2 | 104±2 | 102±1 | 104±3 | 0.29 | 0.92 | 0.42 |
| Day 210 | 103±2 | 106±2 | 104±2 | 104±2 | 102±3 | 108±3 | 105±1 | 107±3 | 0.05 | 0.83 | 0.67 |
| Day 240 | 105±3 | 108±2 | 109±2 | 108±2 | 105±3 | 110±2 | 106±1 | 110±2 | 0.09 | 0.90 | 0.65 |
| Day 270 | 106±3 | 112±2 | 108±3 | 109±2 | 106±3 | 113±2 | 106±2 | 111±2 | **0.01** | 0.98 | 0.54 |
| Day 300 | 107±3 | 114±2 | 110±2 | 111±2 | 107±3 | 115±3 | 106±1 | 112±2 | **0.001** | 0.84 | 0.29 |
| Day 330 | $110±2^{a,b}$ | $116±1^{a,b}$ | $110±2^{a,b}$ | $114±2^{a,b}$ | $110±3^{a,b}$ | $118±1^{a}$ | $108±1^{b}$ | $115±2^{a,b}$ | **<0.0001** | 0.76 | 0.73 |
| Day 360 | $111±2^{b}$ | $116±1^{a,b}$ | $113±2^{b}$ | $117±2^{a,b}$ | $110±3^{b}$ | $121±1^{a}$ | $109±1^{b}$ | $117±2^{a,b}$ | **<0.001** | 0.65 | 0.22 |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. F: female; M: male; CRL: crown-rump length

**Table 3-13. Morphometric measurements include abdominal and chest girth for the treatment groups and controls from day 150 until day 360.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=6) | (F=7) | (M=5) | Sex | Treatment | Interaction |
| **ABDOM (cm)** | | | | | | | | | | | |
| Day 150 | 90±2 | 97±3 | 98±2 | 93±4 | 97±4 | 94±2 | 97±2 | 98±5 | 0.94 | 0.61 | 0.13 |
| Day 180 | 93±4 | 99±1 | 99±3 | 94±3 | 99±4 | 99±2 | 100±1 | 105±5 | 0.52 | 0.12 | 0.21 |
| Day 210 | 93±2$^{b}$ | 99±2$^{a,b}$ | 98±2$^{a,b}$ | 97±2$^{a,b}$ | 99±3$^{a,b}$ | 100±2$^{a,b}$ | 102±2$^{a}$ | 102±4$^{a,b}$ | 0.32 | **0.03** | 0.39 |
| Day 240 | 96±4 | 101±4 | 101±2 | 101±2 | 104±3 | 100±2 | 106±2 | 104±3 | 0.85 | 0.11 | 0.55 |
| Day 270 | 97±4 | 101±5 | 101±3 | 98±4 | 98±5 | 99±2 | 104±3 | 109±3 | 0.49 | 0.18 | 0.75 |
| Day 300 | 93±4 | 104±3 | 103±3 | 101±3 | 99±5 | 101±2 | 100±3 | 102±3 | 0.13 | 0.80 | 0.30 |
| Day 330 | 98±3 | 107±2 | 100±3 | 104±2 | 103±3 | 102±3 | 98±3 | 105±3 | **0.01** | 0.98 | 0.24 |
| Day 360 | 102±2$^{a,b}$ | 106±2$^{a,b}$ | 103±2$^{a,b}$ | 109±2$^{a}$ | 103±3$^{a,b}$ | 104±2$^{a,b}$ | 99±1$^{b}$ | 106±2$^{a,b}$ | **0.002** | 0.54 | 0.36 |
| **CHEST (cm)** | | | | | | | | | | | |
| Day 150 | 80±2 | 89±3 | 85±2 | 82±3 | 85±3 | 84±3 | 86±2 | 88±4 | 0.47 | 0.63 | 0.10 |
| Day 180 | 84±2 | 90±2 | 88±2 | 85±2 | 87±2 | 89±3 | 88±1 | 92±3 | 0.19 | 0.52 | 0.25 |
| Day 210 | 86±2 | 92±1 | 88±2 | 90±2 | 89±2 | 93±2 | 91±1 | 91±2 | **0.03** | 0.41 | 0.54 |
| Day 240 | 90±4 | 94±3 | 92±2 | 93±2 | 92±2 | 94±3 | 95±1 | 93±2 | 0.40 | 0.85 | 0.76 |
| Day 270 | 92±4 | 93±3 | 93±3 | 92±3 | 91±3 | 95±3 | 96±2 | 98±2 | 0.36 | 0.36 | 0.81 |
| Day 300 | 89±3 | 98±2 | 94±3 | 94±3 | 92±3 | 97±3 | 94±2 | 97±2 | 0.05 | 0.89 | 0.43 |
| Day 330 | 92±3 | 102±2 | 95±4 | 99±2 | 95±3 | 97±1 | 94±2 | 99±2 | **0.006** | 0.93 | 0.45 |
| Day 360 | 98±2$^{a,b}$ | 103±1$^{a}$ | 97±2$^{a,b}$ | 102±2$^{a,b}$ | 98±3$^{a,b}$ | 101±2$^{a,b}$ | 95±1$^{b}$ | 101±2$^{a,b}$ | **0.0003** | 0.54 | 0.79 |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. F: female; M: male; ABDO: abdominal girth; CHEST: chest girth

**Table 3-14. Morphometric measurements including hind limb length and hock-to-toe length for the treatment groups and controls from day 150 until day 360.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=6) | (F=7) | (M=5) | Sex | Treatment | Interaction |
| **HL (cm)** | | | | | | | | | | | |
| Day 150 | 59±1 | 62±1 | 61±1 | 63±1 | 60±2 | 63±1 | 61±1 | 64±1 | **0.002** | 0.38 | 0.85 |
| Day 180 | 61±1 | 64±1 | 63±1 | 64±1 | 61±1 | 64±1 | 61±1 | 65±1 | **0.001** | 0.82 | 0.65 |
| Day 210 | 62±1 | 66±1 | 64±1 | 66±1 | 62±2 | 66±2 | 63±1 | 66±1 | **0.0008** | 0.75 | 0.68 |
| Day 240 | 63±2 | 67±1 | 65±1 | 67±1 | 63±1 | 67±2 | 64±1 | 67±1 | **0.0003** | 0.83 | 0.82 |
| Day 270 | 64±1 | 67±1 | 66±1 | 68±1 | 64±1 | 68±1 | 65±1 | 68±1 | **<0.0001** | 0.72 | 0.63 |
| Day 300 | 65±2 | 68±1 | 66±1 | 69±1 | 65±1 | 69±1 | 65±1 | 69±1 | **0.0001** | 0.81 | 0.93 |
| Day 330 | $66\pm1^{a,b}$ | $70\pm1^{a,b}$ | $67\pm1^{a,b}$ | $70\pm1^{a}$ | $66\pm1^{b}$ | $70\pm1^{a,b}$ | $66\pm1^{a,b}$ | $70\pm1^{a,b}$ | **<0.0001** | 0.95 | 0.98 |
| Day 360 | $67\pm1^{c}$ | $71\pm2^{a,b}$ | $68\pm1^{b,c}$ | $71\pm1^{a}$ | $67\pm1^{c}$ | $71\pm1^{a,b}$ | $67\pm1^{c}$ | $71\pm1^{a,b,c}$ | **<0.0001** | 0.87 | 0.94 |
| **HT (cm)** | | | | | | | | | | | |
| Day 150 | 29±1 | 30±1 | 29±1 | 31±0 | 29±1 | 30±1 | 29±0 | 31±1 | **0.002** | 0.55 | 0.86 |
| Day 180 | 29±1 | 31±0 | 30±1 | 31±0 | 29±1 | 31±1 | 29±0 | 31±1 | **0.001** | 0.71 | 0.81 |
| Day 210 | $30\pm1^{a,b}$ | $31\pm0^{a,b}$ | $30\pm1^{a,b}$ | $31\pm0^{a}$ | $29\pm1^{b}$ | $31\pm1^{a,b}$ | $30\pm0^{a,b}$ | $31\pm1^{a,b}$ | **0.0004** | 0.67 | 0.51 |
| Day 240 | $31\pm1^{a,b}$ | $31\pm1^{a,b}$ | $30\pm1^{a,b}$ | $32\pm0^{a,b}$ | $30\pm1^{b}$ | $32\pm0^{a,b}$ | $31\pm0^{a,b}$ | $32\pm1^{a}$ | **<0.0001** | 0.55 | 0.42 |
| Day 270 | $31\pm1^{a,b,c}$ | $32\pm0^{a,b,c}$ | $31\pm1^{b,c}$ | $32\pm0^{a,b}$ | $30\pm0^{c}$ | $32\pm0^{a,b}$ | $31\pm0^{a,b,c}$ | $33\pm1^{a}$ | **<0.0001** | 0.44 | 0.77 |
| Day 300 | $31\pm1^{b}$ | $33\pm0^{a}$ | $31\pm0^{b}$ | $33\pm0^{a}$ | $31\pm0^{b}$ | $33\pm0^{a}$ | $32\pm0^{a,b}$ | $33\pm0^{a}$ | **<0.0001** | 0.07 | 0.91 |
| Day 330 | $31\pm1^{b,c}$ | $33\pm0^{a,b}$ | $31\pm0^{c}$ | $33\pm0^{a}$ | $31\pm1^{c}$ | $33\pm0^{a,b}$ | $32\pm0^{a,b,c}$ | $34\pm0^{a}$ | **<0.0001** | 0.16 | 0.93 |
| Day 360 | $31\pm1^{c}$ | $33\pm0^{a,b}$ | $32\pm0^{b,c}$ | $34\pm0^{a}$ | $31\pm0^{c}$ | $33\pm0^{a,b}$ | $32\pm0^{a,b,c}$ | $34\pm0^{a}$ | **<0.0001** | 0.13 | 0.96 |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. F: female; M: male; HL: hind limb length, HT: hock to toe length

**Table 3-15. Morphometric measurements including biparietal diameter for the treatment groups and controls from day 150 until day 360.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=6) | (F=7) | (M=5) | Sex | Treatment | Interaction |
| **BPD (cm)** | | | | | | | | | | | |
| Day 150 | 8.3±0.1$^{c}$ | 8.9±0.1$^{a,b}$ | 8.4±0.1$^{b,c}$ | 8.6±0.1$^{a,b,c}$ | 8.2±0.1$^{c}$ | 9.0±0.2$^{a,b}$ | 8.4±0.1$^{b,c}$ | 9.0±0.2$^{a}$ | **<0.0001** | 0.37 | 0.16 |
| Day 180 | 8.5±0.1$^{b}$ | 9.0±0.1$^{a}$ | 8.6±0.1$^{a,b}$ | 8.8±0.2$^{a,b}$ | 8.4±0.1$^{b}$ | 9.1±0.2$^{a}$ | 8.6±0.1$^{a,b}$ | 9.1±0.2$^{a}$ | **<0.0001** | 0.70 | 0.21 |
| Day 210 | 8.6±0.1$^{b,c}$ | 9.1±0.2$^{a,b}$ | 8.7±0.1$^{b,c}$ | 8.9±0.2$^{a,b}$ | 8.4±0.2$^{c}$ | 9.4±0.1$^{a}$ | 8.6±0.1$^{b,c}$ | 9.4±0.1$^{a}$ | **<0.0001** | 0.23 | **0.03** |
| Day 240 | 8.7±0.2$^{b,c}$ | 9.3±0.2$^{a,b}$ | 8.8±0.1$^{b,c}$ | 9.1±0.2$^{a,b,c}$ | 8.6±0.2$^{c}$ | 9.5±0.1$^{a}$ | 8.8±0.1$^{b,c}$ | 9.4±0.1$^{a,b}$ | **<0.0001** | 0.75 | 0.24 |
| Day 270 | 8.8±0.1$^{c,d}$ | 9.5±0.2$^{a,b}$ | 8.9±0.1$^{b,c,d}$ | 9.3±0.2$^{a,b,c}$ | 8.6±0.2$^{d}$ | 9.7±0.1$^{a}$ | 8.9±0.1$^{c,d}$ | 9.6±0.1$^{a}$ | **<0.0001** | 0.82 | **0.04** |
| Day 300 | 8.9±0.1$^{b}$ | 9.7±0.2$^{a}$ | 8.9±0.1$^{b}$ | 9.6±0.2$^{a}$ | 8.8±0.1$^{b}$ | 9.7±0.1$^{a}$ | 9.0±0.1$^{b}$ | 9.7±0.1$^{a}$ | **<0.0001** | 0.72 | 0.36 |
| Day 330 | 9.0±0.1$^{b}$ | 9.8±0.2$^{a}$ | 9.0±0.1$^{b}$ | 9.7±0.2$^{a}$ | 8.7±0.1$^{b}$ | 9.8±0.1$^{a}$ | 9.0±0.1$^{b}$ | 10.0±0.1$^{a}$ | **<0.0001** | 0.39 | 0.41 |
| Day 360 | 9.0±0.1$^{b}$ | 10.0±0.2$^{a}$ | 9.2±0.1$^{b}$ | 10.0±0.2$^{a}$ | 9.0±0.2$^{b}$ | 10.1±0.1$^{a}$ | 9.0±0.1$^{b}$ | 10.0±0.1$^{a}$ | **<0.0001** | 0.87 | 0.39 |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. F: female; M: male; BPD: biparietal diameter

**Table 3-16. Growth velocity of weight and crown-rump length for the treatment groups and controls from day 150 until day 360.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=6) | (F=7) | (M=5) | Sex | Treatment | Interaction |
| **GV Weight (g.Kg$^{-1}$.day$^{-1}$)** | | | | | | | | | | | |
| From 120-150 | 6±0 | 7±0 | 7±0 | 6±0 | 7±0 | 6±0 | 6±0 | 6±0 | 0.57 | **0.02** | 0.27 |
| From 150-180 | 5±0$^{a,b}$ | 6±0$^{a}$ | 6±0$^{a,b}$ | 5±0$^{a,b}$ | 6±0$^{a,b}$ | 6±0$^{a,b}$ | 5±0$^{b}$ | 5±0$^{a,b}$ | 0.86 | **0.01** | 0.15 |
| From 180-210 | 5±0 | 5±0 | 5±0 | 5±0 | 5±0 | 5±0 | 4±0 | 5±0 | 0.72 | **0.01** | 0.28 |
| From 210-240 | 4±0 | 5±0 | 4±0 | 4±0 | 4±0 | 4±0 | 4±0 | 4±0 | 0.63 | **0.01** | 0.43 |
| From 240-270 | 4±0$^{a,b}$ | 4±0$^{a}$ | 4±0$^{a,b}$ | 4±0$^{a,b}$ | 4±0$^{a,b}$ | 4±0$^{a,b}$ | 3±0$^{b}$ | 4±0$^{a,b}$ | 0.67 | **0.006** | 0.45 |
| From 270-300 | 4±0$^{a,b}$ | 4±0$^{a}$ | 4±0$^{a,b}$ | 3±0$^{a,b}$ | 4±0$^{a,b}$ | 4±0$^{a}$ | 3±0$^{b}$ | 3±0$^{a,b}$ | 0.27 | **0.001** | 0.49 |
| From 300-330 | 3±0$^{a,b}$ | 4±0$^{a}$ | 3±0$^{a,b}$ | 3±0$^{a,b}$ | 3±0$^{a,b}$ | 3±0$^{a,b}$ | 3±0$^{c}$ | 3±0$^{b,c}$ | **0.009** | **<0.0001** | 0.59 |
| From 330-360 | 3±0$^{a,b,c}$ | 4±0$^{a}$ | 3±0$^{b,c}$ | 4±0$^{a,b}$ | 3±0$^{b,c}$ | 4±0$^{a,b}$ | 3±0$^{d}$ | 3±0$^{c,d}$ | **0.0001** | **<0.0001** | 0.97 |
| **GV CRL (mm.cm$^{-1}$.day$^{-1}$)** | | | | | | | | | | | |
| From 120-150 | 2±0$^{a,b}$ | 2±0$^{a}$ | 2±0$^{a}$ | 2±0$^{a,b}$ | 2±0$^{a}$ | 1±0$^{b}$ | 2±0$^{a,b}$ | 2±0$^{a,b}$ | **0.04** | 0.15 | 0.09 |
| From 150-180 | 2±0$^{a,b}$ | 2±0$^{a}$ | 2±0$^{a}$ | 2±0$^{a,b}$ | 2±0$^{a}$ | 1±0$^{b}$ | 2±0$^{a,b}$ | 2±0$^{a,b}$ | 0.05 | 0.13 | 0.06 |
| From 180-210 | 2±0$^{a,b}$ | 2±0$^{a}$ | 2±0$^{a}$ | 2±0$^{a,b}$ | 2±0$^{a,b}$ | 1±0$^{b}$ | 2±0$^{a,b}$ | 1±0$^{a,b}$ | 0.09 | 0.13 | 0.09 |
| From 210-240 | 1±0$^{a,b}$ | 2±0$^{a}$ | 2±0$^{a}$ | 1±0$^{a,b}$ | 1±0$^{a}$ | 1±0$^{b}$ | 1±0$^{a,b}$ | 1±0$^{a,b}$ | 0.08 | 0.13 | 0.08 |
| From 240-270 | 1±0$^{a,b}$ | 1±0$^{a}$ | 1±0$^{a,b}$ | 1±0$^{a,b}$ | 1±0$^{a,b}$ | 1±0$^{b}$ | 1±0$^{a,b}$ | 1±0$^{a,b}$ | 0.17 | 0.13 | 0.09 |
| From 270-300 | 1±0$^{a,b}$ | 1±0$^{a}$ | 1±0$^{a,b}$ | 1±0$^{a,b}$ | 1±0$^{a,b}$ | 1±0$^{b}$ | 1±0$^{a,b}$ | 1±0$^{a,b}$ | 0.22 | 0.10 | 0.09 |
| From 300-330 | 1±0$^{a,b}$ | 1±0$^{a}$ | 1±0$^{a,b}$ | 1±0$^{a,b}$ | 1±0$^{a,b}$ | 1±0$^{b}$ | 1±0$^{a,b}$ | 1±0$^{a,b}$ | 0.26 | 0.12 | 0.07 |
| From 330-360 | 1±0 | 1±0 | 1±0 | 1±0 | 1±0 | 1±0 | 1±0 | 1±0 | 0.33 | 0.10 | 0.13 |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. GV: growth velocity; GV weight; CRL: crown-rump length; F: female; M: male.

**Table 3-17. Growth velocity of abdominal and chest girth for the treatment groups and controls from day 150 until day 360.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=6) | (F=7) | (M=5) | Sex | Treatment | Interaction |
| **GV ABDOM (mm.cm$^{-1}$.day$^{-1}$)** | | | | | | | | | | | |
| From 120-150 | 2.6±0.1 | 2.8±0.1 | 2.8±0.1 | 2.8±0.1 | 3.0±0.1 | 2.7±0.1 | 2.5±0.1 | 2.6±0.1 | 0.90 | 0.07 | 0.12 |
| From 150-180 | 2.3±0.2 | 2.4±0.1 | 2.3±0.1 | 2.3±0.1 | 2.5±0.1 | 2.4±0.1 | 2.2±0.0 | 2.3±0.1 | 0.72 | 0.34 | 0.54 |
| From 180-210 | 1.9±0.1 | 2.1±0.1 | 2.0±0.0 | 2.1±0.1 | 2.2±0.1 | 2.1±0.1 | 1.9±0.0 | 1.9±0.0 | 0.61 | 0.12 | 0.36 |
| From 210-240 | 1.7±0.1 | 1.8±0.1 | 1.8±0.0 | 1.9±0.0 | 2.0±0.1 | 1.8±0.1 | 1.7±0.0 | 1.7±0.0 | 0.97 | 0.36 | 0.43 |
| From 240-270 | 1.6±0.1 | 1.6±0.1 | 1.6±0.1 | 1.6±0.1 | 1.6±0.1 | 1.6±0.1 | 1.5±0.1 | 1.6±0.1 | 0.65 | 0.92 | 0.85 |
| From 270-300 | 1.4±0.1 | 1.5±0.1 | 1.5±0.1 | 1.5±0.1 | 1.5±0.1 | 1.5±0.1 | 1.3±0.1 | 1.3±0.0 | 0.29 | 0.16 | 0.56 |
| From 300-330 | 1.3±0.1 | 1.4±0.1 | 1.3±0.0 | 1.4±0.1 | 1.4±0.1 | 1.3±0.1 | 1.2±0.0 | 1.3±0.0 | 0.15 | **0.04** | 0.24 |
| From 330-360 | 1.4±0.1 $^{a,b}$ | 1.4±0.1 $^{a,b}$ | 1.3±0.0 $^{a,b}$ | 1.5±0.1 $^{a}$ | 1.4±0.1 $^{a,b}$ | 1.4±0.1 $^{a,b}$ | 1.2±0.0 $^{b}$ | 1.3±0.0 $^{a,b}$ | 0.10 | **0.01** | 0.26 |
| **GV CHEST (mm.cm$^{-1}$.day$^{-1}$).** | | | | | | | | | | | |
| From 120-150 | 2.1±0.1 | 2.3±0.1 | 2.2±0.1 | 2.0±0.1 | 2.3±0.1 | 2.1±0.1 | 2.2±0.0 | 2.1±0.1 | 0.33 | 0.65 | 0.11 |
| From 150-180 | 1.9±0.1 | 2.0±0.1 | 1.9±0.1 | 1.8±0.1 | 2.0±0.1 | 1.9±0.1 | 1.9±0.0 | 1.9±0.1 | 0.50 | 0.51 | 0.45 |
| From 180-210 | 1.6±0.1 | 1.7±0.1 | 1.6±0.0 | 1.7±0.1 | 1.8±0.0 | 1.7±0.0 | 1.7±0.0 | 1.6±0.0 | 0.71 | 0.12 | 0.32 |
| From 210-240 | 1.5±0.1 | 1.5±0.1 | 1.5±0.0 | 1.5±0.0 | 1.6±0.0 | 1.5±0.1 | 1.6±0.0 | 1.4±0.0 | 0.38 | 0.622 | 0.63 |
| From 240-270 | 1.4±0.1 | 1.3±0.1 | 1.4±0.0 | 1.3±0.1 | 1.4±0.1 | 1.4±0.1 | 1.4±0.0 | 1.4±0.0 | 0.56 | 0.83 | 0.99 |
| From 270-300 | 1.2±0.1 | 1.3±0.1 | 1.2±0.0 | 1.2±0.0 | 1.3±0.1 | 1.3±0.0 | 1.2±0.0 | 1.2±0.0 | 0.77 | 0.65 | 0.46 |
| From 300-330 | 1.1±0.1 | 1.2±0.0 | 1.1±0.0 | 1.2±0.0 | 1.2±0.0 | 1.2±0.0 | 1.1±0.0 | 1.1±0.0 | 0.44 | 0.43 | 0.38 |
| From 330-360 | 1.2±0.0 | 1.2±0.0 | 1.2±0.0 | 1.2±0.0 | 1.2±0.0 | 1.2±0.0 | 1.1±0.0 | 1.1±0.0 | 0.33 | **0.02** | 0.66 |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. GV: growth velocity; ABDOM: abdominal girth; CHEST: chest girth; F: female; M: male.

**Table 3-18. Growth velocity of hind limb length and hock-to-toe length for the treatment groups and controls from day 150 until day 360.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=6) | (F=7) | (M=5) | Sex | Treatment | Interaction |
| **GV HL (mm.cm$^{-1}$.day$^{-1}$)** | | | | | | | | | | | |
| From 120-150 | 1.4±0.1 | 1.5±0.0 | 1.4±0.0 | 1.5±0.1 | 1.4±0.1 | 1.5±0.0 | 1.3±0.1 | 1.3±0.0 | 0.17 | **0.02** | 0.76 |
| From 150-180 | 1.2±0.1 $^{a,b}$ | 1.3±0.0 $^{a}$ | 1.2±0.0 $^{a,b}$ | 1.2±0.1 $^{a,b}$ | 1.2±0.1 $^{a,b}$ | 1.3±0.0 $^{a,b}$ | 1.1±0.0 $^{b}$ | 1.1±0.0 $^{a,b}$ | 0.11 | **0.01** | 0.85 |
| From 180-210 | 1.1±0.1 $^{a,b}$ | 1.2±0.0 $^{a}$ | 1.1±0.0 $^{a,b}$ | 1.1±0.0 $^{a,b}$ | 1.1±0.1 $^{a,b}$ | 1.2±0.0 $^{a,b}$ | 1.0±0.0 $^{b}$ | 1.0±0.0 $^{a,b}$ | 0.06 | **0.01** | 0.79 |
| From 210-240 | 1.0±0.1 $^{a,b}$ | 1.1±0.0 $^{a}$ | 1.0±0.0 $^{a,b}$ | 1.0±0.0 $^{a,b}$ | 1.0±0.0 $^{a,b}$ | 1.0±0.0 $^{a,b}$ | 0.9±0.0 $^{b}$ | 0.9±0.0 $^{a,b}$ | **0.04** | **0.01** | 0.86 |
| From 240-270 | 0.9±0.0 $^{a,b}$ | 1.0±0.0 $^{a}$ | 0.9±0.0 $^{a,b}$ | 0.9±0.0 $^{a,b}$ | 0.9±0.0 $^{a,b}$ | 0.9±0.0 $^{a,b}$ | 0.8±0.0 $^{b}$ | 0.8±0.0 $^{a,b}$ | **0.03** | **0.01** | 0.83 |
| From 270-300 | 0.8±0.0 $^{a,b}$ | 0.9±0.0 $^{a}$ | 0.8±0.0 $^{a,b}$ | 0.9±0.0 $^{a,b}$ | 0.8±0.0 $^{a,b}$ | 0.9±0.0 $^{a,b}$ | 0.7±0.0 $^{b}$ | 0.8±0.0 $^{a,b}$ | 0.05 | **0.004** | 0.85 |
| From 300-330 | 0.8±0.0 $^{a,b,c}$ | 0.8±0.0 $^{a}$ | 0.7±0.0 $^{a,b,c}$ | 0.8±0.0 $^{a,b}$ | 0.8±0.0 $^{a,b,c}$ | 0.8±0.0 $^{a,b}$ | 0.7±0.0 $^{c}$ | 0.7±0.0 $^{b,c}$ | **0.009** | **0.0003** | 0.70 |
| From 330-360 | 0.8±0.0 $^{a,b,c}$ | 0.9±0.0 $^{a}$ | 0.8±0.0 $^{a,b,c}$ | 0.8±0.0 $^{a,b}$ | 0.8±0.0 $^{a,b,c}$ | 0.8±0.0 $^{a,b}$ | 0.7±0.0 $^{c}$ | 0.7±0.0 $^{b,c}$ | **0.003** | **0.0001** | 0.74 |
| **GV HT (mm.cm$^{-1}$.day$^{-1}$)** | | | | | | | | | | | |
| From 120-150 | 1.1±0.1 | 1.2±0.1 | 1.2±0.0 | 1.2±0.0 | 1.1±0.1 | 0.8±0.2 | 1.0±0.1 | 1.0±0.0 | 0.37 | 0.12 | 0.31 |
| From 150-180 | 1.0±0.1 | 1.0±0.1 | 1.0±0.0 | 1.0±0.0 | 1.0±0.0 | 0.7±0.2 | 0.9±0.0 | 0.9±0.0 | 0.43 | 0.13 | 0.26 |
| From 180-210 | 0.9±0.1 | 0.9±0.1 | 0.9±0.0 | 0.9±0.0 | 0.8±0.0 | 0.6±0.2 | 0.8±0.0 | 0.8±0.0 | 0.34 | 0.08 | 0.35 |
| From 210-240 | 0.8±0.0 | 0.8±0.0 | 0.8±0.0 | 0.8±0.0 | 0.8±0.0 | 0.6±0.1 | 0.7±0.0 | 0.7±0.0 | 0.41 | 0.12 | 0.25 |
| From 240-270 | 0.7±0.0 | 0.8±0.0 | 0.7±0.0 | 0.7±0.0 | 0.7±0.0 | 0.5±0.1 | 0.7±0.0 | 0.7±0.0 | 0.47 | 0.11 | 0.20 |
| From 270-300 | 0.7±0.0 | 0.7±0.0 | 0.7±0.0 | 0.7±0.0 | 0.7±0.0 | 0.5±0.1 | 0.6±0.0 | 0.6±0.0 | 0.49 | 0.16 | 0.15 |
| From 300-330 | 0.6±0.0 | 0.7±0.0 | 0.6±0.0 | 0.6±0.0 | 0.6±0.0 | 0.4±0.1 | 0.6±0.0 | 0.6±0.0 | 0.48 | 0.12 | 0.14 |
| From 330-360 | 0.6±0.0 | 0.7±0.0 | 0.6±0.0 | 0.7±0.0 | 0.6±0.0 | 0.5±0.1 | 0.6±0.0 | 0.6±0.0 | 0.55 | 0.09 | 0.14 |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. GV: growth velocity; HL: hind limb length; HT: hock to toe length; F: female; M: male.

**Table 3-19. Growth velocity of biparietal diameter for the treatment groups and controls from day 30 until day 120.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=6) | (F=7) | (M=5) | Sex | Treatment | Interaction |
| **GV BPD ($mm.cm^{-1}.day^{-1}$)** | | | | | | | | | | | |
| From 120-150 | 0.9±0.0 $^{a,b}$ | 1.1±0.1 $^{a}$ | 1.0±0.0 $^{a,b}$ | 0.9±0.0 $^{a,b}$ | 1.0±0.1 $^{a,b}$ | 1.0±0.0 $^{a,b}$ | 0.8±0.0 $^{b}$ | 0.9±0.0 $^{a,b}$ | 0.27 | 0.05 | 0.05 |
| From 150-180 | 0.8±0.0 $^{a,b}$ | 0.9±0.1 $^{a}$ | 0.9±0.0 $^{a,b}$ | 0.8±0.0 $^{a,b}$ | 0.9±0.1 $^{a,b}$ | 0.9±0.0 $^{a,b}$ | 0.7±0.0 $^{b}$ | 0.8±0.0 $^{a,b}$ | 0.38 | 0.05 | 0.08 |
| From 180-210 | 0.7±0.0 | 0.8±0.0 | 0.7±0.0 | 0.7±0.0 | 0.7±0.1 | 0.8±0.0 | 0.7±0.0 | 0.7±0.0 | **0.05** | 0.20 | 0.23 |
| From 210-240 | 0.6±0.0 $^{a,b}$ | 0.8±0.0 $^{a}$ | 0.7±0.0 $^{a,b}$ | 0.7±0.0 $^{a,b}$ | 0.7±0.1 $^{a,b}$ | 0.7±0.0 $^{a,b}$ | 0.6±0.0 $^{b}$ | 0.6±0.0 $^{a,b}$ | 0.13 | 0.07 | 0.17 |
| From 240-270 | 0.6±0.0 $^{b}$ | 0.7±0.0 $^{a}$ | 0.6±0.0 $^{a,b}$ | 0.6±0.0 $^{a,b}$ | 0.6±0.1 $^{a,b}$ | 0.7±0.0 $^{a,b}$ | 0.6±0.0 $^{b}$ | 0.6±0.0 $^{a,b}$ | **0.01** | 0.08 | 0.10 |
| From 270-300 | 0.5±0.0 $^{b}$ | 0.7±0.0 $^{a}$ | 0.6±0.0 $^{a,b}$ | 0.6±0.0 $^{a,b}$ | 0.6±0.0 $^{a,b}$ | 0.6±0.0 $^{a,b}$ | 0.5±0 .0$^{b}$ | 0.5±0.0 $^{a,b}$ | **0.003** | **0.03** | 0.28 |
| From 300-330 | 0.5±0.0 $^{b}$ | 0.6±0.0 $^{a}$ | 0.5±0.0 $^{a,b}$ | 0.6±0.0 $^{a,b}$ | 0.5±0.0 $^{a,b}$ | 0.6±0.0 $^{a,b}$ | 0.5±0 .0$^{b}$ | 0.5±0.0 $^{a,b}$ | **0.0005** | **0.05** | 0.38 |
| From 330-360 | 0.5±0.0 $^{b,c}$ | 0.6±0.0 $^{a}$ | 0.5±0.0 $^{a,b,c}$ | 0.6±0.0 $^{a,b}$ | 0.5±0.0 $^{a,b,c}$ | 0.6±0.0 $^{a,b}$ | 0.5±0.0 $^{c}$ | 0.5±0.0 $^{a,b,c}$ | **0.0001** | **0.01** | 0.47 |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. GV: growth velocity; BPD: biparietal diameter; F: female; M: male.

### 3.2.3.5. Post-mortem

Fifty-four sheep (25 male, 29 female, 14 BCAA, 15 Maltodextrin, 13 Water, and 12 Term) underwent post-mortem as described in Chapter 2 ( methods section 2.4.8). In general, there were no differences among treatment groups in the body weight or absolute organ weights (Table 3-20). Males were heavier than females in body weight and carcass weight ($P < 0.001$) and had greater absolute pancreas, liver, kidneys, spleen, lungs, heart, brain, and thyroid weight ($P < 0.01$). The absolute weight of the chest thymus and perirenal fat was greater in females ($P = 0.001$).

When normalised to body weight (Table 3-21), perirenal fat-to-body weight was greatest in the BCAA group ($P = 0.03$). Brain-to-body weight and peri-renal fat: body weight were greater in females than males (F > M, $P = 0.0001$ and $P = 0.03$ respectively). There were no differences among treatment groups in pancreas, liver, kidney (left and right), spleen, lung and heart weight when normalised to body weight.

**Table 3-20. Post-mortem data for body weight and absolute organ weight for adult sheep born Preterm or at Term.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=6) | (F=7) | (M=5) | Sex | Treatment | Interaction |
| **Weight (Kg)** | 71±1$^{b,c,d}$ | 82±4$^{a,b,c}$ | 66±3$^{d}$ | 84±4$^{a,b}$ | 68±3$^{d}$ | 87±3$^{a}$ | 68±2$^{c,d}$ | 84±4$^{a,b}$ | **<0.001** | 0.92 | 0.63 |
| **C.W (Kg)** | 41±1$^{b,c}$ | 50±2$^{a,b}$ | 39±1$^{c}$ | 51±2$^{a}$ | 410±3$^{b,c}$ | 52±2$^{a}$ | 40±1$^{c}$ | 49±2$^{a,b,c}$ | **<0.001** | 0.78 | 0.82 |
| **F. Rump (mm)** | 22±2 | 20±2 | 19±2 | 21±2 | 16±3 | 18±4 | 23±1 | 19±2 | 0.67 | 0.33 | 0.41 |
| **F. Rib (mm)** | 15±2 | 14±1 | 14±2 | 12±2 | 16±3 | 14±3 | 13±2 | 12±2 | 0.33 | 0.72 | 0.98 |
| **Pancreas (g)** | 87±3 | 104±10 | 84±2 | 96±2 | 82±4 | 104±4 | 87±5 | 108±13 | **<0.001** | 0.66 | 0.86 |
| **Liver (Kg)** | 1.3±0.1 | 1.5±0.1 | 1.2±0.1 | 1.5±0.1 | 1.3±0.1 | 1.5±0.1 | 1.2±0.1 | 1.5±0.1 | **<0.001** | 0.75 | 0.79 |
| **Kidney (R) (g)** | 98±3$^{b,c,d}$ | 121±7$^{a}$ | 92±3$^{d}$ | 113±6$^{a,b,c}$ | 94±5$^{c,d}$ | 117±3$^{a,b}$ | 90±4$^{d}$ | 113±5$^{a,b,c,d}$ | **<0.001** | 0.31 | 0.99 |
| **Kidney (L) (g** | 98±2$^{b,c}$ | 124±7$^{a}$ | 92±3$^{c}$ | 117±6$^{a,b}$ | 93±5$^{c}$ | 121±4$^{a}$ | 90±3$^{c}$ | 118±6$^{a,b}$ | **<0.001** | 0.44 | 0.98 |
| **Spleen (g)** | 239±21 | 275±29 | 239±22 | 282±38 | 221±35 | 315±24 | 253±20 | 256±33 | **0.03** | 0.97 | 0.49 |
| **Lungs (g)** | 677±22$^{a,b,c}$ | 766±34$^{a,b}$ | 545±53$^{c}$ | 763±38$^{a,b}$ | 628±28$^{a,b,c}$ | 787±34$^{a}$ | 616±23$^{b,c}$ | 775±47$^{a,b}$ | **<0.001** | 0.26 | 0.35 |
| **Heart (g)** | 294±11$^{b,c}$ | 336±22$^{a,b,c}$ | 285±16$^{b,c}$ | 325±13$^{a,b,c}$ | 267±10$^{c}$ | 350±13.5$^{a,b}$ | 291±18$^{b,c}$ | 375±28$^{a}$ | **<0.001** | 0.38 | 0.39 |
| **Brain (g)** | 78±3 | 91±2 | 81±2 | 88±4 | 82±3 | 87±2 | 81±3 | 80±4 | **<0.01** | 0.45 | 0.16 |
| **Thymus C (g)** | 34±3$^{a,b}$ | 22±3$^{b}$ | 48±4$^{a}$ | 29±4$^{a,b}$ | 40±6$^{a,b}$ | 33±3$^{a,b}$ | 42±5$^{a}$ | 34±6$^{a,b}$ | **<0.001** | 0.06 | 0.52 |
| **Thymus N (g)** | 62±5 | 54±10 | 59±8 | 53±7 | 55±6 | 44±4 | 74±13 | 59±3 | 0.07 | 0.23 | 0.94 |
| **Thyroid (g)** | 10±1 | 12±1 | 9±1 | 11±1 | 9±1 | 11±1 | 9±1 | 12±1 | **<0.01** | 0.75 | 0.89 |
| **Cerebellum (g)** | 12±0 | 13±0 | 12±0 | 13±1 | 12±0 | 12±0 | 12±0 | 12±1 | 0.05 | 0.74 | 0.58 |
| **Brain stem (g)** | 10±1 | 10±1 | 11±0 | 10±1 | 9±1 | 11±1 | 12±2 | 11±2 | 0.86 | 0.31 | 0.64 |
| **TPR-fat (g)** | 893±154 | 560±62 | 651±68 | 454±69 | 734±116 | 370±66 | 661±73 | 285±22 | **<0.001** | 0.05 | 0.73 |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. F: female; M: male; C.W: Carcass weight; F. Rump: Fat depth above rump; F. Rib: Fat depth at 7$^{th}$ rib; Kidney R: right kidney; Kidney L: left kidney; Thymus C: chest thymus; Thymus N: neck thymus; TPR-fat: total perirenal fat.

**Table 3-21. Post-mortem data for organ weight normalised to body weight for adult sheep born Preterm and at Term.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=6) | (F=7) | (M=5) | Sex | Treatment | Interaction |
| **Pancreas (g/Kg)** | 1.2±0.0 | 1.3±0.1 | 1.3±0.1 | 1.2±0.1 | 1.2±0.1 | 1.2±0.0 | 1.3±0.1 | 1.3±0.1 | 0.69 | 0.86 | 0.75 |
| **Liver (g/Kg)** | 19±1 | 18±0 | 18±1 | 18±1 | 19±1 | 17±0 | 18±1 | 17±1 | 0.20 | 0.75 | 0.90 |
| **Kidney (R) (g/Kg)** | 1.4±0.1 | 1.5±0.1 | 1.4±0.0 | 1.3±0.0 | 1.4±0.1 | 1.4±0.0 | 1.3±0.1 | 1.4±0.0 | 0.87 | 0.33 | 0.54 |
| **Kidney (L) (g/Kg)** | 1.4±0.0 | 1.5±0.1 | 1.4±0.0 | 1.4±0.0 | 1.4±0.1 | 1.4±0.0 | 1.3±0.0 | 1.4±0.0 | 0.07 | 0.37 | 0.51 |
| **Spleen (g/Kg)** | 2.9±0.5 | 3.3±0.2 | 3.6±0.2 | 3.4±0.5 | 3.3±0.5 | 3.7±0.4 | 3.7±0.2 | 3.1±0.4 | 0.96 | 0.73 | 0.46 |
| **Lungs (g/Kg)** | 9.6±0.4 | 9.4±0.4 | 8.2±0.8 | 9.2±0.4 | 9.4±0.6 | 9.2±0.6 | 9.1±0.5 | 9.3±0.5 | 0.63 | 0.44 | 0.68 |
| **Heart (g/Kg)** | 4.2±0.1 | 4.1±0.1 | 4.3±0.2 | 3.9±0.1 | 4.0±0.2 | 4.1±0.1 | 4.3±0.2 | 4.5±0.2 | 0.76 | 0.18 | 0.26 |
| **Brain (g/Kg)** | $1.1\pm0.0^{a,b}$ | $1.1\pm0.1^{a,b}$ | $1.2\pm0.1^{a}$ | $1.1\pm0.0^{a,b}$ | $1.2\pm0.1^{a}$ | $1.0\pm0.0^{a,b}$ | $1.2\pm0.0^{a,b}$ | $1.0\pm0.1^{b}$ | **<0.001** | 0.74 | 0.07 |
| **T-Peri fat (g/Kg)** | $12.6\pm2.1^{a}$ | $6.8\pm0.5^{b,c,d}$ | $9.7\pm0.8^{a,b,c}$ | $5.3\pm0.6^{c,d}$ | $10.6\pm1.3^{a,b}$ | $4.2\pm0.6^{d}$ | $9.6\pm0.8^{a,b,c}$ | $3.4\pm0.3^{d}$ | **<0.001** | **0.03** | 0.77 |

Bold font indicates significance on ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. F: female; M: male; Kidney R: right kidney; Kidney L: left kidney; T-Peri fat: total perirenal fat.

### 3.2.4. Results Summary

In the BCAA group, plasma concentrations of BCAA were elevated at 2 and 6 hours after oral administration of BCAA supplements compared with concentrations in Maltodextrin and Term- and Preterm-Water groups. After 15 days of supplementation, Term and BCAA groups had higher plasma isoleucine concentrations than Maltodextrin and Water groups, whereas there was no difference in leucine and valine concentrations among groups. There was, in general, no effect of BCAA supplementation on plasma concentrations of other essential and non-essential amino acids, but concentrations were overall higher in Term than Preterm groups.

Preterm lambs were significantly smaller and lighter on day zero than Term lambs. Oral supplementation of Preterm lambs for the first fifteen days after birth with BCAA or calorie equivalent Maltodextrin did not affect postnatal growth. Milk intake was similar among all groups and positively associated with growth velocity, with the effect stronger in the Preterm groups. Differences in weight and linear measures between Term and Preterm lambs were reduced by the time of weaning and by adulthood had disappeared, but sex differences became more apparent with increasing age.

After the time of expected puberty (between 150 and 180 days in sheep), most growth parameters started to show divergence between sexes, being greater in males thereafter. However, the timing of the divergence was different among groups for some growth parameters, most marked in the Maltodextrin group where body weight between males and females did not diverge until 360 days (other groups from 180 days) and CRL, BPD, and HL also diverged later than other groups. Chest circumference at 360 days was different between sexes in the Term group but not in any of the Preterm groups.

There was no long-term effect of postnatal BCAA or Maltodextrin supplements on adult weight, linear measures, or organ weight compared to control groups. When normalized to body weight, perirenal fat-to-body weight was greatest in the BCAA group.

## 3.3. Discussion

### 3.3.1. BCAA Supplementation Did Not Affect Plasma Amino Acid Concentration

BCAA supplementation did not affect the plasma concentration of other essential and non-essential amino acids during the period of supplementation. Our experiment was done on sheep, which are ruminants. Increasing plasma amino acid concentration through oral supplementation is challenging in ruminants [231,232]. In adult sheep, plasma amino acid concentrations generally do not rise after feeding because dietary protein and amino acids are mainly metabolised by ruminal microbiota [232]. Despite being ruminants as adults, newborn lambs have a digestive system similar to non-ruminant (monogastric) animals. Milk is digested by an enzymatic process in the abomasum and intestine [232]. When a protein or amino acids enter the abomasum directly through oral feeding, plasma amino acid concentrations increase [231]. Newborn ruminants can absorb BCAA in a manner comparable to that of monogastric animals [233]. We found higher plasma BCAA concentrations two hours post oral BCAA supplementation but a decrease toward the baseline four hours later. BCAA concentrations have been shown to be elevated after a meal containing BCAA [234], but clearance from the circulation occurs within hours of intake [235]. BCAA enter the circulation by absorption from the intestines, bypass the liver, and are delivered to peripheral tissues [236]. About 95 % of all circulating BCAA are reabsorbed in the kidney [235]. The skeletal muscles extract more than half of the circulating BCAA, representing the largest reservoir of BCAA in the body [160]. In contrast with other amino acids, BCAA catabolism resides in skeletal muscle, brain, heart, and adipose tissue [158,234,236]; therefore, measuring BCAA in the tissues rather than plasma would more likely reflect body BCAA content more accurately.

Increased BCAA intake can affect other amino acid concentrations by stimulating their utilization in skeletal muscle for protein accretion [237] or reducing amino acid efflux from muscle due to inhibition of muscle protein degradation [152]. Other plasma amino acid concentrations did not change during supplementation, despite the increase in BCAA intake in the BCAA-supplemented group. The Term group had the highest concentration of all amino acids compared to the three Preterm groups regardless of their supplementation. Other studies have found a positive relationship between amino acid intake and plasma amino acid concentration, particularly when amino acid supplements were given parenterally in preterm infants [238] and preterm baboons [239], but also in some animal studies using oral supplements. In

weaning pigs, supplementing BCAA orally together with a low protein diet increased leucine, isoleucine, and valine concentration during the supplementation period [240] Male term calves received a mixture of BCAA (1:1:1 of valine, leucine, and isoleucine) with either whole or skimmed milk orally from 3-28 days of age, and concentrations of valine, isoleucine, and leucine increased after supplementation [232]. It seems that the correlation between BCAA supplementation and the increase in plasma amino acid concentration can be affected by many factors. The way the supplementation is given (oral or intravenous) and other dietary components (for example, low or high protein) can affect the intestinal absorption of BCAA, transport between the tissues and blood, or alter protein synthesis.

### 3.3.2. BCAA Supplementation Did Not Affect Growth

We found no effect of BCAA supplements on growth parameters during the period of supplementation, juvenile life, or young adulthood. Several studies have similarly shown no effect of amino acid or BCAA supplementation on growth in preterm born humans and animals [241-243]. A study to determine if leucine alone or combined BCAA supplementation enhanced muscle growth in neonatal piglets showed that neither affected body weight [244]. Leucine supplementation was given to enhance pancreatic development in milk-fed calves and showed no effect on body weight [21]. However, not all studies showed similar outcomes, and some found either positive or negative growth outcomes. In preterm babies, a higher amino acid intake given parenterally improved a range of growth parameters [245] resulting in larger head circumference [145] and increased weight gain [246]. In contrast, one study showed decreased early head growth and poor growth after parenteral amino acid supplementation [247]. In animals, dietary BCAA supplementation improved short-term growth performance in calves [232], and improved growth in immature dogs [248]. Kittens that received 5% and 10 % leucine in their standard diet showed an increase in their growth [249]. Conversely, rats fed diets containing excess leucine, isoleucine, or valine showed growth depression [249]. It seems that the relationship between BCAA supplementation and growth is not straightforward. The interaction might be affected by the degree of immaturity, the way the supplementation is given (oral or parenteral), the rest of the diet (low or high protein), the study methods, species differences, and different growth responses in males and females.

In general, male infants have greater weight, length, head circumference, and growth rate than females [106]. In our study, there was no difference in growth parameters between males

and females during the 15 days of supplementation except in the Maltodextrin group, in which growth velocity in the first 4-days was less in males than females.

Nutritional intake and energy requirements are different between the sexes [250]. Mothers of male infants produce milk with higher energy content than mothers of female infants [251], indicating that breastmilk composition varies according to sex. This suggests that preterm males and females may also have different requirements for, and physiological responses to, neonatal nutritional supplements [209,229,252].

Nutritional intervention may have different effects on males and females due to differences in the relative developmental maturity of their organs at the time of birth. A study on neonatal baboons showed that a high caloric diet increased males' weight from the first week of supplementation, while an increase in weight started after 15-days of supplementation in females [253]. However, few reports in the literature indicate that growth patterns of females and males are affected differently by manipulations of early life nutrition [16,252]. Furthermore, most studies do not report their findings by sex.

### 3.3.3. BCAA Supplementation did not affect the Milk Intake

Milk intake was used to assess appetite since lambs were suckling from their mothers. Milk intake regulates postnatal growth, and any alteration in milk intake secondary to appetite suppression by nutritional supplements may cause modification of postnatal growth. In our study, both milk intake and growth velocity were similar among groups, and there was a positive relationship between the two. Therefore, there was no apparent effect of BCAA or Maltodextrin supplements on appetite. Findings were similar to those of a study on weaning pigs fed a low protein diet supplemented with BCAA which showed no effect of the supplementation on feed intake [240]. However, some studies found that neonatal BCAA supplementation does affect milk or feed intake. A 5% and a 10% excess leucine in a standard diet increased food intake in kittens [249], while dietary BCAA supplementation increased feed intake in piglets [254] and immature dogs [248], respectively. Conversely, two- and four-week-old lambs who received excess leucine in their diet decreased their milk intake [156] and, in rats, excess leucine, isoleucine, or valine in their diet caused food intake depression [249]. So there are different outcomes from different studies. It might be that the interaction between the supplementation and the milk intake can be affected by the type and amount of

supplementation, whether it consists of BCAA alone or combined with other amino acids, and sex- and species-specific interactions.

### 3.3.4. BCAA Supplementation Did Not Affect Organ Weight

We found no difference among groups in organ weight, suggesting no effect of preterm birth or nutritional supplements. Similarly, a study in calves found that pancreas weight was not affected by milk supplemented with leucine [21]. Interestingly, pigs that received oral BCAA supplementation in their restricted diet had lower liver and heart weight [244]. In another study, rats had decreased liver weight when fed a low protein diet (6%) with excess leucine (4 and 8%) [192]. The weight and size of the organs can reflect the health of the body [21] to a certain extent. However, organ size does not necessarily reflect function [21].

The pancreas comprises both exocrine and endocrine cells, with the endocrine component being only 2 % of the pancreatic mass [26]. Islet cells, function, and mass are the critical characteristics that determine the efficacy of the endocrine pancreas. However, pancreas weight has been shown to correlate with number of islets [255] and, therefore, β-cell function. In adult preterm-born lambs, the pancreas weight relative to live body weight was higher than in adult term-born lambs, and it was correlated positively with the fasting plasma insulin concentration [209].

### 3.3.5. Maltodextrin Supplementation

Maltodextrin, an iso-caloric carbohydrate supplement, was given to control any effect on pancreatic development and function due to increased energy intake. However, additional carbohydrates given during the neonatal period can also potentially affect metabolism and growth.

Most formulas now fed to preterm infants include a mixture of lactose and maltodextrin based on the assumption of lactose malabsorption and the ability to use maltodextrin as a nutrient source [228,256]. In preterm neonates who received intravenous dextrose for the first 24 to 72 hours, carbohydrate intake was directly associated with increased later weight [226]. However, in mice, maltodextrin-dominant infant formula reduced the overall body weight compared to control mice [257], while maltodextrin-enriched preterm formula given to preterm pigs did not affect their body or pancreas weight [258].

Our study found no long-term effect of Maltodextrin supplementation on growth, growth velocity, milk intake, or plasma amino acid concentration. However, the pattern of growth velocity between the males and females in this group was different. The females showed a higher growth velocity in the first four days after birth than males, and the male-female divergence in growth around the time of expected puberty happened later in this group compared with the others. The reasons for this are not clear.

## 3.4. Strengths and Limitations

The current study was performed in a consistent breeding group produced during extended breeding seasons over two years. Lambing assistance, supplement preparation and administration and serial growth measurements were performed by a small number of people, maximising data consistency.

Body composition was not assessed; these data would have helped detect if BCAA supplements had increased relative lean mass or adipose tissue, since there was an increase in the perirenal fat in the BCAA males. We measured BCAA in plasma. However, since BCAA affects muscle growth, it might have been informative to measure BCAA in the muscle as well as in plasma to see if that correlated with lean mass, for example, in muscle biopsies collected on day 15 at the end of the supplementation period.

Overall survival of Preterm lambs in our study was high. In humans, there is a sex-specific difference in neonatal mortality, with the males having a higher rate than the females [259]. Females are smaller than males at day zero; however, female fetuses are relatively more mature than males [260], which may be a contributing factor to male susceptibility to perinatal mortality [259,260]. In our study, three male lambs from the Water groups died (two after day-60 and one after day-90). However, no deaths occurred in the Preterm supplemented groups. There were no recorded female deaths in any groups (Preterm and Term).

## 3.5. Conclusion

BCAAs (isoleucine, leucine, and valine) were absorbed when given orally to the lambs, with a rapid but short increase in plasma concentration. However, no effect of nutritional supplementation with BCAA on growth in preterm lambs was found. Therefore, any subsequent effects of supplementation on endocrine pancreatic development or function are unlikely to be due to altered growth patterns in early life.

# Chapter 4. Effect of Branched-Chain Amino Acid Supplementation on the Function of the Endocrine Pancreas in Preterm-Born Lambs at One-Year of Age

In a healthy fasting state, there is a balanced relationship between basal insulin secretion rate, tissue glucose uptake, and hepatic glucose release. Following glucose ingestion (eating or drinking) or intravenous infusion, this balance between hepatic glucose production and tissue glucose consumption is disturbed. The body maintains normal glucose homeostasis through several mechanisms: 1. stimulation of insulin secretion by hyperglycaemia; 2. increase in glucose utilization by tissues (liver, gut, muscle, and fat) driven by hyperglycaemia and hyperinsulinemia; 3. suppression of hepatic glucose release; and 4. inhibition of lipolysis to reduce the availability of free fatty acids, thereby enhancing muscle glucose utilization [30]. The inability to process these steps in response to hyperglycaemia, mainly due to the loss of insulin secretion due to β-cell dysfunction, leads to insulin resistance and continuous hyperglycaemia [261]. Hyperglycaemia becomes clinically significant when increased insulin secretion can no longer balance the impaired glucose uptake due to insulin resistance, leading to β-cell deterioration and ultimately cell death [261].

During fasting, glucagon maintains glucose availability [23] by stimulating hepatic gluconeogenesis [262]. Glucagon secretion can be regulated by glucose, amino acids, and hormones [263]. Glucagon secretion is stimulated during hypoglycaemia and suppressed during hyperglycaemia [263], providing the first line of defence in glucose counter-regulation [264]. However, the interaction of glucagon and insulin in the prandial state is responsible for normal glucose tolerance during hyperglycaemia [265]. Glucose homeostasis depends on the cooperation between α- and β-cells, not exclusively on the β-cells [264]. Rather than having opposing roles in glucose homeostasis, α- and β-cells cooperate in the prandial state to regulate the metabolic control of nutrients [266].

Islet cells exist in an environment in which they respond to signals from neighbouring (paracrine) cells, as well as from the cells themselves (autocrine), including influencing hormone secretion [76,23]. To sustain the glycaemic set point, paracrine glucagon signalling in the islet is critical for the secretion of the appropriate amount of insulin [264]. Glucagon stimulates insulin secretion, despite being a counterregulatory hormone that opposes insulin action [266], and within the islet, glucagon potentiates insulin secretion during hyperglycaemia

[267]. However, the increase in glucagon concentration during hyperglycaemia is enough to potentiate insulin secretion to a certain level without increasing hepatic glucose output [267]. In comparison between a whole isolated islet and isolated α-cells from mice, isolated islets secretes insulin when stimulated with glucose while glucagon secretion is inhibited; however, isolated α-cells secrete glucagon under glucose stimulus [268]. α-cell-ablated islets showed a blunted insulin response to glucose, suggesting that α-cell-glucagon secretion is necessary for β-cell function [269]. Different responses to glucose were found between single and aggregated β-cells [270]. Aggregates comprising a β-cell coupled to α-cell (β–α pairs) secreted significantly more insulin than β-cells alone [270], and the quantity of glucagon secreted by the α-cell, even at high glucose concentration, is sufficient to affect β-cell insulin secretion [270]. Removing the cells from the islet environment will affect cell-to-cell contact paracrine communication, which is vital for glucose homeostasis.

Decreased communication between cells reduces paracrine-mediated effects on insulin and glucagon secretion and/or suppression [76]. Diabetes is believed to be a bi-hormonal disease with impaired insulin secretion and/or action and altered glucagon secretion [271]. In type-2 diabetes, over-secretion of glucagon during meals due to α-cell "hypoglycaemic blindness" [272] results in excessive glucose release, leading to hyperglycaemia, contributing to eventual β-cell death [76,272]. On the other hand, glucagon secretion is lost in type 1 diabetic patients, supporting the requirement for β-cell function for glucagon secretion [271].

Studying the regulation of glucose homeostasis during the fed state is essential to understand glucose utilisation and insulin production, but it is difficult to measure accurately the continuous changes in glucose and insulin concentrations following gastrointestinal absorption of glucose [30]. Glucose homeostasis has therefore been investigated using intravenous glucose (glucose infusion), and clamp studies have provided comprehensive information about whole body and organ-specific glucose uptake [30].

## 4.1. Hyperglycaemic Clamp

Hyperglycaemic clamp (HGC) is a sophisticated and reproducible technique to evaluate glucose homeostasis by assessing β-cell function, insulin sensitivity, and quantifying the amount of glucose metabolised by the body under maximal stimulatory conditions (a controlled hyperglycaemic stimulus) [30,214,261,273]. Using intravenous glucose infusion (25% dextrose), blood glucose concentration is raised above basal levels quickly (within minutes) with a fixed

desired hyperglycaemic plateau concentration maintained for two hours [214,261]. The hyperglycaemic clamp technique is sometimes paired with subsequent arginine stimulation to evaluate non-glucose insulin secretagogues and measure maximum insulin secretory capacity at a steady-state glucose concentration [261,273].

In normal physiological conditions and under constant hyperglycaemia, a biphasic insulin response is observed in the plasma depending on the type and magnitude of the glucose stimulus [214,261,273,274]. First phase insulin response (FIR) is the first or immediate plasma insulin concentration rise that occurs during the first 20 min following the glucose infusion with a peak response at 5 min, representing the release of preformed insulin that is stored within β-cell granules by which β-cell function can be measured [30,214,261,273]. The second phase, or the 'steady-state' insulin response (SIR), is the gradual linear increase in plasma insulin concentration from 60-120 min of glucose infusion in response to ongoing hyperglycaemic stimulus [261,273]. This phase represents the release of newly synthesized insulin by which insulin sensitivity can be measured [30]. Arginine challenge stimulates maximal insulin secretion [261].

The HGC test has many advantages as it enables measurement of the amount of glucose metabolised [214], calculation of insulin sensitivity since the glucose infusion rate is an index of glucose metabolism when the glucose concentration is held constant [261], quantification of β-cell response, assessing FIR and SIR separately [214], and examination of the same subject over time to observe any improvement or deterioration in the pancreatic insulin response [261].

Sheep are ruminants, and in adulthood they absorb very little glucose from their normal diet, but instead produce nearly all their glucose requirements through gluconeogenesis [275]. However, as seen in humans, they show similar glucose elevation and insulin biphasic responses after intravenous glucose infusion [13], enabling the use of HGC to assess insulin secretion and sensitivity.

We hypothesised that postnatal oral supplementation of preterm lambs with BCAA, key nutrients for pancreatic development, would mitigate the immaturity of the endocrine pancreas due to preterm birth thereby reducing the incidence of permanent long-term sequelae on glucose metabolism.

## 4.2. Methods

### 4.2.1. Hyperglycaemic Clamp (HGC)

Hyperglycaemic clamp was performed at 12 months of age (Methods section 2.4.7). Briefly, catheters were inserted in both jugular veins, one for glucose infusion and another for blood collection. Blood samples were collected to measure glucose, insulin, and glucagon concentration at set time points throughout the test. Baseline blood samples were taken at -20, -10, and 0 minutes and averaged. Samples were processed immediately on a YSI 2300 (Yellow Springs Instruments, Dayton, OH, USA) to determine glucose concentration. At 0 minutes, a priming dose (25% glucose) was infused to increase the blood glucose concentration to 10 mmol/L, with an infusion rate calculated according to body weight and surface area. This increase in blood glucose concentration was maintained throughout the study by altering the maintenance dose using a computer algorithm based on blood glucose concentration measurements on samples collected every 5 minutes. At 135 minutes, a bolus infusion of arginine was given to achieve maximal insulin secretion.

### 4.2.2. Hormone Measurement

#### 4.2.2.1. Plasma Insulin Concentration

Plasma insulin concentrations were measured using an ELISA kit (cat #10-1202-01, Mercodia AB, Sweden) following the manufacturer's instructions. The enzyme conjugate solution and buffer solution were prepared according to the table provided in the manual. First, 25 µL of the calibrators and samples were pipetted into the wells, then 100 µL of the enzyme conjugate was added to each well. The microplate was incubated on a plate shaker (700-900 rpm) for 2 hours at room temperature, then washed with 700 µL wash buffer 1 × solution per well using an automatic plate washer. After the final wash, the plate was inverted, discharging the remaining washing buffer, and tapped firmly against absorbent paper to remove excess liquid. Then, 200 µL of the substrate was added to each well, and the microplate was incubated for 15 minutes at room temperature. 50 µL of stop solution was added to each well, and the microplate was incubated for 5 minutes on the plate shaker. The microplate's optical density was read at 450 nm by a plate reader (BioTek Instruments, Inc, Winooski, Vermont, USA) (Methods section 2.4.9.1).

#### 4.2.2.2. Plasma Glucagon Concentration

Plasma glucagon concentrations were measured using a commercially available ELISA kit (DuoSet Glucagon, cat # DY1249, R&D Systems, Minneapolis, MN). The working concentration of the glucagon capture antibody, glucagon detection antibody, glucagon standard, and streptavidin-HRP solutions were prepared according to the certificate of analysis provided by the manufacturer. The washing buffer, reagent diluent, and substrate solutions were prepared according to the information provided in the manual. The plate was coated with 100 µL per well of the glucagon capture antibody. Wells were aspirated and washed with 1 × washing buffer solution using an automatic plate washer. Then, the plate was blocked by adding 300 µL of the reagent diluent to each well. The aspiration/wash was repeated, then 150 µL of sample and 100 µL of the standard were added per well, then the plate was sealed and incubated overnight at room temperature on a plate shaker (300 rpm). The aspiration/wash was repeated, and 100 µL of the glucagon detection antibody was added to each well, followed by adding 100 µL streptavidin-HRP to each well. Finally, 100 µL of the substrate solution was added per well, then 50 µL of the stop solution was added to each well. The plate's optical density was read at 450 nm by a plate reader (BioTek Instruments, Inc, Winooski, Vermont, USA) (Methods section 2.4.9.2).

### 4.2.3. Statistical Analysis

Hyperglycaemic clamp samples were analysed if the blood glucose coefficient of variation (CV) was < 10% during steady-state insulin response (60-120 minutes) [13]. A linear-linear spline fit curve of the standard curve was used to measure insulin concentration, while the plasma glucagon concentration was extrapolated from the standard curve using the four-parameter logistic (4-PL) curve-fit. The baseline plasma insulin, glucagon, and blood glucose concentrations were calculated as the mean of samples taken at -20, -10 and 0 minutes. The first phase insulin response was calculated from the plasma insulin concentration during the first 20 minutes of the clamp. The steady-state insulin response was calculated from the insulin concentration during the maintenance phase (60-120 minutes). Arginine challenge (AC) was calculated from the insulin concentration during arginine infusion (140-165 minutes). The area under the curve (AUC) for the FIR, SIR, and AC were calculated using the trapezoid rule.

Insulin sensitivity (IS) was calculated from the formula [13]:

$$\text{IS } (\text{mmol.L}^{-1}.\text{Kg}^{-1}.\text{min}^{-1}.\mu\text{g}^{-1}) = \frac{\text{mean glucose infused during the steady-state } (\text{mmol.Kg}^{-1}.\text{min}^{-1})}{\text{mean plasma insulin concentration during the steady-state } (\mu\text{g.L}^{-1})}$$

Data were analysed by one-way ANOVA between groups followed by Tukey's post hoc test. Pearson's correlation was used to examine bivariate relationships, and the correlation coefficient (r) was calculated. $P \leq 0.05$ was considered statistically significant. Results are reported as mean ± SEM.

## 4.3. Results

Data from two sheep (Maltodextrin 1F & BCAA 1F) were excluded from the analysis as the coefficients of variation (CV) for glucose concentration during the steady-state period were > 10% [276]. Fifty-two animals were included in the glucose and insulin results. Results are shown in Figure 4-1 and Table 4-1. One sheep (Term 1F) was excluded from glucagon analysis due to a high CV. Fifty-one animals were included in the glucagon results (Figure 4-2 and Table 4-2).

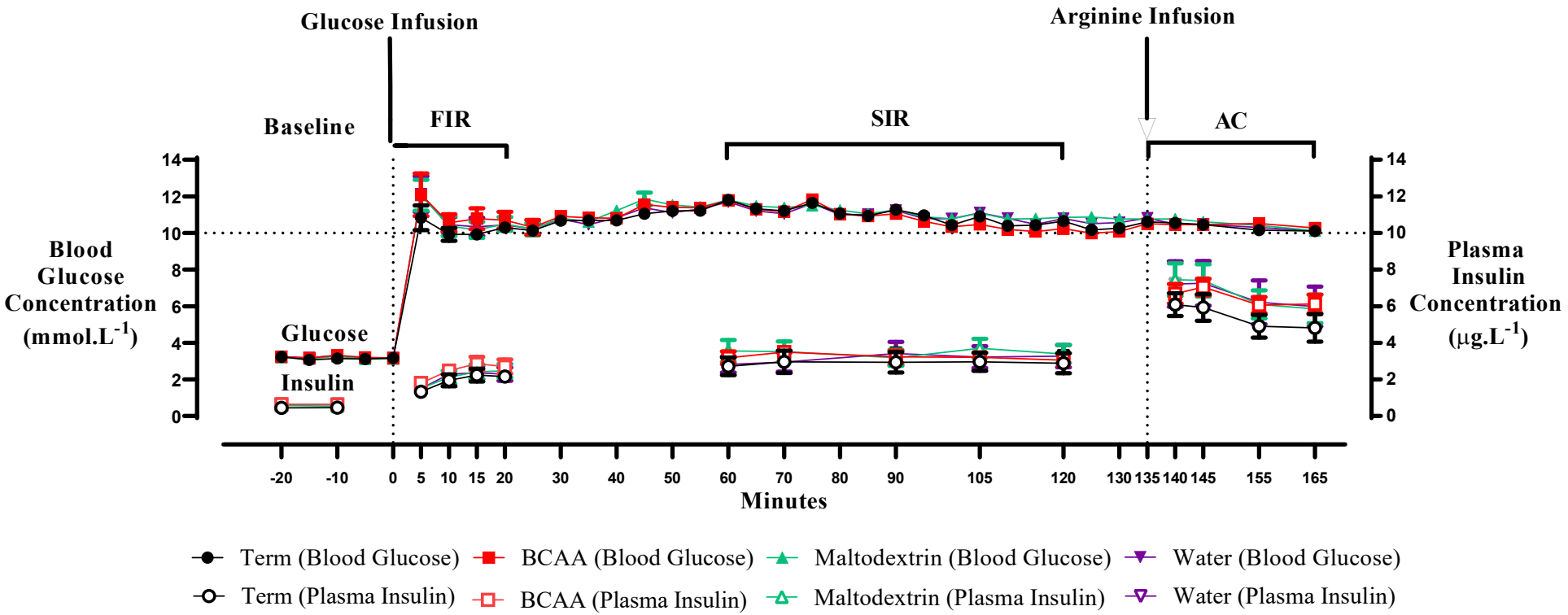



**Figure 4-1. Blood glucose and plasma insulin concentration during HGC and arginine challenge in sheep at 12 months of age.**
FIR: first insulin response (0-20 minutes), SIR: steady-state insulin response (60-120 minutes), AC: arginine challenge (135-165 minutes). Data are mean with ± SEM.

**Table 4-1. Blood glucose and plasma insulin concentration during HGC at 12 months of age.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=6) | (M=7) | (F=7) | (M=7) | (F=7) | (M=6) | (F=7) | (M=5) | Sex | Treatment | Interaction |
| **Baseline** | | | | | | | | | | | |
| Glucose (mmol.L$^{-1}$) | 3.1±0.1$^{a,b}$ | 3.3±0.1$^{a,b}$ | 3.0±0.1$^{b}$ | 3.4±0.1$^{a}$ | 3.2±0.1$^{a,b}$ | 3.2±0.1$^{a,b}$ | 3.2±0.1$^{a,b}$ | 3.0±0.1$^{a,b}$ | 0.10 | 0.86 | **0.02** |
| Insulin (µg.L$^{-1}$) | 0.4±0.1 | 0.7±0.1 | 0.5±0.1 | 0.7±0.1 | 0.6±0.2 | 0.7±0.2 | 0.4±0 | 0.6±0.2 | **0.04** | 0.69 | 0.92 |
| **Glucose Infusion** | | | | | | | | | | | |
| PD (mmol.Kg$^{-1}$.min$^{-1}$) | 701±10$^{b}$ | 805±22$^{a}$ | 695±21$^{b}$ | 798±25$^{a}$ | 690±24$^{b}$ | 813±16$^{a}$ | 682±16$^{b}$ | 756±32$^{a,b}$ | **<0.001** | 0.39 | 0.73 |
| MD (mmol.Kg$^{-1}$.min$^{-1}$) | 67±3 | 70±6 | 79±12 | 90±4 | 74±11 | 80±9 | 73±7 | 87±21 | 0.21 | 0.40 | 0.94 |
| **5 min post Glucose Infusion** | | | | | | | | | | | |
| Glucose (mmol.L$^{-1}$) | 14±2 | 10±0.2 | 11±1.3 | 14±1 | 11±0.5 | 13±2 | 11±1 | 10±1 | 0.68 | 0.47 | 0.06 |
| Insulin (µg.L$^{-1}$) | 1.4±0.2 | 2.2±0.2 | 1.1±0.2 | 2.0±0.4 | 1.3±0.3 | 1.7±0.3 | 1.2±0.2 | 1.6±0.4 | **0.002** | 0.67 | 0.59 |
| Insulin: Glucose ratio | 0.1$^{b}$ | 0.2$^{a}$ | 0.1$^{b}$ | 0.1$^{a,b}$ | 0.1$^{a,b}$ | 0.1$^{a,b}$ | 0.1$^{b}$ | 0.2$^{a,b}$ | **0.002** | 0.48 | 0.13 |
| **First Insulin Response (0-20min)** | | | | | | | | | | | |
| Mean (µg.L$^{-1}$) | 2.0±0.3 | 3.0±0.4 | 1.5±0.2 | 3.0±0.6 | 2.0±0.4 | 2.4±0.5 | 2.0±0.3 | 2.2±0.6 | **0.005** | 0.84 | 0.52 |
| AUC (µg.L$^{-1}$.min$^{-1}$) | 27±4 | 48±8 | 21±3 | 43±9 | 27±5 | 34±7 | 27±6 | 33±8 | **0.003** | 0.76 | 0.45 |
| **Steady-State Insulin Response (60-120min)** | | | | | | | | | | | |
| Mean (µg.L$^{-1}$) | 3.0±0.5 | 3.5±0.4 | 3.0±0.4 | 4.4±1.0 | 3.0±1.0 | 3.4±1.0 | 3.0±0.5 | 3.3±1.1 | 0.06 | 0.75 | 0.83 |
| AUC (µg.L$^{-1}$.min$^{-1}$) | 179±28 | 213±23 | 161±24 | 263±53 | 178±52 | 203±43 | 158±32 | 199±68 | 0.07 | 0.78 | 0.85 |
| **Arginine Challenge (135-165min)** | | | | | | | | | | | |
| Mean (µg.L$^{-1}$) | 3.1±0.4 | 3.6±0.4 | 3.0±0.4 | 3.7±1.0 | 3.6±1.0 | 3.2±1.0 | 2.7±0.4 | 2.2±0.3 | 0.86 | 0.38 | 0.55 |
| AUC (µg.L$^{-1}$.min$^{-1}$) | 148±15 | 174±16 | 134±19 | 199±34 | 160±46 | 169±40 | 130±21 | 137±31 | 0.22 | 0.65 | 0.80 |
| Peak (µg.L$^{-1}$) | 6.2±1 | 7.2±1.0 | 6.0±1.0 | 9.1±1.5 | 7.2±2.0 | 7.3±1.4 | 6.2±1.0 | 6.0±1.0 | 0.27 | 0.61 | 0.62 |
| **IS** (mmol.L$^{-1}$.Kg$^{-1}$.min$^{-1}$.µg$^{-1}$) | 26±4 | 22±3 | 30±2.5 | 31±9 | 35±8.5 | 28±6 | 31±3 | 31±6 | 0.47 | 0.65 | 0.85 |
| **Weight at HGC** (Kg) | 68±1$^{b}$ | 84±3$^{a}$ | 67±3$^{b}$ | 82±4$^{a}$ | 66±3$^{b}$ | 85±3$^{a}$ | 65±2$^{b}$ | 76±5$^{a,b}$ | **<0.001** | 0.38 | 0.72 |

Bold font indicates significance in ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. F: female; M: male; PD: priming dose; MD: maintenance dose; AUC: area under the curve; IS: insulin sensitivity).

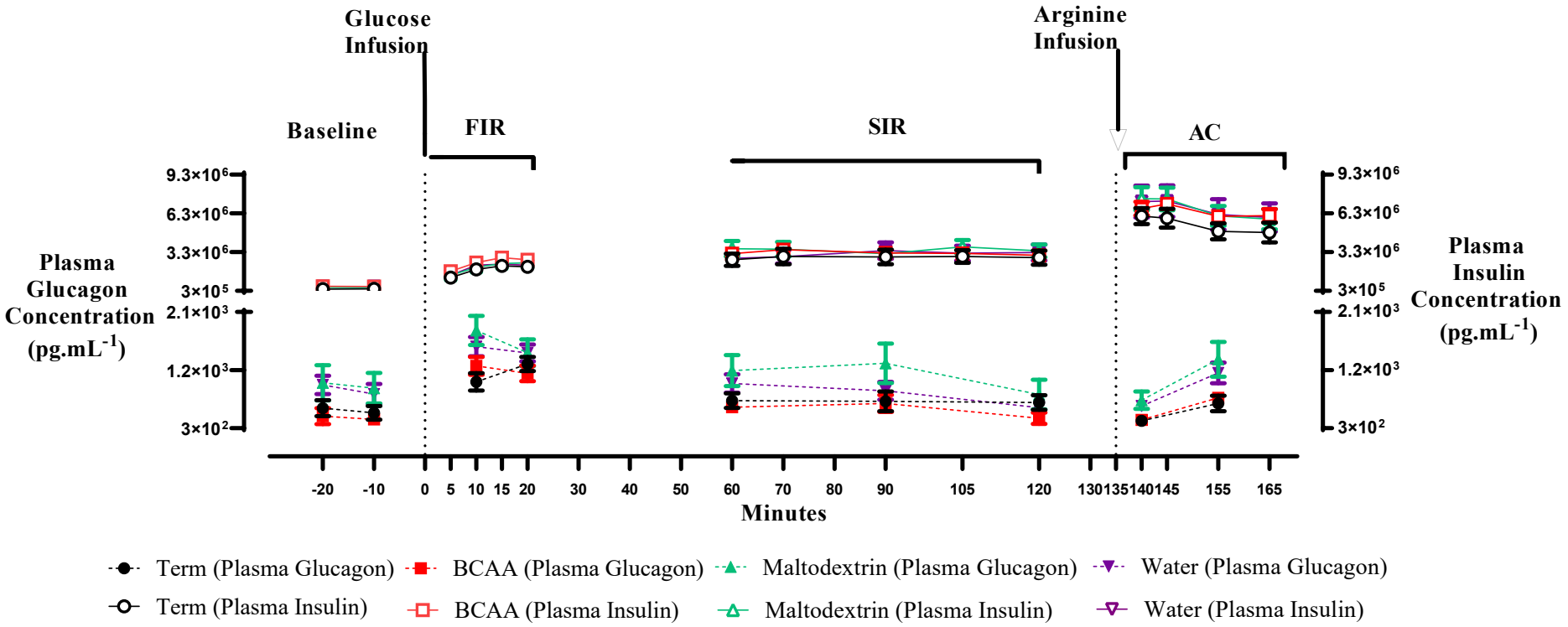



**Figure 4-2. Plasma insulin and glucagon concentration during HGC and arginine challenge in sheep at 12 months of age.**
FIR: first insulin response (0-20 minutes), SIR: steady-state insulin response (60-120 minutes), AC: arginine challenge (135-165 minutes). Data are mean with ± SEM.

**Table 4-2. Plasma glucagon concentration, glucagon-to-insulin and glucagon-to-glucose ratios calculated from HGC.**

| | Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=6) | (M=7) | (F=7) | (M=7) | (F=7) | (M=6) | (F=6) | (M=5) | Sex | Treatment | Interaction |
| **Glucagon** (pg.mL$^{-1}$) | | | | | | | | | | | |
| Baseline | 437±139 | 482±76 | 1316±479 | 609±86 | 959±208 | 829±217 | 693±184 | 434±85 | 0.12 | 0.10 | 0.40 |
| *(10-20min) | 1129±154$^{b}$ | 1277±135$^{a,b}$ | 2048±324$^{a}$ | 1236±108$^{b}$ | 1487±149$^{a,b}$ | 1548±171$^{a,b}$ | 1270±119$^{a,b}$ | 1022±158$^{b}$ | 0.10 | **0.02** | **0.04** |
| *(60-120min) | 650±127$^{a,b}$ | 537±66$^{b}$ | 1538±443$^{a}$ | 675±98$^{a,b}$ | 919±170$^{a,b}$ | 726±133$^{a,b}$ | 914±146$^{a,b}$ | 474±86$^{b}$ | **0.009** | 0.08 | 0.25 |
| *(140-155min) | 661±81$^{a,b}$ | 548±78$^{a,b}$ | 1326±379$^{a}$ | 775±83$^{a,b}$ | 958±183$^{a,b}$ | 831±139$^{a,b}$ | 713±144$^{a,b}$ | 350±55$^{b}$ | **0.03** | **0.02** | 0.57 |
| **Glucagon : Insulin Ratio** | | | | | | | | | | | |
| At Baseline | 1.3±0.5 | 0.9±0.3 | 2.5±0.8 | 1.3±0.4 | 2.0±0.3 | 1.3±0.4 | 2.1±0.7 | 0.9±0.2 | **0.01** | 0.45 | 0.76 |
| At 10 minutes | 0.7±0.1$^{a,b}$ | 0.5±0.1$^{b}$ | 1.6±0.3$^{a}$ | 0.6±0.2$^{b}$ | 0.9±0.2$^{a,b}$ | 0.8±0.2$^{a,b}$ | 1.0±0.3$^{a,b}$ | 0.5±0.2$^{b}$ | **0.001** | 0.11 | 0.09 |
| At 20 minutes | 0.7±0.2 | 0.5±0.1 | 1.2±0.2 | 0.5±0.2 | 0.9±0.2 | 0.7±0.2 | 0.9±0.2 | 0.6±0.2 | **0.02** | 0.47 | 0.50 |
| At 60 minutes | 0.3±0.1$^{a,b}$ | 0.2±0.0$^{b}$ | 0.8±0.3$^{a}$ | 0.3±0.1$^{a,b}$ | 0.4±0.0$^{a,b}$ | 0.3±0.1$^{a,b}$ | 0.5±0.1$^{a,b}$ | 0.2±0.0$^{a,b}$ | **0.088** | 0.15 | 0.25 |
| At 90 minutes | 0.4±0.1$^{a,b}$ | 0.2±0.0$^{b}$ | 0.8±0.3$^{a}$ | 0.2±0.1$^{b}$ | 0.4±0.1$^{a,b}$ | 0.3±0.1$^{a,b}$ | 0.5±0.1$^{a,b}$ | 0.2±0.1$^{a,b}$ | **0.004** | 0.15 | 0.22 |
| At 120 minutes | 0.2±0.1$^{a,b}$ | 0.1±0.0$^{b}$ | 0.5±0.1$^{a}$ | 0.2±0.0$^{b}$ | 0.3±0.1$^{a,b}$ | 0.2±0.0$^{a,b}$ | 0.5±0.1$^{a}$ | 0.2±0.1$^{a,b}$ | **0.0002** | 0.10 | 0.19 |
| At 140 minutes | 0.1±0.0 | 0.1±0.0 | 0.2±0.1 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.0±0.0 | **0.01** | 0.29 | 0.34 |
| At 155 minutes | 0.2±0.1 | 0.1±0.0 | 0.4±0.2 | 0.2±0.0 | 0.2±0.0 | 0.2±0.1 | 0.3±0.1 | 0.1±0.0 | **0.01** | 0.24 | 0.56 |
| **Glucagon : Glucose Ratio** | | | | | | | | | | | |
| At Baseline | 148±54 | 142±23 | 419±153 | 179±30 | 292±62 | 245±60 | 212±55 | 139±26 | 0.08 | 0.12 | 0.38 |
| At 10 minutes | 116±12$^{a,b}$ | 134±26$^{a,b}$ | 211±37$^{a}$ | 133±18$^{a,b}$ | 129±24$^{a,b}$ | 181±15$^{a,b}$ | 115±12$^{a,b}$ | 88±27$^{b}$ | 0.58 | **0.02** | **0.04** |
| At 20 minutes | 100±18 | 122±14 | 173±30 | 101±19 | 146±13 | 139±31 | 133±12 | 120±20 | 0.24 | 0.43 | 0.14 |
| At 60 minutes | 51±3$^{a,b}$ | 55±7$^{a,b}$ | 131±35$^{a}$ | 70±6$^{a,b}$ | 96±21$^{a,b}$ | 75±16$^{a,b}$ | 78±15$^{a,b}$ | 42±4$^{b}$ | **0.03** | **0.03** | 0.32 |
| At 90 minutes | 76±20$^{a,b}$ | 50±14$^{b}$ | 158±42$^{a}$ | 68±13$^{a,b}$ | 91±20$^{a,b}$ | 65±16$^{a,b}$ | 84±23$^{a,b}$ | 44±12$^{b}$ | **0.007** | 0.10 | 0.41 |
| At 120 minutes | 51±19 | 40±7 | 104±34 | 43±10 | 64±12 | 50±10 | 89±8 | 38±15 | **0.007** | 0.39 | 0.35 |
| At 140 minutes | 43±5$^{a,b}$ | 40±6$^{a,b}$ | 86±23$^{a}$ | 51±6$^{a,b}$ | 64±11$^{a,b}$ | 59±9$^{a,b}$ | 52±9$^{a,b}$ | 24±4$^{b}$ | **0.03** | **0.02** | 0.42 |
| At 155 minutes | 81±10$^{a,b}$ | 65±10$^{a,b}$ | 165±48$^{a}$ | 98±10$^{a,b}$ | 121±24$^{a,b}$ | 102±18$^{a,b}$ | 86±18$^{a,b}$ | 44±7$^{b}$ | **0.04** | **0.02** | 0.66 |

Bold font indicates significance in ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. F: female; M: male; * mean of the timepoints; AC: Arginine Challenge

## 4.3.1. HGC: Baseline Concentrations

### 4.3.1.1. Glucose

The mean blood glucose concentration at baseline was (3.0 mmol.$L^{-1}$) (Table 4-1). Overall, there was no difference among groups or between sexes; however, there was a sex × treatment interaction ($P$ = 0.02) (Table 4-1), with Maltodextrin-males having a higher fasting blood glucose concentration than Maltodextrin-females ($P$ = 0.03). There was a positive correlation between blood glucose Blood glucose concentration at baseline was not associated with early growth velocity (day 1-15), bodyweight at the of the HGC, or pancreas weight (data not shown).

### 4.3.1.2. Insulin

Plasma insulin concentration at baseline was not different among groups (Table 4-1). However, there was a difference between sexes, with males having higher baseline plasma insulin concentration than females (male = 0.7±0.1 µg.$L^{-1}$, female = 0.5±0.1 µg.$L^{-1}$, $P$ = 0.04) (Figure 4-4-A). In the Term group, plasma insulin concentration at baseline was positively associated with birthweight ($P$ = 0.04, r = 0.59) and pancreas weight ($P$ = 0.01, r = 0.69), while in the Maltodextrin group, baseline plasma insulin concentration was positively associated with sheep bodyweight at HGC ($P$ = 0.01, r = 0.41). No relationship was found with early or later growth velocity (data not shown).

### 4.3.1.3. Glucagon

Plasma glucagon concentration was not different among groups or between sexes (Table 4-2). No association was found between plasma glucagon concentration and birthweight, early growth velocity (day 1-15), bodyweight at the time of the HGC, or pancreas weight (data not shown). No effect of treatment was found on the glucagon-to-insulin ratio at baseline. However, there was a sex effect, with the females having higher ratios than males ($P$ < 0.05). No effect of treatment nor sex was found on the glucagon-to-glucose ratio at baseline.

## 4.3.2. HGC: Post Glucose Infusion

### 4.3.2.1. Five Minutes Post Glucose Infusion

4.3.2.1.1. Blood Glucose Concentration

Blood glucose concentration five minutes post glucose infusion was not different among groups or between sexes (Figure 4-3 and Table 4-1) and was not associated with birthweight, early growth velocity (day 1-15), bodyweight at the time of the HGC, or pancreas weight (data not shown).

4.3.2.1.2. Plasma Insulin Concentration

The plasma insulin concentration five minutes post glucose infusion was similar among groups (Figure 4-3 and Table 4-1**)** but different between sexes, with males (1.9±0.2 $\mu g.L^{-1}$) having higher plasma insulin concentration than females (1.2±0.1 $\mu g.L^{-1}$) ($P$ = 0.002). A positive correlation was found between plasma insulin concentration five minutes post glucose infusion and bodyweight at HGC in supplemented groups (BCAA ($P$ = 0.009, r = 0.68) and Maltodextrin ($P$ = 0.01, r = 0.63)), and with pancreas weight in the Term group ($P$ = 0.003, r = 0.76). There was no relationship between plasma insulin concentration five minutes post glucose infusion and birthweight or early growth velocity (day 1-15) (data not shown).

4.3.2.1.3. Insulin-to-Glucose Ratio

The insulin-to-glucose ratio five minutes post glucose infusion was similar among groups but different between sexes, with the males higher than females ($P$ = 0.002) (Table 4-1 and Figure 4-4-B). Post hoc analysis showed a higher insulin-to-glucose ratio five minutes post glucose infusion in the BCAA-male group in comparison to females in all groups (Term-female ($P$ = 0.02), BCAA-female ($P$ = 0.01), Maltodextrin-female ($P$ = 0.04)). Birthweight in the Term group ($P$ = 0.04, r = 0.59) and current bodyweight in the BCAA ($P$ = 0.01, r = 0.66) group were positively correlated with the insulin-to-glucose ratio. There was no relationship between insulin-to-glucose ratio and early growth velocity (day 1-15) or pancreas weight (data not shown).

### 4.3.2.2. First Insulin Response (FIR 0-20 Minutes)

4.3.2.2.1. Plasma Insulin Concentration

The $FIR_{AUC}$ was similar among groups (Figure 4-3), but there was a difference between the sexes, with the males (39.5±3 $\mu g.L^{-1}.min^{-1}$) having higher $FIR_{AUC}$ than females (25.4±3 $\mu g.L^{-}$

$^{1}$.min$^{-1}$) ($P = 0.003$) (Table 4-1 and Figure 4-4-C). A positive relationship was found between $FIR_{AUC}$ and pancreas weight in the Term group ($P = 0.01$, r = 0.66), and between $FIR_{AUC}$ and bodyweight at HGC in the Maltodextrin group ($P = 0.02$, r = 0.60). No association was found between $FIR_{AUC}$ and birthweight or early growth velocity (day 1-15) (data not shown).

4.3.2.2.2. Plasma Glucagon Concentration

Post glucose infusion, there was an increase from baseline to 10 minutes in plasma glucagon concentration in all groups (Term ($P = 0.008$), BCAA, Maltodextrin, and Water (all $P < 0.0001$) (Figure 4-2). The plasma glucagon concentration (mean 10-20 minutes) was different among groups (Maltodextrin $P = 0.02$). No difference between sexes was found, but there was a sex × treatment interaction ($P = 0.04$). On post hoc analysis, the Maltodextrin-female group had higher plasma glucagon concentration during this period than males in Term ($P = 0.009$) and Maltodextrin ($P = 0.03$) groups and females in the BCAA group ($P = 0.01$) (Table 4-2 and Figure 4-3). No association was found between plasma glucagon concentration and birthweight, early growth velocity (day 1-15), bodyweight at the time of the HGC, or pancreas weight (data not shown).

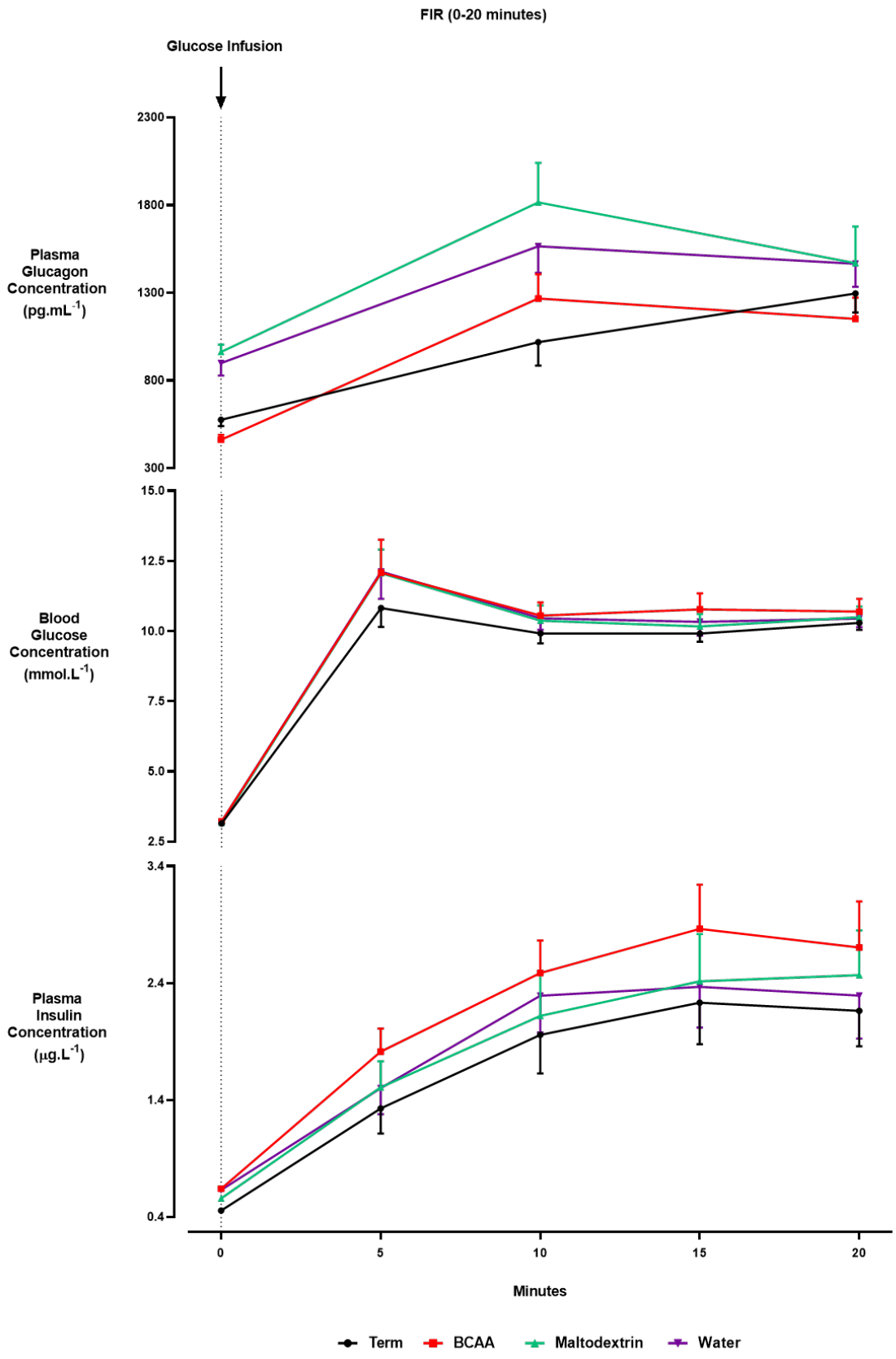



**Figure 4-3. Plasma insulin, glucagon, and blood glucose concentrations during FIR.** Data are mean with ± SEM. Timepoint zero represents the mean from -20 to -10 minutes.

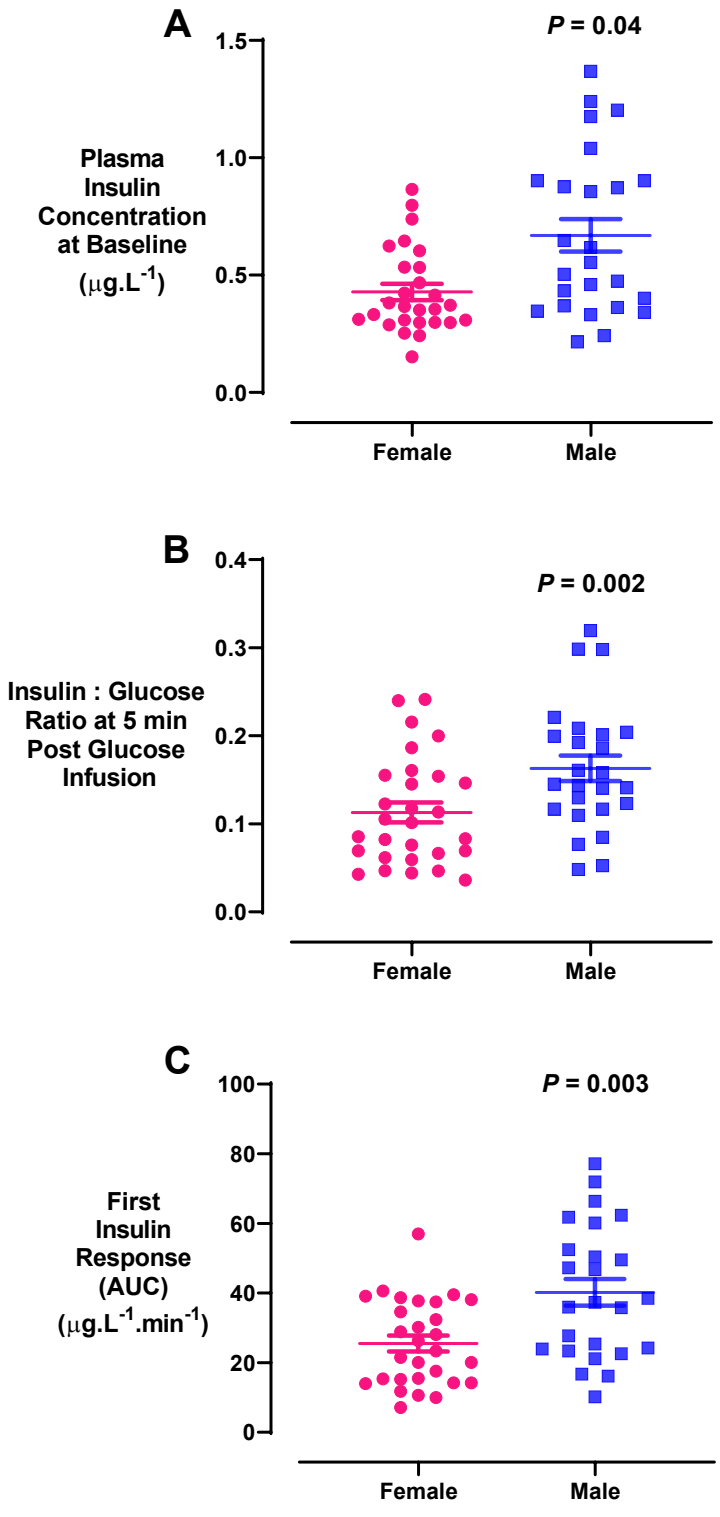



**Figure 4-4. The difference between males and females in insulin secretion.**
A. plasma insulin secretion at baseline. B. insulin-to-glucose ratio at 5 minutes post glucose infusion. C. $FIR_{AUC}$. Scatter plot with mean ± SEM. *P*-values for differences between groups by ANOVA.

4.3.2.2.3. Glucagon-to-Insulin Ratio

The glucagon-to-insulin ratio was similar among groups. Females had higher glucagon-to-insulin ratios than males at 10 and 20 minutes ($P < 0.05$) (Figure 4-5). At 10 minutes post glucose infusion, the Maltodextrin-female group had higher glucagon: insulin ratio than males in Term ($P = 0.007$), BCAA ($P = 0.005$), and Maltodextrin ($P = 0.01$) groups (Table 4-2).

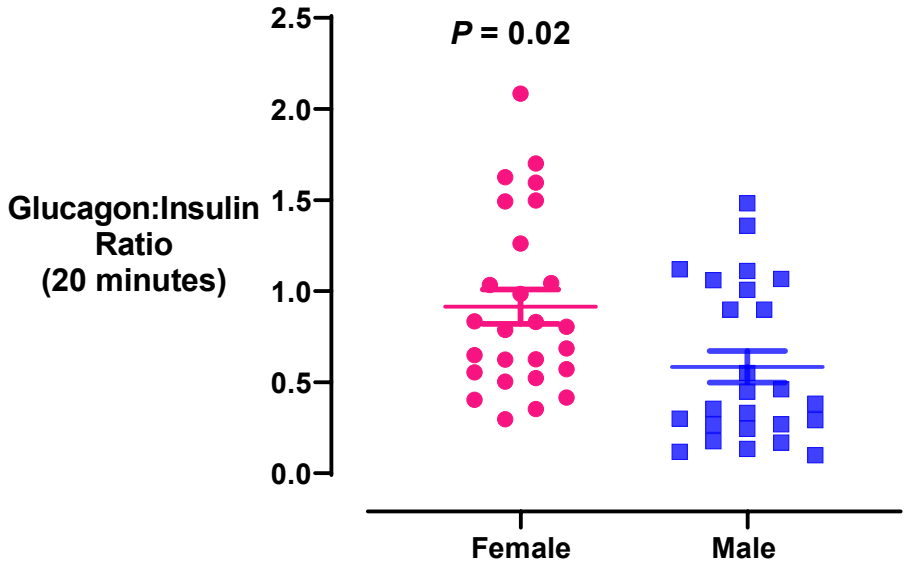



**Figure 4-5. The difference between males and females in glucagon-to-insulin ratio at 20 minutes post glucose infusion.**
Scatter plot with mean ± SEM. *P*-values for differences between groups by ANOVA.

4.3.2.2.4. Glucagon-to-Glucose Ratio

There was a difference among groups in glucagon-to-glucose ratio at 10 minutes post glucose infusion, with the Maltodextrin group having a higher ratio than the Term group ($P = 0.02$). No difference between the sexes was found. On post-hoc analysis, the Maltodextrin-females had a higher ratio than Term-males ($P = 0.02$). Glucagon-to-glucose ratio at 20 minutes post glucose infusion was not different between sexes or among groups (Table 4-2).

### 4.3.2.3. Steady-State Insulin Response (SIR 60-120 Minutes)

4.3.2.3.1. Plasma Insulin Concentration

Plasma insulin concentration was not different among groups or between sexes (Table 4-1 and Figure 4-6). No association was found between plasma insulin concentration during SIR

and birthweight, early growth velocity (day 1-15), bodyweight at the time of the HGC, or pancreas weight (data not shown).

4.3.2.3.2. Plasma Glucagon Concentration

Plasma glucagon concentration during SIR was greater in females than males ($P = 0.009$) but not different among groups. On post hoc analysis, the Maltodextrin-females had higher plasma glucagon concentration than Term ($P = 0.02$) and BCAA ($P = 0.01$) males. (Table 4-2 and Figure 4-6). No association was found between plasma glucagon concentration and birthweight, early growth velocity (day 1-15), bodyweight at the time of the HGC, or pancreas weight (data not shown).

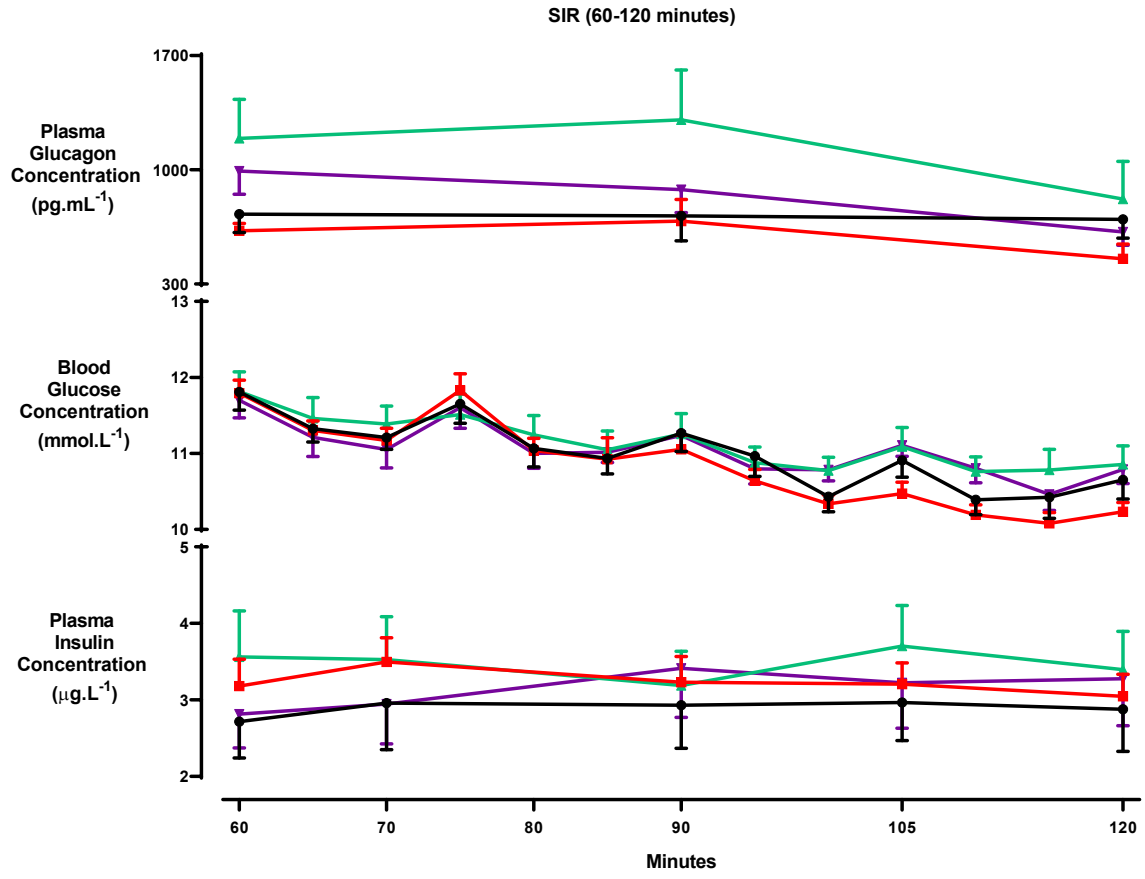



**Figure 4-6. Plasma insulin, glucagon, and blood glucose concentration during SIR.** Data are mean with ± SEM.

4.3.2.3.3. Glucagon-to-Insulin Ratio

The glucagon-to-insulin ratio during SIR was similar among groups. Females had higher glucagon-to-insulin ratios than males at 60, 90, and 120 minutes (all $P < 0.05$) (Figure 4-7). The glucagon-to-insulin ratio was higher in Maltodextrin-females than BCAA-males at 60 minutes post glucose infusion ($P = 0.02$), and then BCAA-males ($P = 0.009$) and Maltodextrin-males ($P = 0.03$) at 90 minutes post glucose infusion. At 120 minutes post glucose infusion, glucagon-to-insulin ratio was the highest in Term-female and Maltodextrin-female groups compared with BCAA-male ($P = 0.01$and $P = 0.02$ respectively) and Maltodextrin-male ($P = 0.03$ and $P = 0.04$, respectively) (Table 4-2).

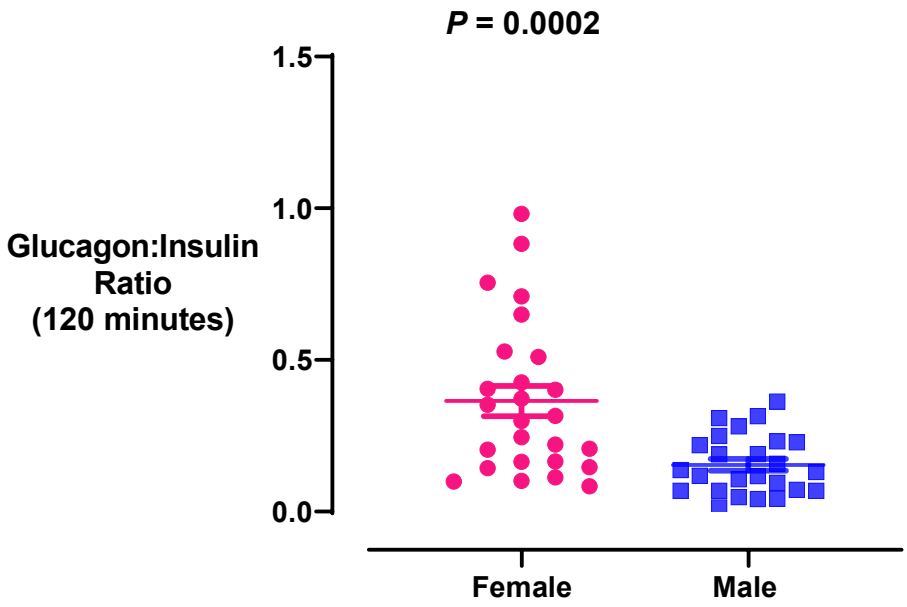



**Figure 4-7. The difference between males and females in glucagon-to-insulin ratio at 120 minutes post glucose infusion.**
Scatter plot with mean ± SEM. *P*-values for differences between groups by ANOVA.

4.3.2.3.4. Glucagon-to-Glucose Ratio

The glucagon-to-glucose ratio at 60, 90 and 120 minutes was higher in females than males ($P = 0.03$, $P = 0.007$ and $P = 0.007$ respectively). On post hoc analysis, Maltodextrin-females had a higher ratio than the Term-males at 60 minutes ($P = 0.03$) and than Term ($P = 0.03$) and BCAA ($P = 0.02$) males at 90 minutes. At 60 minutes, the ratio was higher in the Maltodextrin group than in the BCAA group ($P = 0.04$). The glucagon-to-glucose ratio was not different among groups at 90 and 120 minutes (Table 4-2).

#### 4.3.2.4. Arginine Challenge (135-165 Minutes)

##### 4.3.2.4.1. Plasma Insulin Concentration

After arginine infusion, there was an increase in insulin secretion in all groups, followed by a decline in plasma insulin concentration. There was no difference between sexes or among groups in mean, AUC, or peak plasma insulin concentration during the arginine challenge (Table 4-1 and Figure 4-8). No association was found between plasma insulin concentration AUC and birthweight, early growth velocity (day 1-15), bodyweight at the time of the HGC, or pancreas weight (data not shown).

##### 4.3.2.4.2. Plasma Glucagon Concentration

After arginine infusion, plasma glucagon concentration at 140 minutes decreased in all groups. However, there was an increase in the plasma glucagon concentration at 155 minutes in all groups (Figure 4-2 and Figure 4-8). During the arginine challenge, mean plasma glucagon concentration differed among groups ($P = 0.02$), with the Maltodextrin group having higher plasma glucagon concentration than the Term group ($P = 0.04$). Plasma glucagon concentration was higher in females than in males ($P = 0.03$). The Maltodextrin-female group had a higher plasma glucagon concentration than the Term-male group ($P = 0.01$) (Table 4-2). No association was found between plasma glucagon concentration at either timepoints and birthweight, early growth velocity (day 1-15), bodyweight at the time of the HGC, or pancreas weight (data not shown).

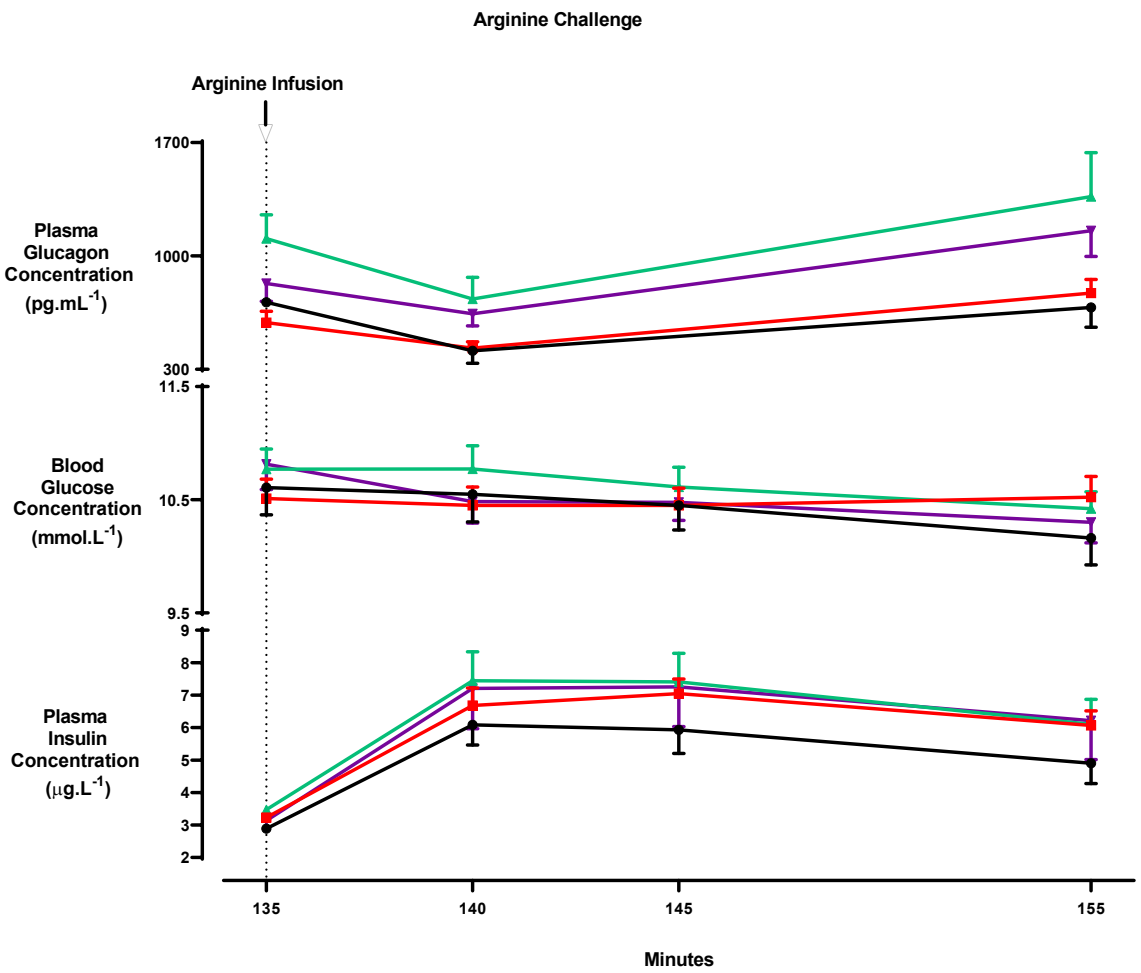



**Figure 4-8. Plasma insulin, glucagon, and blood glucose concentrations during arginine challenge.**
Data are mean with ± SEM. Timepoint 135 represents the mean from 60 to 120 minutes.

4.3.2.4.3. Glucagon-to-Insulin Ratio

The glucagon-to-insulin ratio was similar among groups (Table 4-2). Females had higher glucagon-to-insulin ratios than males at 140 and 155 minutes ($P < 0.05$) (Figure 4-9).

4.3.2.4.4. Glucagon-to-Glucose Ratio

The Glucagon-to-Glucose ratio at 140 and 155 minutes was higher in the Maltodextrin group than in the Term group ($P = 0.02$ for both time points). The ratio was greater in females than males at both timepoints ( $P = 0.03$ and $P = 0.04$, respectively). Maltodextrin-females had a higher ratio at both time points than Term-males ($P = 0.01$ and $P = 0.02$, respectively) (Table 4-2).

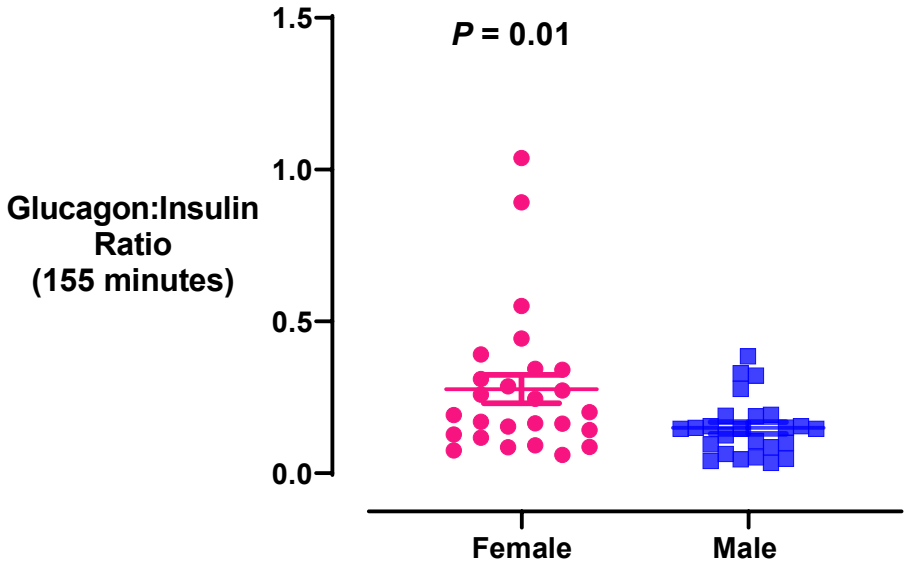



**Figure 4-9. The difference between males and females in glucagon-to-insulin ratio at 155 minutes post glucose infusion.**
Scatter plot with mean ± SEM. *P*-values for differences between groups by ANOVA.

### 4.3.3. Insulin Sensitivity (IS)

IS was not different between sexes or among groups (Table 4-1). There was no association between IS and birthweight, early growth velocity (day 1-15), bodyweight at the time of the HGC, pancreas weight, or mean plasma glucagon concentration at baseline, FIR, SIR, and AC (data not shown).

### 4.3.4. Results Summary

Baseline blood glucose concentration was highest in males in the Maltodextrin group. Baseline plasma insulin concentration was higher in males than females and similar among groups. Plasma insulin concentration five minutes post glucose infusion and during FIR were affected only by sex, with males having higher concentrations. The ratio of insulin-to-glucose five minutes post glucose infusion was higher in males, especially BCAA, compared to Term-females, BCAA-females, and Maltodextrin-females. No effect of sex or treatment was found on plasma insulin secretion during SIR and AC or insulin sensitivity. Baseline plasma glucagon concentration was similar among groups at baseline. After glucose infusion, there was an increase in glucagon concentration in all groups. Females had higher plasma glucagon

concentration and glucagon-to-insulin ratio throughout the clamp, particularly in the Maltodextrin group. All groups had a decrease in glucagon concentration post arginine infusion.

## 4.4. Discussion

In this study, BCAA supplements were given to preterm born lambs based on the hypothesis that this would enhance pancreatic islet development to mitigate the adverse effects of preterm birth on glucose metabolism in later life.

There is an association between preterm birth and pancreatic β-cell dysfunction, low insulin secretion, and insulin resistance, causing impaired glucose homeostasis [8]. Preterm birth reduced β-cell mass in adult sheep and was associated with reduced insulin secretion in juvenile sheep and reduced insulin mRNA expression in adult sheep [13]. Adults born preterm have low insulin sensitivity [100,101] and are more susceptible to becoming diabetic, presumed due to poor fetal β-cell growth and short gestational age [6].

HCG was used to observe insulin and glucagon responses to prolonged hyperglycaemia. In all groups, a biphasic insulin response was observed. The first response (FIR) during the first 20 minutes represents the release of stored insulin. The second phase, between 20 and 120 minutes (SIR), during which the plasma insulin concentration was maintained in response to ongoing hyperglycaemia, represents the release of newly synthesised insulin. The pattern of insulin response is critical because it has been suggested that the absence of a biphasic response in insulin secretion is an indication of β-cell impairment [214,274,277,278], and that healthy β-cells show a biphasic response while unhealthy β-cells show impaired FIR [274]. A reduction in FIR is found in individuals with impaired glucose tolerance or early stages of type-2 diabetes [274,278]. The biphasic response in our sheep (Term/Preterm and supplemented/Water) suggests that β-cells in all groups are healthy at this age.

### 4.4.1. Baseline Concentrations

BCAA supplementation during the postnatal period after preterm birth was not associated with increased or decreased fasting blood glucose concentration in adult sheep. However, Maltodextrin supplementation was associated with increased fasting blood glucose concentration, but only in males, raising the possibility of a mild disturbance of glucose metabolism in this group. The Preterm Water (control) group had similar fasting blood glucose

concentration to the Term group, suggesting no effect of preterm birth *per se.* Contrary to our findings, others have reported that blood glucose concentration at baseline was higher in adult preterm than term sheep, but only in females [108] and higher in adult female sheep that received neonatal nutritional supplements (milk fortifier) [209] when an intravenous glucose tolerance test (IVGTT) was performed. However, consistent with our work, Bansal *et al.* reported that both preterm and term sheep had similar baseline plasma glucose concentrations during IVGTT and HGC at 12 months of age [13]. In a clinical study on preterm-born adults, fasting plasma glucose concentration during HGC was similar in adults born preterm or term [100].

Insulin plays a vital role in glucose homeostasis. It inhibits glucose production and stimulates glucose uptake. Neither preterm birth nor BCAA or Maltodextrin supplementation affected fasting plasma insulin and glucagon concentration in this study. However, there were sex-specific findings, with males having higher fasting insulin concentrations than females and females having greater fasting glucagon-to-insulin ratio than males. Some studies report interactions between nutritional supplementation, sex and fasting insulin concentrations but findings are inconsistent. Fasting insulin concentration in adult male preterm-born sheep supplemented with milk fortifier containing protein, carbohydrate and fat was almost double that of preterm controls, whereas supplemented term sheep had decreased fasting insulin compared with term controls [209]. Juvenile term-born piglets fed a high protein diet had increased fasting insulin concentration compared to controls, but the data were not analysed by sex [279]. Glucagon measurements are rarely reported in animal studies of glucose metabolism. One study investigating glucagon responses to ante- and postnatal nutritional interventions found greater glucagon concentrations in females in the fed but not fasted state [66]. The underlying mechanisms for these sex differences remain unclear.

### 4.4.2. First Insulin Response & Steady State Insulin Response

Many studies associate preterm birth with impaired glucose/insulin homeostasis [5,100,199]. However, our findings suggest that preterm birth did not affect insulin synthesis or secretion, consistent with other studies in sheep, which found that preterm birth did not impair insulin secretion during IVGTT and hyperinsulinaemic euglycaemic clamp in adulthood [108,209].

Our findings also showed that BCAA supplementation did not affect insulin response during FIR and SIR; however, BCAA males had a higher insulin-to-glucose ratio at 5 minutes post glucose infusion. This may suggest that BCAA males had more sensitive β-cells by which they

displayed an exaggerated response to the sudden increase in blood glucose concentration. Another possibility is that BCAA affected β-cell secretory function, increasing secretory capacity in the BCAA males by synthesising more insulin. Measuring proinsulin and C-peptide would likely give further information about the observed increase, but may not fully explain the response in insulin secretion. In piglets, a high protein diet during the suckling period did not affect insulin secretion [279], while in adult preterm-born sheep that received fortified formula, males had higher insulin response during the second phase of the IVGTT compared with preterm controls [209]. Our findings suggest that by young adulthood, there is no effect of preterm birth on insulin secretion during HGC; however, the supplementation may affect the initial insulin response to a glucose load.

Hyperglycaemia is known to inhibit glucagon secretion by α-cells. However, glucagon is released in a sufficient amount under hyperglycaemia (meal/glucose infusion) to potentiate insulin secretion as a local amplifier [267, 280], meaning that glucagon balances insulin response to match the strength of glucose stimulation [267]. In addition, a sharp increase in insulin secretion will locally affect glucagon secretion, increasing glucagon level as an anticipatory control mechanism to ensure that the islet is prepared for upcoming disturbances (hypoglycaemia) [264]. In our study, post glucose infusion, there was an initial increase in plasma glucagon concentration observed in all groups at 10 minutes, then a decrease by 20 minutes post glucose infusion. Plasma glucagon concentration was raised during HGC when blood glucose concentration was raised. A study in adult sheep found that plasma insulin and glucagon concentrations increased simultaneously 2-4 hours after oral feeding [281]. However, plasma insulin concentration increased after glucose infusion while glucagon decreased [281,282]. Mixed-nutrient meals stimulate both glucagon and insulin secretion. In mice, oral gavage of a mixed-nutrient solution (Ensure) significantly increased both insulin and glucagon [265]. However, an oral glucose gavage increased plasma insulin only, but not glucagon [265]. In humans, mixed-nutrient meals increased glucagon levels in both lean and obese subjects [283], but glucagon levels were suppressed by the oral glucose [283]. It seems that the amino acid content in the mixed meal can stimulate both insulin and glucagon, changing the glucagon/insulin ratio to favour glucose removal [284]. However, glucagon secretion is regulated not only by amino acids or the autonomic nervous system in case of hypoglycaemia, but also by glucose at the islet level [271]. Our finding suggests that glucagon may have physiologic actions induced by intravenous glucose infusion during hyperglycaemia. A glycaemic increase response is different if the same bolus of glucose is given orally or intravenously (due to gastric

emptying and intestinal absorption of glucose). In addition, isocaloric amounts of glucose combined with other macronutrients (mixed-nutrient meal) enhances insulin and glucagon secretion [266]. It is somewhat unknown which physiological conditions stimulate α- to β-cell communication, thereby stimulating insulin/glucagon secretion during hyperglycaemia.

Usually, the physiology of glucagon action is described and studied only during the fasting or hypoglycaemic states. Understanding the mechanisms by which glucagon-stimulated insulin secretion occurs during hyperglycaemia, is an essential next step toward further understanding the complexity of glucagon's actions in metabolism. Furthermore, the effect of preterm birth on α- to β-cell communication has not been fully explored.

During the HGC, we could not find any evidence to suggest that preterm birth or BCAA supplementation were related to changes in glucagon secretion, glucagon-to-insulin ratio, or glucagon-to-glucose ratio. However, Maltodextrin supplementation affected all these factors but only in females. This was an unexpected finding. It has been suggested that over-secretion of glucagon during meals might be due to α-cell "hypoglycaemic blindness," by which α-cells continue to secrete glucagon in the face of the rising substrate rather than being suppressed [272]. This may explain the increase in glucagon secretion in the Maltodextrin group, possibly due to altered paracrine communication between α- and β-cells, so that rather than secreting only enough glucagon to potentiate insulin secretion during hyperglycaemia, gluconeogenesis is also induced.

### 4.4.3. Arginine Challenge

Arginine is an amino acid that stimulates both insulin and glucagon secretion [186,285,286]. *In vitro*, arginine has been shown to increase insulin secretion in β-cell lines from humans [287] and rodents [49]. In sheep, intravenous arginine infusion stimulated insulin [54,285] and glucagon [285] secretion. During HGC, insulin response to arginine infusion is greater than glucagon [263], and glucagon secretion does not precede insulin release [53].

In our study, a maximum release of insulin was observed post arginine infusion followed by a fall in insulin concentration similarly in all groups. Neither preterm birth nor supplementation affected insulin response to arginine challenge. With the increase in arginine and insulin, one would expect suppression of glucagon, and this was observed. This decrease

is presumed to be due to communication between the cells within the islet, signalling β-cells to secrete more insulin and inhibiting α-cells from secreting glucagon.

### 4.4.4. BCAA Supplementation Did Not Affect Insulin Sensitivity

There was no impairment of IS after preterm birth nor BCAA supplementation in preterm-born adult sheep. A study of adults born preterm [288] reported that preterm birth was not associated with reduced IS in young adulthood. Other studies in preterm-born adult sheep reported that IS did not differ significantly between groups (term/preterm and supplemented/water) or sexes [13,108,209].

On the contrary, other studies showed reduced IS in preterm-born children [3] and adults [4,6,100,101]. The reduction in IS might be influenced by gestation length, or related to other factors associated with preterm birth, rather than preterm birth itself, such as neonatal complications or nutrition during and beyond the neonatal period [135,139]. Our preterm lambs received maternal milk from birth onwards simultaneously with the supplementation, unlike preterm infants, for whom nutrition is often based on fortified human milk or non-human milk. Term infants fed on formula have higher rates of type 2 diabetes and higher fasting insulin concentrations in adulthood than those fed on breastmilk [140]. Obesity in adults can be another factor associated with preterm birth since adult weight, rather than weight at birth, might affect IS [288].

Experimental studies that used early nutritional supplements (similar to human milk fortifier) found no effect on IS in adult term or preterm sheep [209]. Leucine supplements were found to increase IS [289] and improve insulin signalling [290] in mice fed a high protein diet. However, it was also reported that enhanced nutrition with an enriched preterm formula might increase markers of insulin resistance in childhood [16]. This is discussed further in Chapter 6.

## 4.5. Strength and Limitations

All the interventions, including the HGC (from birth to adulthood), were conducted in a research facility located at a farm where the sheep have matured in a relatively normal farm environment. During the HGC, sheep were isolated and restrained; however, stress was minimised since the sheep were acclimatised to indoor conditions and underwent regular blood collection.

Labour was induced using glucocorticoids, which can also affect organ maturation. Antenatal steroids are routinely given to women at risk of preterm labour to hasten lung maturation, so most babies born preterm have been exposed to glucocorticoids shortly before birth. However, in an experimental setting, the question always arises whether any alterations in organ development are due to preterm birth *per se* or to glucocorticoid exposure. A previous study showed impaired insulin secretion in adult sheep when preterm birth was induced by dexamethasone [13]. However, De Matteo *et al.* reported that betamethasone did not impair insulin secretion in adult sheep born preterm [108]. To separate the effects of preterm birth from those of dexamethasone, we used dexamethasone to induce labour in both Term and Preterm groups. Our findings showed that preterm birth did not alter insulin secretion during HGC when the glucocorticoid-exposed Preterm Water (control) group was compared with the glucocorticoid-exposed Term group.

The inclusion of a term, non-corticosteroid exposed group (spontaneous birth), would have been advantageous to isolate the effect of glucocorticoid exposure on insulin secretion. Performing serial HGC tests at different ages, including older ages than the sheep in the current study, would provide information about the longitudinal changes in insulin and glucagon secretion from early postnatal life until late adulthood.

Our preterm lambs were born at 137 days of gestation (late-preterm), and supportive care was focused on reducing thermal stress and aiding intake of maternal milk without other interventions, unlike preterm infants, who may require other more invasive support or alternative nutrition.

Measuring C-peptide was not included in the study aims. However, clinically, C-peptide concentration is measured instead of insulin concentration. The liver metabolises insulin but does not metabolise C- peptide, meaning that C-peptide stays longer in the blood, with a half-life of 30-35 minutes, while that of insulin is 3-6 minutes. Therefore, including C-peptide measurements in our study may have yielded additional information about insulin response.

## 4.6. Conclusion

The pancreatic endocrine cells in sheep from all the groups were healthy, and their response to an acute change in blood glucose concentration (via intravenous glucose infusion) was rapid and adequate to cope with the glucose load. During the HGC, sheep in all groups responded

appropriately to the glucose load and maintained insulin secretion throughout the HCG with no signs of β-cell function impairment.

Preterm birth *per se* did not influence insulin and glucagon secretion; however, there were interactions between supplementation and sex in the preterm groups, with BCAA males having higher insulin-to-glucose ratios and Maltodextrin females having higher plasma glucagon concentrations throughout the HGC.

The influence of preterm birth and early nutrition on endocrine pancreatic function is complex and likely to involve altered communication between islet cells as well as hormonal signalling pathways.

# Chapter 5. Effect of Branched Chain Amino Acid Supplementation on Endocrine Pancreas Morphology in Preterm-Born Lambs at One-Year of Age

Although the islets of Langerhans are small regions in the pancreas, occupying only 1–2% of the pancreatic volume, they produce hormones that play a critical role in blood glucose homeostasis [291]. Islets mainly contain β-(insulin-producing), α-(glucagon-producing), and δ-(somatostatin-producing) cells that exist within a paracrine environment [76].

There are interspecies differences in the structure of islets of Langerhans, for example, the location of the cell type within the islet. The islet architecture facilitates paracrine-mediated interactions among different cell types and the hormones they secrete [292]. It has been suggested that a decrease in the interaction among the cells, *i.e.,* reduction in the paracrine interaction, can cause a decrease in insulin secretion and glucagon suppression [76].

Islet size ranges from a small cluster of few cells to a large cluster of thousands of cells [291]. There is a strong suggestion that a relationship exists between islet size and islet function, with small islets producing more insulin than large islets. In addition, small islets have better survival than large islets both *in vivo* and *in vitro* [293]. It has been suggested that islet number rather than islet size is the major determinant of cell mass in humans [294].

Cell mass is calculated from the cell size; however, the mass can be increased due to proliferation and hypertrophy or decreased by apoptosis and atrophy [97,46]. Under normal conditions during adulthood, cells have a steady low rate of proliferation and apoptosis [97]. Maintaining cell mass during adulthood is vital to maintain blood glucose homeostasis and prevent diabetes [97]. Cell mass, especially β-cell mass, is able to expand during adulthood in response to increased body weight and insulin resistance [97,46], thereby displaying an ability to adapt to increased insulin demand [46].

An imbalance between the rate of proliferation and apoptosis can lead to an inability to maintain islet cell mass and cellular function. In rodent models of type-2 diabetes and insulin resistance, an increase in islet mass was found due to an increase in β-cell proliferation [23]. However, autopsy studies of the human pancreas have consistently found that individuals with

longstanding type-2 diabetes have reduced β-cell mass, hypothesized to be due to decreased proliferation-to-apoptosis ratio [295,296].

An altered intrauterine or extrauterine environment, including preterm birth, can disturb the development of β-cell number [98] and β-cell mass [8]. There is an association between preterm birth and type-2 diabetes, likely rising from a direct effect of preterm birth on endocrine pancreatic development [11].

We hypothesized that postnatal BCAA supplementation to preterm-born lambs would enhance endocrine pancreatic development reflected in normalized islet cell count, size, and mass during adulthood.

## 5.1. Methods

Ten days after the clamp study, sheep were euthanised by intravenous pentobarbitone overdose (Pentobarb 300, Provet, Auckland, NZ) injection. Organs were identified, removed, weighed, and preserved. The pancreas was sectioned along the head-tail axis and placed in formalin for immunohistochemistry analysis. Tissues were processed for paraffin embedding, microtomy, and immunofluorescent multiple labelling (methods section 2.4.10).

### 5.1.1. Immunohistochemistry

Five μm sections were prepared from paraffin-embedded tissue, placed on glass slides, and left to dry at 37°C overnight. The wax was melted by incubating the slides at 60ºC for one hour. Slides were then deparaffinized in xylene, rehydrated in serial concentrations of alcohol (100, 90, 70, and 50%), and finally washed with 1 × phosphate buffer saline (PBS). Antigen retrieval was carried out as described in method section 2.4.10.5. Slides were blocked with 10% goat serum and incubated for one hour at room temperature. The immunohistochemical staining was performed over three days as described in methods section 2.4.10.5.

#### 5.1.1.1. Triple Staining for Insulin, Glucagon, and Somatostatin Antibodies

Due to the cross-reactivity, antibodies were applied at different incubation times. The following primary antibodies were used for each slide: guinea pig anti-insulin (1:50), mouse anti-glucagon (1:200), and rabbit anti-somatostatin (1:300). Secondary antibodies were goat anti-guinea pig Alexa Flour 647 (1:400), goat anti-mouse Alexa flour 555 (1:300), and donkey anti-rabbit IRDye 800CW (1:400) (methods section 2.4.10.5.3.1).

#### 5.1.1.2. Quadruple Staining for Insulin, Glucagon, Ki67, and Active-Caspase-3 Antibodies

The following primary antibodies were used for each slide: mouse anti-insulin conjugated with Alexa Flour 647 (1:250), mouse anti-glucagon (1:200), mouse anti-Ki67 (1:100), and rabbit anti-caspase-3 (1:200). Secondary antibodies were goat anti-mouse Alexa Flour 555 (1:500), goat anti-rabbit Alexa Flour 488 (Tyramide SupperBoost Kit), and goat anti-mouse IRDye 800CW (1:500) (methods section 2.4.10.5.3.2).

### 5.1.2. Quantification

The antibodies were visualised using a VSlide slide scanner system1.1.128. Five single band filters were used (DAPI, FITC, TRITC, Cy5, and Cy7) to acquire the images (methods section 2.4.10.6). Insulin, glucagon, somatostatin, Ki67, and active-caspase-3 positive areas were digitally quantified using a macro written in ImageJ Macro programming language and performed using FIJI (Fiji is just ImageJ version 1.53f51, Maryland, USA) (Appendix I).

To measure the nuclear and whole-cell diameter, insulin-, glucagon-, somatostatin-, Ki67-, and active-caspase-3-stained sections were counterstained with DAPI. For the determination of proliferation, the total number of Ki67 positive cells per section and the number of β-cells or α-cells co-staining with Ki67 were quantified. For the determination of apoptosis, the total number of active-caspase-3 positive cells and the number of β-cells or α-cells co-staining with active-caspase-3 were quantified.

To measure islet size and number, positive insulin area, positive glucagon area, and positive somatostatin area were combined. Islet density was quantified by measuring the number of islets contained within the pancreatic area (islet/$mm^2$).

### 5.1.3. Endocrine Cell Mass Calculation

β-cells, α-cells, and δ-cells were marked and calculated as areas positive with insulin, glucagon, and somatostatin staining, respectively. The β-cell area is an area of both positive insulin area and β-cell number. β-Cell mass was calculated by the formula [13]:

$$\beta\text{-cell mass (mg)} = \frac{\beta\text{-cell area per field of view } (\mu m^2) \times \text{pancreas weight (mg)}}{\text{Tissue area per field of view } (\mu m^2)}$$

The α-cell mass and δ-cell mass were measured from a similar equation, using glucagon positive area and α-cell, somatostatin positive area, and δ-cell, respectively. The total islet area was calculated as a sum of the β-cell, α-cell, and δ-cell area per field of view.

### 5.1.4. Statistical Analysis

Statistical analysis was performed using JMP (version 15.2.1, SAS Institute Inc., North Carolina, USA) and GraphPad Prism (version 8.2.1, GraphPad Software, Inc., California, USA). Data were analysed by two-way analysis of variance (ANOVA) to determine the effects of sex and treatment on each parameter by post hoc analysis using either the student t-test for paired analysis or Tukey's test for multiple comparisons. Data are presented for each sex separately if there were significant differences between the sexes and with both sexes combined when no difference was found.

Pearson's correlation was used to examine bivariate relationships, and the correlation coefficient (r) was calculated. $P \leq 0.05$ was considered statistically significant. Results are reported as mean ± SEM.

## 5.2. Results

### 5.2.1. Pancreas Morphology and Islet Structure

When pancreas sections were triple stained for insulin, glucagon, and somatostatin, the endocrine sections of the pancreas represented by the islets of Langerhans were distributed within the exocrine section of the pancreas. The islets were highly variable in appearance, size, structure, and composition. They appeared as round, oval, or irregular shapes of different sizes (large, medium, and small). Islet structures were asymmetric. In some islets, insulin-,

glucagon- and somatostatin-staining cells were intermingled (Figure 5-1), while in others, insulin-positive cells were situated in the core and glucagon- and somatostatin-positive cells in the periphery (Figure 5-1). There was also considerable variation in the composition of the islet. The relative abundance of insulin-, glucagon- and somatostatin-staining cells within individual islets varied, either β-cells, α-cells, or δ-cells could dominate (Figure 5-1).

The endocrine cells of the islets representing β-cells stained yellow appeared large with prominent nuclei and were located both at the periphery and core of the islets. Cells representing α-cells stained pink and were located mainly at the periphery of the islets, as were the cells staining light blue representing δ-cells (Figure 5-1).

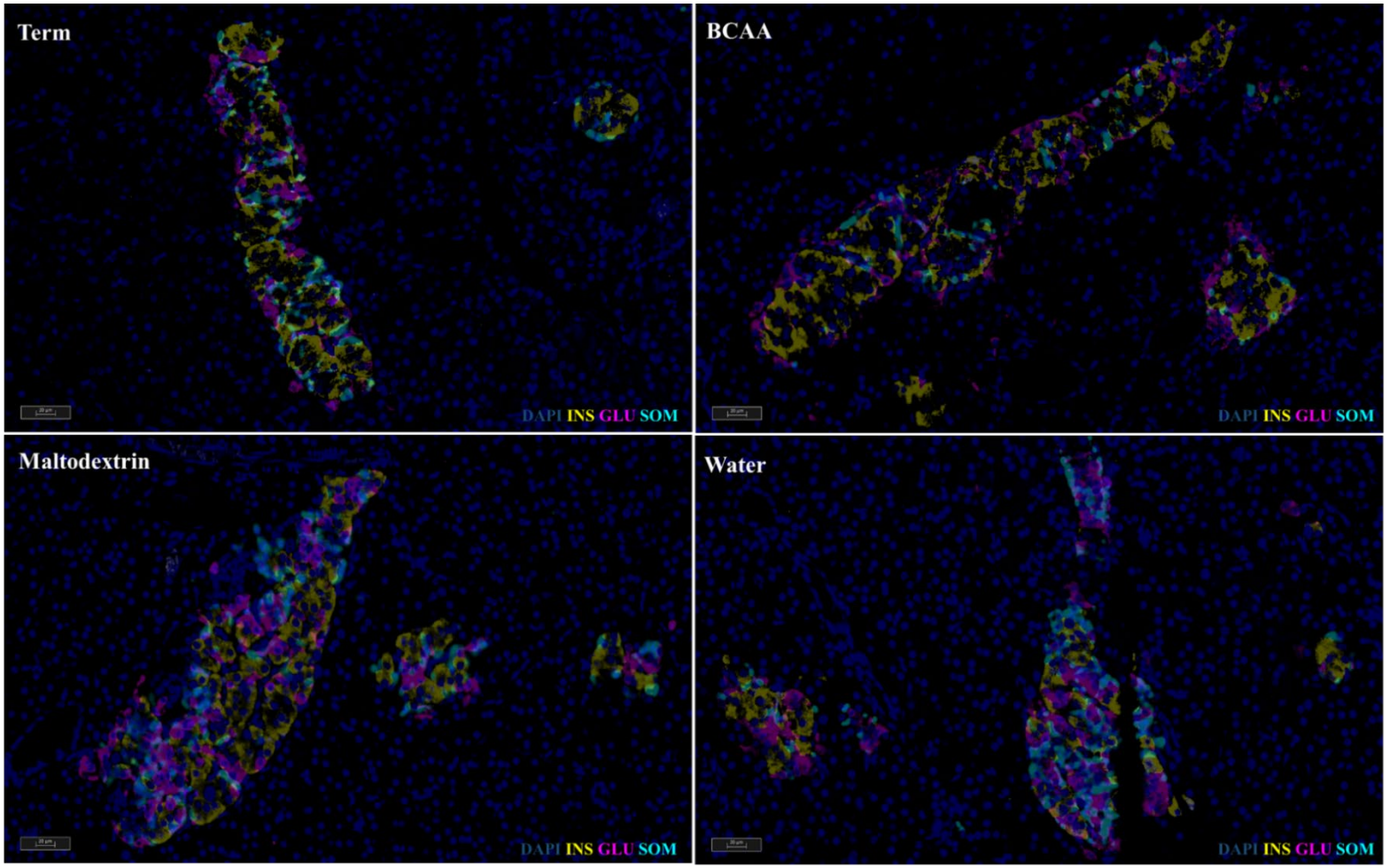



**Figure 5-1. Representative photomicrographs of the islet of Langerhans observed in 12-month-old sheep pancreas from the four experimental groups.**

Insulin-positive β-cells (yellow), glucagon-positive α-cells (pink), somatostatin-positive δ-cells (light blue), and nuclei (dark blue). Islet size ranges from large to small, with β-, α-, and δ-cells intermingled. Scale bar 20 µm. https://figshare.com/s/6b3a5a7ba694e7228aa8

## 5.2.2. Cell Count and Area

β-cell, α-cell, δ-cell, and islet counts were similar among groups and sexes (Table 5-1). Within each group, the β-cell count was higher than the α-cell and δ-cell count (Figure 5-2). Insulin-, glucagon-, and somatostatin-positive areas were similar among groups and between sexes (Table 5-1). The insulin-positive area was the highest in each group compared to glucagon- and somatostatin-positive areas (Figure 5-3). Islet area was similar among groups and between sexes (Table 5-1 and Figure 5-4).

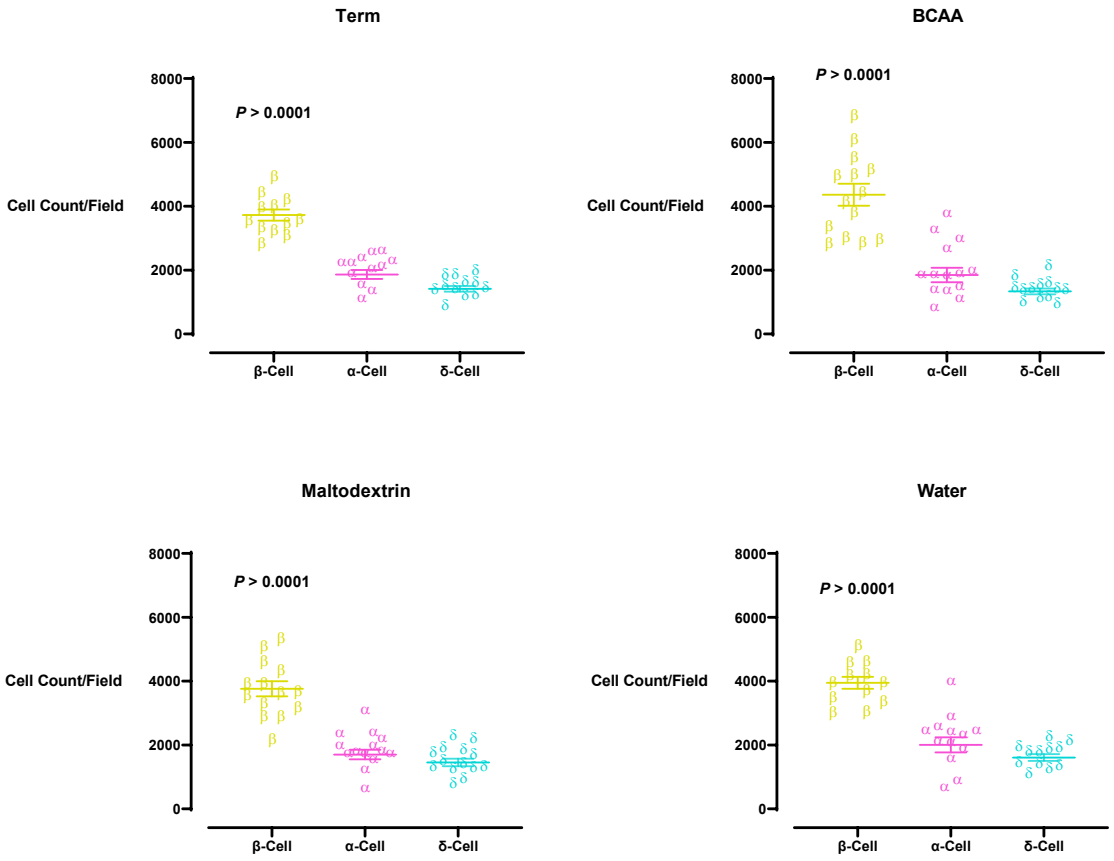



**Figure 5-2. β-cell, α-cell, and δ-cell count of the islet of Langerhans observed in 12-month-old sheep pancreas from the four experimental groups (Term, BCAA, Maltodextrin, and Water).**

Data are mean ± SEM. *P*-values for differences between groups by ANOVA.

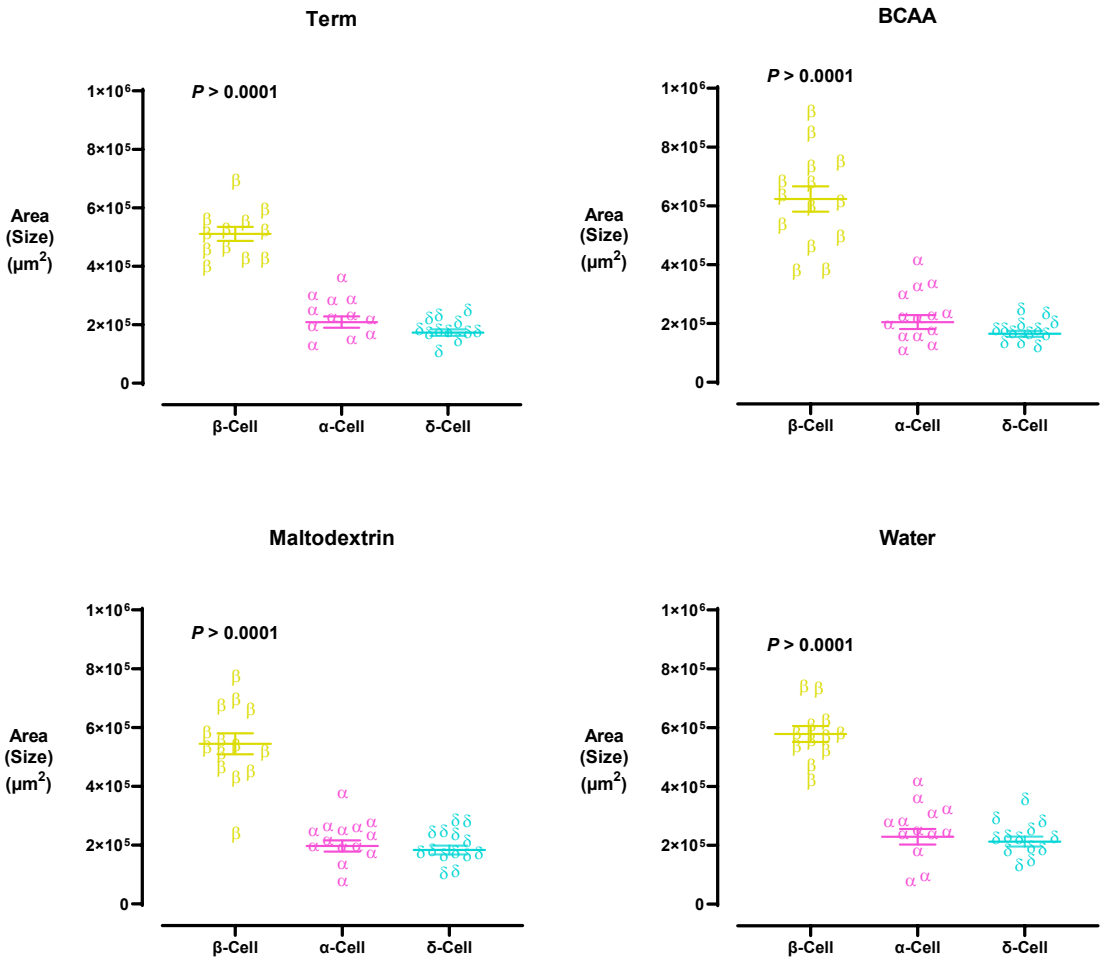



**Figure 5-3. Insulin-, glucagon, and somatostatin-positive areas of the islet of Langerhans observed in 12-month-old sheep pancreas from the four experimental groups (Term, BCAA, Maltodextrin, and Water).**

Data are mean ± SEM. *P*-values for differences between groups by ANOVA.

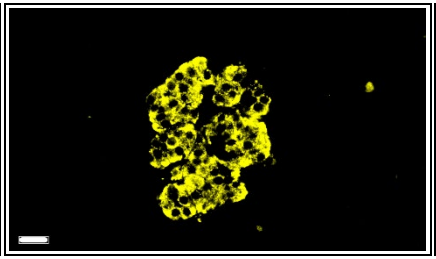
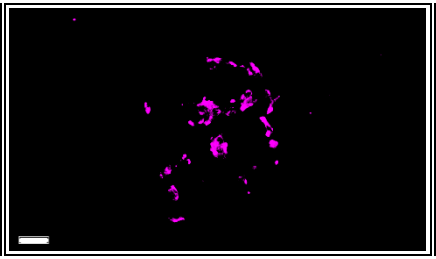
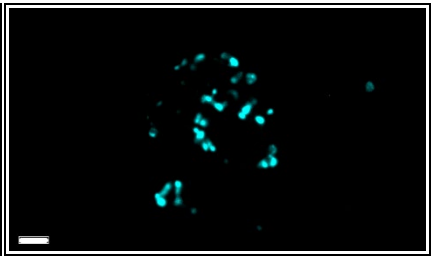
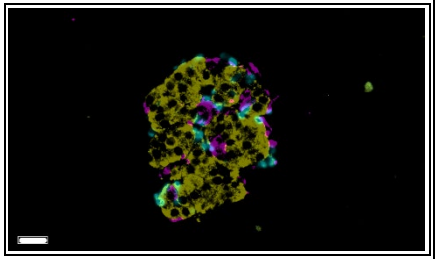
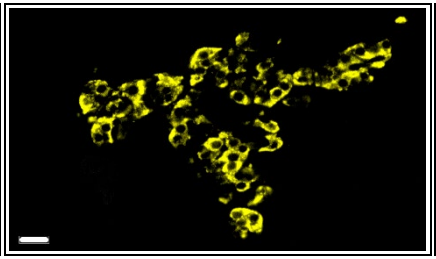
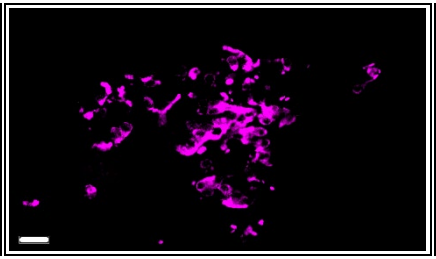
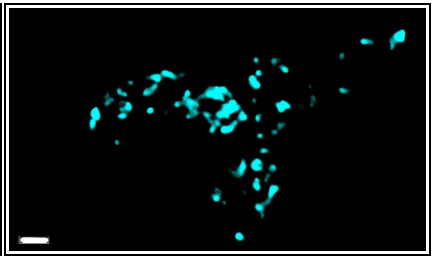
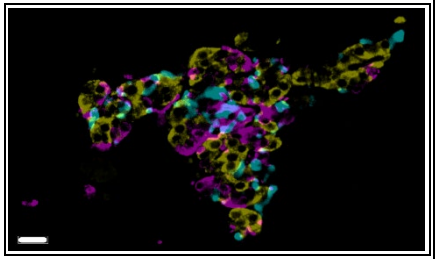
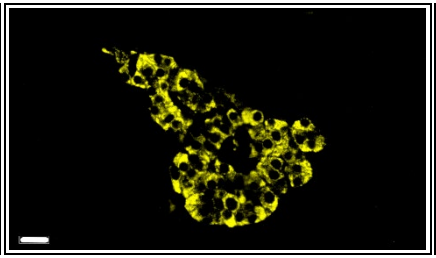
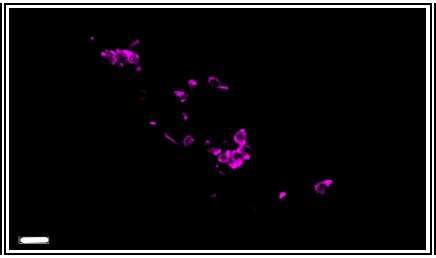
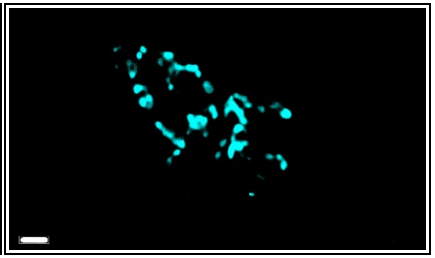
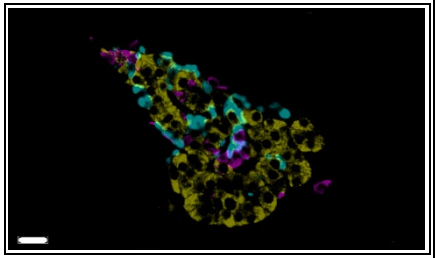
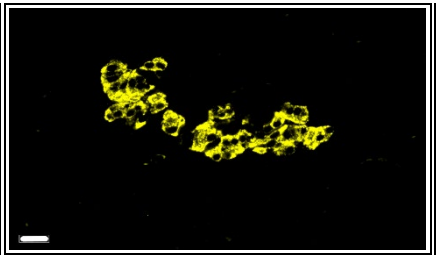
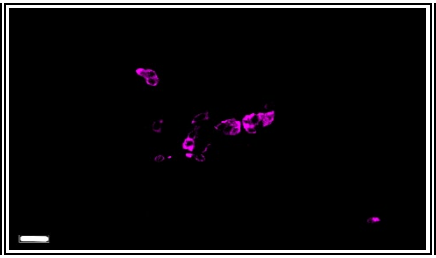
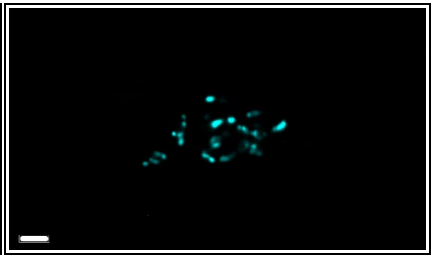
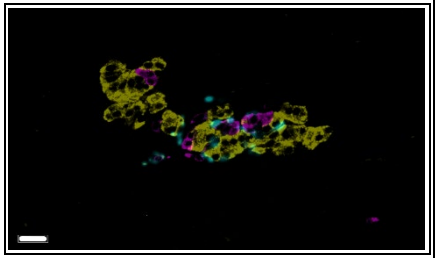
| | Insulin (β-cell) | Glucagon (α-cell) | Somatostatin (δ-cells) | Islet |
|---|---|---|---|---|
| Term |  |  |  |  |
| BCAA |  |  |  |  |
| Maltodextrin |  |  |  |  |
| Water |  |  |  |  |

**Figure 5-4. Representative photomicrographs of triple staining of insulin, glucagon, and somatostatin.**

Scale bar 20 µm. Term https://figshare.com/s/5714d7fb02a89bf61764 BCAA https://figshare.com/s/cf999a498c7c395038f5, Maltodextrin https://figshare.com/s/4130126ff2a8791c8b3f, and Water https://figshare.com/s/a741d3923d35c383f002.

**Table 5-1. Pancreas morphology.** β-cell, α-cell, δ-cell, and islet count, positive area, and mass from 12-month-old sheep.

| | Sheep Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=6) | (F=7) | (M=5) | Sex | Treatment | Interaction |
| **Cell Count/Field ($10^2$)** | | | | | | | | | | | |
| β-cell | 44±4 | 43±5 | 37±2 | 45±9 | 45±6 | 39±2 | 37±1 | 36±3 | 0.98 | 0.65 | 0.55 |
| α-cell | 17±2 | 19±3 | 15±2 | 21±2 | 18±4 | 21±1 | 19±1 | 16±2 | 0.45 | 0.91 | 0.57 |
| δ-cell | 14±1 | 15±3 | 13±1 | 18±3 | 16±2 | 18±1 | 14±0.8 | 14±1 | 0.22 | 0.52 | 0.72 |
| Islet | 75±7 | 77±11 | 65±4 | 84±14 | 81±13 | 79±3 | 71±2 | 68±6 | 0.23 | 0.56 | 0.08 |
| **$^+$Area ($10^4$) ($\mu m^2$)** | | | | | | | | | | | |
| Insulin | 62±4 | 63±8 | 54±3 | 68±15 | 63±8 | 59±3 | 49±2 | 53±5 | 0.49 | 0.46 | 0.66 |
| Glucagon | 20±3 | 21±4 | 18±3 | 24±3 | 22±5 | 24±2 | 22±3 | 19±3 | 0.46 | 0.87 | 0.54 |
| Somatostatin | 17±1 | 19±4 | 17±2 | 24±4 | 20±3 | 23±2 | 17±1 | 18±2 | 0.15 | 0.46 | 0.75 |
| Islet | 99±6 | 103±14 | 90±5 | 116±22 | 105±14 | 106±4 | 88±4 | 90±9 | 0.46 | 0.53 | 0.68 |
| **Mass (g)** | | | | | | | | | | | |
| β-cell | 1.2±0.1 | 1.4±0.1 | 1.0±0.1 | 1.4±0.3 | 1.2±0.2 | 1.4±0.1 | 1.1±0.1 | 1.4±0.1 | **0.008** | 0.87 | 0.93 |
| α-cell | 0.4±0.1 | 0.5±0.1 | 0.4±0.1 | 0.5±0.1 | 0.4±0.1 | 0.6±0.0 | 0.5±0.0 | 0.5±0.1 | **0.01** | 0.77 | 0.69 |
| δ-cell | 0.3±0.0 | 0.4±0.1 | 0.3±0.0 | 0.5±0.1 | 0.4±0.1 | 0.5±0.0 | 0.4±0.0 | 0.5±0.1 | **0.003** | 0.77 | 0.86 |
| Islet | 1.4±0.1 | 1.7±0.2 | 1.3±0.1 | 1.7±0.3 | 1.4±0.2 | 1.8±0.1 | 1.4±0.1 | 1.6±0.2 | **0.004** | 0.91 | 0.91 |
| **Mass (%)** | | | | | | | | | | | |
| β-cell | 0.014±0.001 | 0.015±0.002 | 0.013±0.001 | 0.015±0.003 | 0.014±0.002 | 0.014±0.001 | 0.012±0.000 | 0.013±0.002 | 0.78 | 0.35 | 0.96 |
| α-cell | 0.004±0.001 | 0.005±0.001 | 0.004±0.001 | 0.005±0.001 | 0.005±0.001 | 0.006±0.000 | 0.006±0.001 | 0.005±0.001 | 0.61 | 0.79 | 0.64 |
| δ-cell | 0.004±0.000 | 0.004±0.001 | 0.004±0.000 | 0.005±0.001 | 0.004±0.001 | 0.005±0.000 | 0.004±0.000 | 0.004±0.001 | 0.23 | 0.64 | 0.91 |
| Islet | 0.017±0.001 | 0.018±0.002 | 0.015±0.001 | 0.018±0.003 | 0.017±0.002 | 0.017±0.001 | 0.016±0.000 | 0.016±0.002 | 0.79 | 0.86 | 0.95 |

Bold font indicates significance in ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. $^+$Area: positively stained area; Mass (g): mass in absolute; Mass (%): mass as a percentage of body weight.

## 5.2.3. Cell Mass

β-cell, α-cell, δ-cell, and islet absolute masses were similar among groups but different between sexes ($P = 0.008$, $P = 0.01$, $P = 0.003$, and $P = 0.004$, respectively) (Table 5-1). The difference between the sexes was not seen when the mass percentage of body weight was calculated (Table 5-1). There was a positive correlation between cell mass and weight at birth in the Water preterm group only (Figure 5-5).

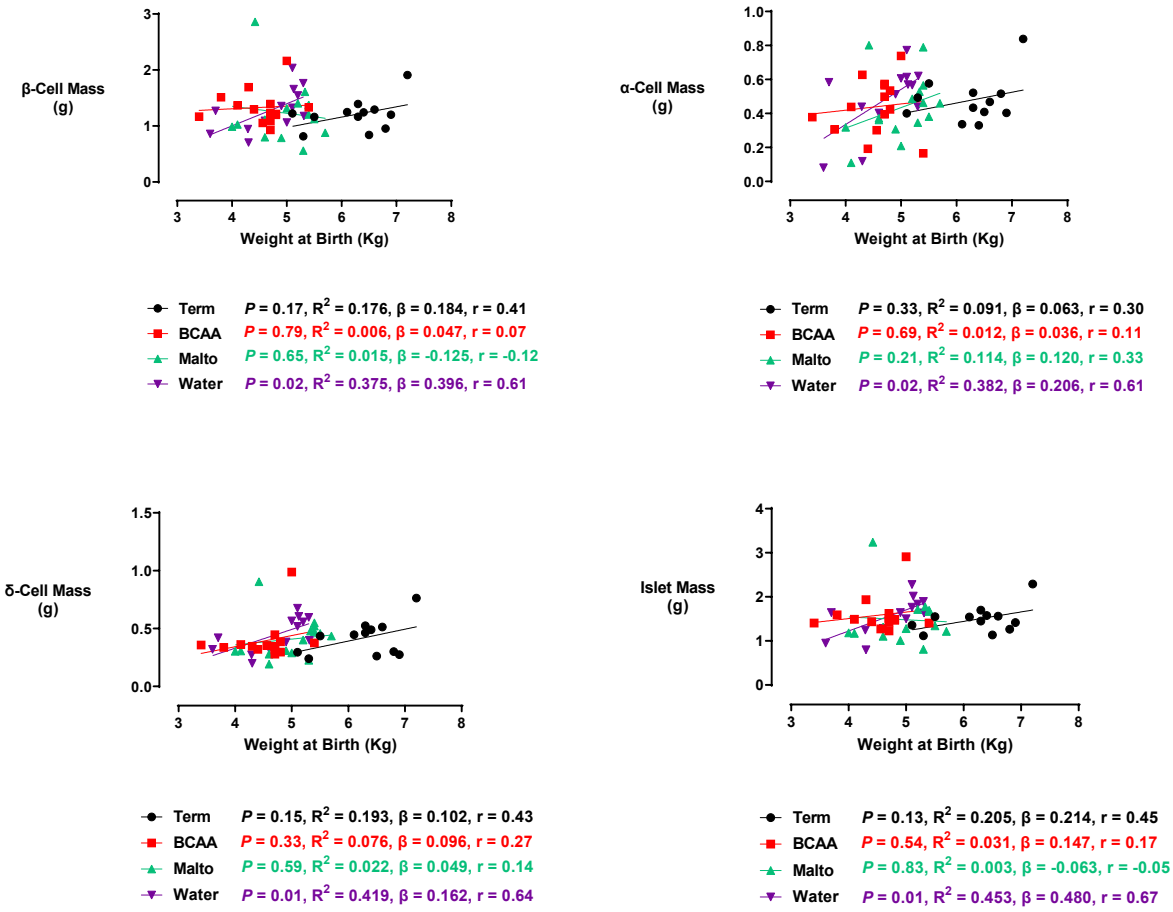



**Figure 5-5. The correlation between cell mass at 12-months-old and weight at birth.**

Pearson's correlation was used to examine bivariate relationships and calculate the correlation coefficient (r) r and β-coefficient.

### 5.2.4. Islet and Cell Density

β-cell, α-cell, and δ-cell densities were similar among groups and between sexes (Table 5-2). Islet density was similar among groups and between sexes; however, there was a sex × treatment interaction ($P = 0.04$), with the female-Term group having higher islet density than the male-Term group ($P = 0.001$).

### 5.2.5. Percentile of Islet Size

The 90$^{th}$ percentile of islet size was similar among groups but different between sexes, with the males having larger islets than females ($P = 0.03$). On post hoc analysis, the male-Term group had larger islets than the female-Term group ($P = 0.01$). The 10$^{th}$ percentile of islet size was not different among groups nor between sexes (Table 5-2).

**Table 5-2. β-cell, α-cell, δ-cell, and islet densities and the percentile of islet size from 12-month-old sheep.**

| | Sheep Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term-Water | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=6) | (F=7) | (M=5) | Sex | Treatment | Interaction |
| **Density (cell/mm$^2$)** | | | | | | | | | | | |
| β-cell | 102±12 | 101±12 | 87±7 | 102±18 | 103±15 | 91±8 | 95±7 | 93±11 | 0.99 | 0.92 | 0.70 |
| α-cell | 40±7 | 45±8 | 36±6 | 47±5 | 43±10 | 49±3 | 50±3 | 42±6 | 0.43 | 0.86 | 0.58 |
| δ-cell | 32±3 | 36±7 | 32±4 | 41±7 | 37±6 | 42±3 | 36±3 | 36±5 | 0.22 | 0.77 | 0.80 |
| Islet | 104±7$^{a,b}$ | 105±18 $^{a,b}$ | 89±5 $^{a,b}$ | 100±9 $^{a,b}$ | 109±9 $^{a,b}$ | 104±12 $^{a,b}$ | 127±7$^{a}$ | 79±7$^{b}$ | 0.17 | 0.66 | **0.04** |
| **Percentile of Islet Size %** | | | | | | | | | | | |
| $^{*}$90$^{th}$ (10$^2$) | 15±1$^{a,b}$ | 17±2$^{a,b}$ | 17±1$^{a,b}$ | 16±2$^{a,b}$ | 15±1$^{a,b}$ | 15±1 $^{a,b}$ | 10±0.8$^{b}$ | 21±3$^{a}$ | **0.03** | 0.80 | **0.02** |
| $^{*}$10$^{th}$ (10$^2$) | 0.64±0.1 | 0.64±0.2 | 0.64±0.1 | 0.66±0.1 | 0.66±0.2 | 0.65±1 | 0.63±0.2 | 0.66±0.2 | 0.46 | 0.89 | 0.44 |
| **Pancreas W (g)** | 87±3 | 104±10 | 84±2 | 96±2 | 82±4 | 104±4 | 87±5 | 108±13 | **0.0001** | 0.66 | 0.86 |
| **Body W (Kg)** | 71±1$^{b,c,d}$ | 82±4$^{a,b,c}$ | 66±3$^{d}$ | 84±4$^{a,b}$ | 68±3$^{d}$ | 87±3$^{a}$ | 68±2$^{c,d}$ | 84±4$^{a,b}$ | **<0.0001** | 0.92 | 0.63 |

Bold font indicates significance in ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. $^{+}$Area: positively stained area; ID: Islet density; $^{*}$90$^{th}$ percentile of islet size; Large islets were defined as islets greater than the 90$^{th}$ percentile of islet size. $^{*}$10$^{th}$ percentile of islet size; Small islets were defined as islets smaller than the 10$^{th}$ percentile of islet size. W: weight.

### 5.2.6. Cell Replication and Apoptosis

Pancreas sections were quadruple stained for insulin, glucagon, Ki67, and active-caspase-3. Both β-cells and α-cells co-stained positively with Ki67 and active-caspase-3 were similar among groups and between sexes. The ratio of proliferation-to-apoptosis was similar among groups and between sexes (Figure 5-6 and Table 5-3).

## 5.3. Summary

β-cell, α-cell, δ-cell, and islet counts, areas, and masses were similar among groups. However, there was a sex difference in all cell absolute masses, related to males having greater pancreatic weight than females. Cell mass in early adult life was positively correlated with weight at birth only in the Water preterm group. The islet density was different between sexes by which the females had higher islet density than the males only in the Term group. Cell proliferation and apoptosis were similar among groups and between sexes.

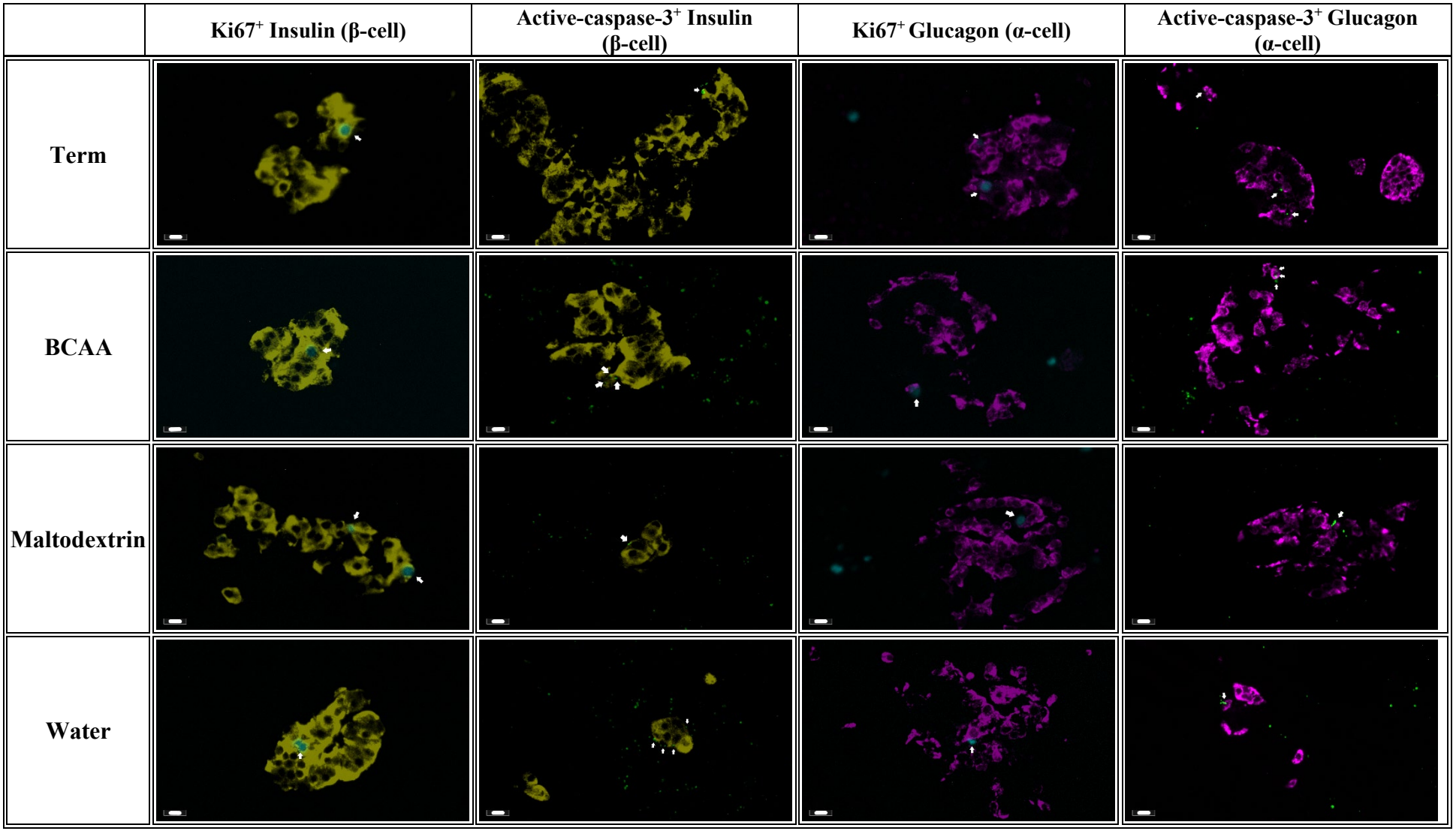



**Figure 5-6. Representative photomicrographs of quadruple staining of insulin, glucagon, Ki67 and active caspase-3.**

Scale bar 10 µm. Term https://figshare.com/s/3b95fa3442e3ed4fd063, BCAA https://figshare.com/s/948e1d6c12c646b238b3, Maltodextrin https://figshare.com/s/17d6cd5819fa97b795ed, and Water https://figshare.com/s/18fd3c9ce874d347f9af.

**Table 5-3. Cell proliferation and apoptosis.**

| | Sheep Group | | | | | | | | Significance (*P*-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm-BCAA | | Preterm-Maltodextrin | | Preterm-Water | | Term | | | | |
| | (F=7) | (M=7) | (F=8) | (M=7) | (F=7) | (M=6) | (F=7) | (M=5) | Sex | Treatment | Interaction |
| **[+]Ki67** | | | | | | | | | | | |
| β-cell | 23±5 | 16±4 | 21±4 | 18±5 | 14±2 | 13±2 | 10±2 | 22±7 | 0.82 | 0.33 | 0.13 |
| α-cell | 3±1 | 5±1 | 3±1 | 4±1 | 5±2 | 5±1 | 3±1 | 4±1 | 0.53 | 0.69 | 0.88 |
| **[+]Caspase-3** | | | | | | | | | | | |
| β-cell | 12±3 | 24±11 | 12±5 | 18±6 | 18±4 | 12±6 | 16±4 | 18±7 | 0.38 | 0.94 | 0.49 |
| α-cell | 10±3 | 22±10 | 9±2 | 18±6 | 11±2 | 9±3 | 12±2 | 15±5 | 0.12 | 0.72 | 0.47 |
| **Ki67:Caspase-3 Ratio** | | | | | | | | | | | |
| β-cell | 4.1±2 | 1.8±0.8 | 4.3±1.3 | 2.9±1.3 | 1.4±0.6 | 3.3±1.1 | 1.1±0.4 | 2.1±1 | 0.81 | 0.38 | 0.24 |
| α-cell | 0.7±0.2 | 0.4±0.1 | 0.5±0.1 | 0.5±0.1 | 0.8±0.4 | 0.8±0.3 | 0.4±0.1 | 0.4±0.1 | 0.68 | 0.27 | 0.88 |

Bold font indicates significance in ANOVA. Non-matching letters indicate a significant difference ($P < 0.05$) among groups on *post hoc* analysis. Data are mean ± SEM. [+]Area: positively stained area; ID: Islet density; [*]$90^{th}$ percentile of islet size; Large islets were defined as islets greater than the $90^{th}$ percentile of islet size. [*]$10^{th}$ percentile of islet size; Small islets were defined as islets smaller than the $10^{th}$ percentile of islet size.

## 5.4. Discussion

We examined the effect of BCAA supplementation on endocrine pancreas development and structure in preterm-born lambs. We found that islet cell count, area, and mass were not affected by BCAA supplementation, preterm birth, or sex.

### 5.4.1. Islet Structure and Morphology

A distinct islet structure was identified by triple staining with insulin, glucagon, and somatostatin. The islets from preterm-born sheep, whether supplemented with BCAA, Maltodextrin, or water, had a similar structure to islets from Term-born sheep and were variable in appearance, structure, and cell composition.

There are interspecies differences in the structure of islets of Langerhans, for example, the location of the cell type within the islet [297]. In the islets of mice and other rodents, β-cells are located in the core, while α-cells and δ-cells are aggregated in the peripheral areas [292,297,298], and islets are mainly composed of β-cells clusters [297]. In cattle, β-cells are primarily located in the core of the islets, whereas α-cells and δ-cells are located in the periphery; however, β-cells can also be seen in this area [299]. Pig islets resemble mouse islets [297].

In human and non-human primate islets, β-, α-, and δ-cells are distributed throughout the islet [297,298]. In the human islet, β-cells are intercalated between α-cells and display cytoplasmic extensions that spread between α-cells [292]. This is similar to studies in sheep, including the current study, in which the appearance of the islets is variable [300]. β-cells are found as clusters and single cells, distributed peripherally and centrally [301]. α-cells have similar distribution as β-cells but are fewer in number [301]. δ-cells are found as clusters and single-cell and located peripherally and centrally [301,302].

Given the variable morphology, it is difficult to draw any conclusions regarding function from the appearance of sheep islets; however, the distribution of cells within the islet is more similar to human islets than those of rodents [300].

### 5.4.2. Cell Count, Area, Density, and Mass

Measuring the cell and islet count and area is essential to define a healthy islet. Increased cell/islet area (size) might be a compensatory response to decreased cell/islet count (number). In our study, no difference was found in β-, α-, δ-cell, and islet counts and areas regardless of

birth and supplementation. A study of β-cell function in adult sheep, who were also preterm-born, reported that the average number of β-cells per islet to be similar between term and preterm-born male and female sheep, but other cell types were not examined [108].

Expansion of endocrine cell mass occurs during late gestation through neogenesis, and then after birth, undifferentiated precursor cells will proliferate [98]. Cell mass is maintained under normal conditions in a healthy pancreas due to normal cell size and number. Maintaining cell mass during adulthood is vital to maintaining glucose homeostasis, preventing diabetes, and altering cell mass according to the requirement for insulin. β-cell mass can also be affected by factors after birth [11], such as perinatal malnutrition [98] or overfeeding [114]. Higher concentrations of amino acids, particularly BCAA, can stimulate an increase in pancreatic β-cell mass [117], although if this had occurred in our study, it was no longer evident by early adulthood when the pancreas was examined.

β-, α-, δ-cell, and islet mass was different between sexes regardless of birth and supplementation, but this difference was due to the greater pancreatic weight in males and disappeared when normalized to body weight. There was a sex difference in islet density, but only in the Term group. Other studies have not demonstrated a sex difference in β-cell mass after preterm birth [13,108], but α-cell mass was higher in male than female sheep after *in utero* treatment of fetal growth restriction with insulin-like growth factor [303].

Maternal exposure to corticosteroids may affect fetal growth and organ maturation [106]. Glucocorticoids are important in regulating cell proliferation and apoptosis by preparing the fetal tissues for the extrauterine environment [202]. However, an increased maternal concentration of corticosteroids can reduce fetal β-cell mass in rats [97] and mice [107].

De Matteo *et al.* found no effect of preterm birth on β-cell mass in sheep after antenatal betamethasone [108]. Bansal *et al.* found a reduction in β-cell mass in adult sheep after preterm birth was induced with glucocorticoid, compared to spontaneously-born term sheep [13]. However, induction of preterm birth without exogenous glucocorticoid exposure resulted in a further reduction in β-cell mass [111], suggesting that in sheep, glucocorticoids can partially mitigate the reduction of β-cell mass caused by preterm birth [111]. In our study, both term and preterm groups received antenatal dexamethasone; there were no differences between groups in islet cell mass.

### 5.4.3. Cell Proliferation and Apoptosis

Cell mass can be regulated by cell proliferation and apoptosis [98], assessed by measurement of Ki67 and cleaved active caspase-3, respectively. In our study, the rate of proliferation and apoptosis was similar among groups and between sexes. Contrary to our findings, Bansal *et al.* found decreased proliferation-to-apoptosis ratio of β-cells in preterm-born compared to term-born sheep at 12 months of age [13]. The reason for these different results is unclear since the same immunohistochemistry technique was used in both studies, apart from the difference in the caspase used to measure apoptosis. However, in that study, control lambs underwent surgical intervention in the neonatal period with some associated morbidity, which could potentially have adversely impacted on pancreatic development [13].

## 5.5. Strength and Limitations

In this study, we minimized potential inaccuracies by the handling, fixation, and paraffin embedding of the tissues that may affect the cellular structure of the islets.

Measuring insulin and glucagon was repeated when a quadruple antibody staining was done to measure Ki67 and active-caspase-3. The cell count, size, and mass were similar in both assays, confirming the accuracy of the staining techniques and macros used for analysis.

Only cells that were stained for insulin and glucagon were counted to guarantee that the frequency of apoptosis and proliferation measured was explicitly related to relevant cells and not to other endocrine or nonendocrine cell types. Therefore, it is likely that both of these were underestimated because cells that have not yet started to produce insulin and those that are completely degranulated were not counted.

## 5.6. Conclusion

Although postnatal abnormalities in endocrine pancreas cell and islet development depend to some extent on gestation length and nutrition, none were found to be associated with early nutritional supplementation in our study.

It is possible that 12 months of age was too young for any effects of ageing to appear, and future studies could be carried out at an older age when any such changes may be more apparent. However, the good health and nutrition of the ewes, induction of labour with glucocorticoids, late (rather than early) preterm birth, facilitated access to maternal milk from

birth, and the health management of the lambs postnatally and for their first year of life might have mitigated the effects of preterm birth and/or resulted in any benefits of nutritional supplementation being less apparent.

# Chapter 6. Thesis Discussion

The experiments described in this thesis were designed to examine the effects of preterm birth and postnatal nutrition on postnatal growth and later endocrine pancreatic function and structure. These experiments investigated, in preterm lambs, the effects of postnatal BCAA supplementation for 15 days after birth on short- and long-term growth, milk intake during the supplementation period, and endocrine pancreas structure and function in adulthood including insulin and glucagon secretion, insulin sensitivity, β-, α-, δ-cell, and islet number and mass and the ratio of proliferation-to-apoptosis in β-cells and α-cells. We demonstrated that effects on growth and endocrine pancreatic function and structure that persist into adulthood resulted from a combination of pre- and postnatal factors in addition to sex.

In our study, preterm birth did not alter endocrine pancreas function or insulin sensitivity. This was surprising as there is increasing evidence from different studies suggesting an association between preterm birth and pancreatic β-cell dysfunction presumed due to poor fetal β-cell growth [6]. A previous study reported reduced β-cell mass, insulin secretion and mRNA expression in adult sheep born preterm [13]. In human, adults born preterm have low insulin sensitivity [100,101] associated with impaired glucose homeostasis [8] and are more susceptible to becoming diabetic. Preterm lambs in our study were born only 10 days earlier than the Term lambs, i.e. moderately preterm. Pancreatic maturity at birth may have been relatively greater than, for example, that of an infant born preterm (< 37 weeks). Furthermore, preterm lambs were relatively independent from birth, were able to feed on maternal milk and did not require parenteral nutrition or other supportive interventions that are commonly given to preterm infants. Given the effects of nutrient supplementation on glucose-insulin axis function, it is possible that the reported effects of preterm birth on this axis arise from differences between preterm and term-born in the content of nutritional supplements, the way the supplementation is given, additional interventions, and the delay in enteral feeding.

The preterm-born sheep in our study did not differ from Term-born sheep in pancreatic structure, but there were interactions between preterm birth, growth, nutritional supplementation, sex and pancreatic function. No difference was found among groups in islet morphology, β-, α-, δ-cell and islet mass regardless of being born Term or Preterm, or receiving nutritional supplements (BCAA/Maltodextrin). The only difference in absolute islet cell mass was between the sexes. Males had higher β-, α-, δ-cell and islet mass than females, which was

due to the bigger pancreas in males proportionate to their greater body size. No difference was found in the function of the endocrine pancreas (insulin and glucagon response to HCG) between the Term- and Preterm-Water groups during the HGC. Our findings suggest that at young adulthood, there is no effect of preterm birth on insulin secretion during a hyperglycaemic challenge. However, insulin and glucagon responses during the HGC differed among the supplemented groups and between sexes.

## 6.1. Neonatal Nutritional Supplementation

Nutritional supplements are often given to preterm babies to enhance growth and development. However, these may induce rapid early growth that can itself be associated with adverse metabolic outcomes. We hypothesised that, in lambs, targeted nutritional supplementation with BCAA, nutrients essential for the development of β-cells in the endocrine pancreas but which have a supportive rather than a direct role in growth, would enhance pancreatic islet development and mitigate the adverse effects of preterm birth on glucose metabolism in later life without accelerating postnatal growth.

BCAA are essential elements in fetal development [147]. In fetal sheep [166,167] and humans [168], leucine infusion stimulates insulin secretion. BCAA activate the mTOR signalling pathway controlling β-cell differentiation during fetal life [155] and stimulating protein synthesis [147,155]. Amino acids, especially BCAA, are essential for glucose and insulin activation of mTOR in β-cells [169]. Accumulating studies indicate that protein-rich diets containing BCAA improve muscle protein synthesis and body composition [151], especially important in preterm babies. The stimulatory effect of leucine and isoleucine on insulin secretion is more effective in young people than in adults [152]. Beyond the neonatal period, there are positive effects on glucose metabolism [167] and muscle growth from high BCAA intake, particularly with leucine [189].

Newborn ruminants can absorb BCAA in a manner comparable to that of monogastric animals [233]. We found higher plasma BCAA concentrations 2 hours post oral BCAA supplementation but a decrease toward the baseline 4 hours later. In humans, BCAA concentrations are elevated after a meal containing BCAA [234], but clearance from the circulation occurs within hours of intake [235]. BCAA enter the circulation by absorption from the intestines, bypass the liver, and are delivered to peripheral tissues [236]. About 95 % of all circulating BCAA are reabsorbed in the kidney [235]. The skeletal muscles extract more than half of the circulating BCAA, representing the largest reservoir of BCAA in the body [160]. In

contrast with other amino acids, BCAA catabolism resides in skeletal muscle, brain, heart, and adipose tissue [158,234,236]; therefore, measuring BCAA in the tissues rather than plasma would have likely reflected body BCAA content more accurately. There was, in general, no effect of BCAA supplementation on plasma concentrations of other essential and non-essential amino acids, but concentrations were overall higher in Term than Preterm groups

Maltodextrin, an iso-caloric carbohydrate supplement, was given to control any effect on pancreatic development and function due to increased energy intake derived from BCAA carbon skeletons in the BCAA group. However, additional carbohydrates given during the neonatal period can also potentially affect metabolism and growth. Most formulas now fed to preterm infants include a mixture of lactose and maltodextrin based on the assumption of lactose malabsorption and the ability to use maltodextrin as a nutrient source [228,256]. Our study found no long-term effect of maltodextrin supplementation on growth, growth velocity, milk intake, or plasma amino acid concentration. However, the pattern of growth during the period of supplementation differed between males and females in this group. The females showed a higher growth velocity in the first 4 days after birth than males. The reasons for this are not clear; however, nutritional intake and energy requirements are different between the sexes [250], so that giving additional carbohydrates alone rather than a balanced macronutrient supplement may have affected males more than females.

## 6.2. Growth

Preterm lambs were significantly smaller and lighter at birth than Term lambs. As we hypothesised, despite demonstrated absorption, we found no effect of nutritional supplementation with BCAA on the growth of preterm lambs. Several studies have similarly shown no effect of amino acid or BCAA supplementation on growth in preterm born humans and animals [241-243]. However, not all studies showed similar outcomes, and some found either positive or negative growth outcomes. It is apparent from the literature that the relationship between BCAA supplementation and growth is not straightforward. The interaction might be affected by the degree of immaturity, the way the supplementation is given (oral or parenteral), other dietary components (low or high protein), the study methods, species differences, and different growth responses in males and females.

In our study, there was no difference in growth parameters between males and females during the 15 days of supplementation except in the Maltodextrin group, in which growth

velocity in the first 4 days was less in males than females as mentioned above. Mothers of male infants produce milk with higher energy content than mothers of female infants [251], indicating that breastmilk composition varies according to sex. This suggests that preterm males and females may also have different requirements for, and physiological responses to, neonatal nutritional supplements [209,229,252].

The lambs in this experimental study had free access to the ewe at all times and were able to feed *ad libitum*. The nutritional supplements were given in addition to, not instead of, maternal milk. Milk intake analysis confirmed no difference in milk intake between supplemented and un-supplemented groups, suggesting no effect of supplementation on appetite. The milk intake was positively associated with growth velocity, with the effect more robust in the preterm groups.

Differences in weight and linear measures between term and preterm lambs were reduced by the time of weaning and by adulthood had disappeared, but as expected, sex differences became more apparent with increasing age. As expected, after puberty (between 150 and 180 days in sheep), most growth parameters started to show divergence between sexes, being greater in males from then on. However, the timing of the divergence was different among groups for some growth parameters, most marked in the Maltodextrin group, where body weight between males and females did not diverge until 360 days (other groups from 180 days), and CRL, BPD, and HL also diverged later than other groups. The reasons for this remain unclear.

At post-mortem, organs including the pancreas, heart, liver, and kidney were collected and weighed. We found no difference among groups in organ weight, suggesting there was no effect of preterm birth or nutritional supplements. The weight and size of the organs may relate to the health of the animal [21] to a certain extent, even though organ size does not necessarily reflect function [21].

Because no effect of BCAA supplementation on preterm lamb growth was found, any subsequent effects of supplementation on endocrine pancreatic development or function were unlikely to be due to altered growth patterns in early life.

## 6.3. Pancreatic Endocrine Function and Structure

HGC was performed at 12-months of age to assess both α and β-cell function, and IS. At post-mortem, the pancreas was collected and tissues were prepared for IHC. Slides then were triple stained with insulin, glucagon and somatostatin antibodies to measure β-, α-, δ- and islet counts, area, and mass. Another set of slides were quadruple stained with insulin, glucagon, Ki67 and caspase-3 to measure the proliferation:apoptosis ratio.

Islet cells exist in an environment where they respond to signals from neighboring (paracrine) cells and the cells themselves (autocrine), influencing hormone secretion [76,23]. Insulin is secreted continuously in a pulsatile oscillation manner. This oscillation arises from the feedback cycle between the pancreas and liver [40] and recurs every 5 minutes [41,42]. Amino acids and glucose amplify the signal, increasing insulin secretion in an ultradian oscillation that ranges from 50-135 minutes [40-42]. Endocrine pancreatic cells can be characterised as functional when the cells rapidly and adequately cope with the acute changes in blood glucose concentration due to meal or glucose infusion. β-cell changes from amino acid-stimulated insulin secretion *in utero* to a dynamic glucose-responsive insulin secretion after birth. The shift from amino acid to glucose responsiveness is gradual, takes 10-15 days, via mTOR [206], therefore, we supplemented our lambs during their first 15-days after birth.

A key question to address is whether insulin/glucagon release is controlled directly by β-/α-cells, that is a direct effect upon receptor sites on the cell itself sensing and metabolising glucose, or indirectly through interactions between β-, α-, and δ-cells? This is not easy to elucidate *in vivo* with certainty. *In vitro,* isolated mouse pancreatic islets secret insulin when stimulated with glucose while glucagon secretion is inhibited; however, isolated α-cells secrete glucagon under glucose stimulus [268]. α-cell-ablated islets showed a blunted insulin response to glucose, suggesting that α-cell-glucagon secretion is necessary for β-cell function [269]. Different responses to glucose were found between single and aggregated β-cells [270]. Aggregates comprising of a β-cell coupled to α-cell (β–α pairs) secreted significantly more insulin than β-cells alone [270], and the quantity of glucagon secreted by the α-cell, even at high glucose concentration, is sufficient to affect β-cell insulin secretion [270]. Clearly, removing cells from the islet environment in an *in vitro* examination will affect cell-to-cell contact paracrine communication, which is vital for glucose homeostasis. Although we measured the insulin and glucagon response to an hyperglycaemic challenge during HCG in the systemic circulation giving an indication of key components of pancreatic function, assessing the effect of

somatostatin or the functional relationship between islet cells was beyond the scope of this thesis.

To sustain the glycaemic set point, paracrine glucagon signalling in the islet is critical for the secretion of the appropriate amount of insulin [264]. Glucagon stimulates insulin secretion, despite being a counterregulatory hormone that opposes insulin action [266], and within the islet, glucagon potentiates insulin secretion during hyperglycaemia [267]. Glucagon is released in a sufficient amount during hyperglycaemia (meal/glucose infusion) to potentiate insulin secretion as a local amplifier [267, 280], meaning that glucagon balances insulin response to match the strength of glucose stimulation [267]. In addition, a sharp increase in insulin secretion will locally increase as an anticipatory control mechanism to ensure that the islet is prepared for upcoming disturbances (hypoglycaemia) [264]. However, the increase in circulating glucagon concentration during hyperglycaemia is enough to potentiate insulin secretion to a certain level without increasing hepatic glucose output [267].

In our study, no difference was found in the function of the endocrine pancreas (insulin and glucagon response) between the Term and Preterm-Water groups during the HGC. Preterm birth *per se* did not influence insulin and glucagon secretion. Our findings suggest that by young adulthood, there is no effect of preterm birth on insulin secretion during HGC. However, insulin and glucagon responses during the HGC differed among the supplemented groups and between sexes.

Preterm-born adult sheep supplemented with BCAA had normal basal glucose concentration, high insulin response during the first 5-minutes post glucose infusion, normal insulin response during SIR and AC, and normal glucagon concentration throughout the clamp test. A high insulin-to-glucose ratio 5-minutes post glucose infusion was found in the BCAA males. This increase was unrelated to the β-cell count, area, and mass since all groups had similar β-cell count, area, and mass. Exposure to supplemental BCAA during the postnatal period may affect β-cell sensitivity to glucose, so that BCAA males had an exaggerated insulin response to the sudden increase in blood glucose concentration. Another possibility is that BCAA affected β-cell secretory function, increasing secretory capacity in the BCAA males by synthesising more insulin. Measuring proinsulin and C-peptide would likely give further information about the observed increase but may not fully explain the response in insulin secretion.

Preterm-born adult sheep supplemented with Maltodextrin had high basal glucose concentration, normal insulin response during FIR, SIR, and AC, and high glucagon concentration throughout the clamp test. Maltodextrin males had higher fasting blood glucose concentration than Maltodextrin females, raising the possibility of a mild disturbance of glucose metabolism in this group. A high plasma glucagon concentration during all phases of the HGC was observed in the Maltodextrin females only. Maltodextrin supplementation affected the standard response of glucagon during hyperglycaemia. This was an unexpected finding for which the underlying explanation is unclear since no difference in the α-cell count, area, and mass was found among groups. It has been suggested that over-secretion of glucagon during meals might be due to α-cell "hypoglycaemic blindness," by which α-cells continue to secrete glucagon in the face of the rising substrate rather than being suppressed [272]. This may explain the increase in glucagon secretion in the Maltodextrin group, possibly due to altered paracrine communication between α- and β-cells. In addition, perhaps the Maltodextrin group is crossing the threshold between secreting enough glucagon to potentiate insulin secretion during hyperglycaemia and inducing gluconeogenesis.

In our study, there was no IS impairment after preterm birth nor BCAA supplementation in preterm-born adult sheep. However, many studies showed reduced IS in preterm-born children [3] and adults [4,6,100,101]. The reduction in IS might be influenced by gestation length, or related to other factors associated with preterm birth, rather than preterm birth itself, such as neonatal complications or nutrition during and beyond the neonatal period [135,139]. Unlike preterm infants, our preterm lambs received maternal milk from birth onwards simultaneously with the supplementation. Preterm human infant nutrition is often based on fortified human milk or non-human milk. Term infants fed on formula have higher rates of type 2 diabetes and higher fasting insulin concentrations in adulthood than those fed on breastmilk [140]. Obesity in adults can be another factor associated with preterm birth since adult weight, rather than weight at birth, might affect IS [288]. Experimental studies that used early nutritional supplements (similar to human milk fortifier) reported no effect on IS in adult term or preterm sheep [209]. Leucine supplements were found to increase IS [289] and improve insulin signalling [290] in mice fed a high protein diet. However, it was also reported that enhanced nutrition with an enriched preterm formula might increase markers of insulin resistance in childhood [16]. These factors cannot explain the wide variability in IS. Perhaps the variation is due to the interaction among factors such as gestational length, nutrition, hormones, and supportive care.

In our study, triple staining with insulin, glucagon, and somatostatin identified a distinct islet structure. Whether supplemented with BCAA, Maltodextrin, or water, the islets from preterm-born sheep had a similar structure to islets from Term-born sheep. Islets were variable in appearance, structure, and cell composition. β-, α-, and δ-cells were either intermingled within the islets or β-cells were found in the core, and α-and δ-cells were located at the periphery. The dominance of the cells varied among β-, α-, and δ-cells. Knowing the location of the cells is essential since the blood flow through the islet impacts the ability of cells to intercommunicate within the islet [304]. The direction of the blood flow through the islet would expose the cell, for example, the β-cell in the core, to hormones released by the outer peripheral α- and δ-cells [305].

All groups had similar β-, α-, δ- and islet counts, area, and mass, in addition to proliferation:apoptosis ratio; however, there was a difference in insulin and glucagon secretion in the preterm supplemented groups. A possible reason could have been that the combination of preterm birth and the nutritional supplementation affected the communication between cells reducing paracrine-mediated effects on insulin and glucagon secretion and/or suppression. Usually, the physiology of glucagon action is described and studied only during fasting or hypoglycaemic states. Understanding the mechanisms by which glucagon-stimulated insulin secretion during hyperglycaemia is an essential next step toward further understanding the complexity of the actions glucagon has within metabolic regulation. Furthermore, as of the time of these studies, α- to β-cell communication has not been explored in great depth in either preterm born humans or sheep.

A previous study on preterm-born sheep found that preterm birth reduced β-cell mass [13]. This was not observed in the current study. Our preterm Water group had similar β-, α-, δ- and islet cell mass as the Term group. Both the compared studies had utilised dexamethasone to induce labour for all study preterm groups. The lambs in the study by Bansal *et al.* underwent postnatal interventions with some associated morbidity, which could have potentially affected their early development. De Matteo *et al.* found no effect of preterm birth on β-cell mass in sheep after antenatal betamethasone [108]. The nutrition and health management of the ewes used for the studies was excellent and they all had at least two successful previous pregnancies prior to this one. In the immediate postnatal period, intake of maternal milk by lambs was carefully monitored and facilitated if required. Importantly, the 137 day-born groups of lambs were only

moderately preterm. There is the possibility that any of these factors alone or combined, led to us no observed effect on β-cell mass at the age studied.

## 6.4. Strength and limitations

The current study was performed in a consistent breeding group produced during extended breeding seasons over 2 years. All the interventions (from birth to adulthood) were conducted in a research facility located at a farm where the sheep have matured in a relatively normal farm environment. A small number of people performed lambing assistance, supplement preparation and administration, and serial growth measurements, maximising data consistency. During the HGC, sheep were isolated and restrained; however, stress was minimised since the sheep were acclimatised to indoor conditions and underwent regular blood collection.

Labour was induced using glucocorticoids, which can also affect organ maturation. Antenatal steroids are routinely given to women at risk of preterm labour to hasten lung maturation, so most preterm babies have been exposed to glucocorticoids shortly before birth. However, in an experimental setting, the question always arises whether any alterations in organ development are due to preterm birth *per se* or to glucocorticoid exposure. A previous study showed impaired insulin secretion in adult sheep when preterm birth was induced by dexamethasone [13]. However, De Matteo *et al.* reported that betamethasone did not impair insulin secretion in adult sheep born preterm [108]. To separate the effects of preterm birth from those of dexamethasone, we used dexamethasone to induce labour in both Term and Preterm groups. Our findings showed that preterm birth did not alter insulin secretion during HGC when the glucocorticoid-exposed Preterm Water (control) group was compared with the glucocorticoid-exposed Term group. The inclusion of a term-born, non-corticosteroid exposed group (spontaneous birth) would have been advantageous to isolate the effect of glucocorticoid exposure on insulin secretion. Performing serial HGC tests at different ages would have provided information about the longitudinal changes in insulin and glucagon secretion from early postnatal life until adulthood.

Our preterm lambs were born at 137 days of gestation (late-preterm), and supportive care was focused on reducing thermal stress and aiding intake of maternal milk without other interventions, unlike preterm infants, who may require other more invasive support or alternative nutrition.

Overall survival of Preterm lambs in our study was high. There is a sex-specific difference in neonatal mortality in humans, with the males having a higher rate than the females [259]. Females are smaller than males at day zero; however, female fetuses are relatively more mature than males [260], contributing to male susceptibility to perinatal mortality [259,260]. In our study, three male lambs from the Water groups died (two after day-60 and one after day-90). However, no deaths occurred in the Preterm supplemented groups (BCAA and Maltodextrin). There were no recorded female deaths in any group (Preterm and Term).

Body composition was not assessed; these data would have helped detect if BCAA supplements had increased relative lean mass or adipose tissue since there was an increase in the perirenal fat in the BCAA males.

We measured BCAA in plasma. However, since BCAA affects muscle growth, it might have been informative to measure BCAA in the muscle as well as in plasma to see if that correlated with lean mass, for example, in muscle biopsies collected on day 15 at the end of the supplementation period.

Measuring C-peptide was not included in the study aims. However, clinically, C-peptide concentration is measured instead of insulin concentration. The liver metabolises insulin but does not metabolise C- peptide, meaning that C-peptide stays longer in the blood, with a half-life of 30-35 minutes, while that of insulin is 3-6 minutes. Therefore, including C-peptide measurements in our study may have yielded additional information about insulin response.

In this study, we minimized potential inaccuracies by the handling, fixation, and paraffin embedding of the tissues that may affect the cellular structure of the islets during the immunohistochemistry study.

Measuring insulin and glucagon was repeated when a quadruple antibody staining was done to measure Ki67 and active-caspase-3. The cell count, size, and mass were similar in both assays, confirming the accuracy of the staining techniques and macros used for analysis.

## 6.5. Future directions

In sheep, the micro-vascularisation of the pancreas is not well studied. Knowing if the direction of hormone secretion is from the core to the periphery, vice versa, or bidirectional is

needed to understand the communication between the islet cells and if this communication is disturbed by preterm birth or affected by nutritional supplementation.

## 6.6. Conclusion

Postnatal supplementation with BCAA did not affect the growth in preterm-born lambs. Preterm birth *per se* did not influence insulin and glucagon secretion; however, there were interactions between supplementation and sex in the preterm groups, with BCAA males having higher insulin-to-glucose ratios and Maltodextrin females having higher plasma glucagon concentrations throughout the HGC.

The influence of preterm birth and early nutrition on endocrine pancreatic function is complex and likely to involve altered communication between islet cells as well as hormonal signalling pathways.

# Appendix I :Macro Analysis

The image analysis was performed on raw images from each field of view. The analysis was supported by a macro written in ImageJ Macro programming language and performed using FIJI (Fiji is just ImageJ version 1.53f51, Maryland, USA).

## I.1. Triple Staining for Insulin, Glucagon, and Somatostatin Antibodies

The stainings were visualised using a VSlide slide scanner system1.1.128. DAPI, FITC, TRITC, Cy5, and Cy7 were used to acquire the images (methods section 2.4.10.6). Each channel generated one raw image for each individual field of view (FOV). The images were then processed using an ImageJ Fiji (Figure I-1 and Table I-1) https://figshare.com/s/7866c60be28b84c3792a.

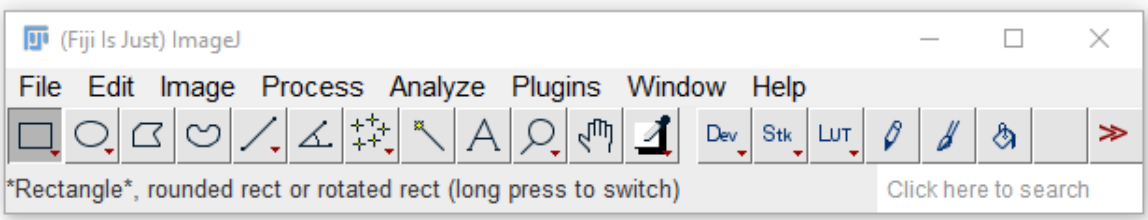



**Figure I-1. Image J (Fiji is Just) software was used to write the macro.**

**Table I-1. Commands written in ImageJ macro to analyse the images.**

| Lines | Command |
|---|---|
| 1-33 | Batch process command for processing files in the directory |
| 35-40 | Extract assay name, folder name, file name, and image name |
| 42-48 | Create a folder for image output and islet area summary for each slide |
| 51-70 | Define colour code letter for each stain and open image files for image analysis |
| 72-73 | Set scale in microns |
| 75-113 | Crop images (cropping areas overlapped between neighbouring FOV)<br>Merge channels (combining each image stack to create a composite image) |
| 116-127 | Measure FOV area (used for counting the total number of FOVs for each slide) |
| 129-149 | Measure the pancreatic tissue area within each FOV |
| 151-174 | Measure autofluorescence (for background subtraction) |
| 176-202 | Count nuclei |
| 204-228 | Measure insulin area |
| 230-270 | Count β-cells |
| 272-298 | Measure the total area of insulin and β-cells (used for β-cell mass calculation) |
| 300-324 | Measure glucagon area |
| 326-367 | Count α-cells |
| 369-395 | Measure the total area of glucagon and α-cells (used for α-cell mass calculation) |
| 397-421 | Measure somatostatin area |

| | |
|---|---|
| 423-464 | Count delta-cells |
| 466-492 | Measure the total area of somatostatin and delta-cells (used for delta-cell mass calculation) |
| 494-507 | Measure total islet count and each islet area within the FOV |
| 509-522 | Save islet measurements generated from line 494 ~507 in the "Output Islet Area" folder |
| 524-529 | Close images and clear memory before starting the next loop |
| 531- | Save all measurement results under the corresponding filename in the "Output Summary" folder |

## I.2. Quadruple Staining for Insulin, Glucagon, Ki67, and Active Caspase-3 Antibodies

Slides were imaged using six single band filters (DAPI, A430, FITC, TRITC, Cy5, and Cy7) to acquire the images (section 2.1.10.6.1). The analysis command is as described previously with the addition of measuring and counting for Ki67 and active caspase-3 https://figshare.com/s/7cda7b2980d4d295e72c. Table I-2 describe the additional commands used for Ki67 and active caspase-3.

**Table I-2. Commands used to measure and count K67 and active caspase-3.**

| Lines | Command |
|---|---|
| 1-8 | Define colour code letter for each stain and open image files for image analysis |
| 12-37 | Crop images (cropping areas overlapped between neighbouring FOV)<br>Merge channels (combining each image stack to create a composite image) |
| 40-65 | Count Ki67 cells |
| 67-90 | Measure Ki67 positive β-cell count |
| 92-115 | Measure Ki67 positive α-cell count |
| 117-139 | Measure the active caspase-3 area |
| 141-166 | Measure active caspase-3 positive cell count |
| 169-192 | Measure active caspase-3 positive β-cell count |
| 194-217 | Measure active caspase-3 positive α-cell count |

## I.3. Data Collection and Analysis

Power Query in Excel was used to combine data from multiple summary files into a single table

$$^{*}\ \beta\text{-cell mass (mg)} = \frac{\text{insulin-positive area } (\mu m^2) \times \text{pancreas weight (mg)}}{\text{total tissue area } (\mu m^2)}$$

The α-cell mass and δ-cell mass were measured from a similar equation [13], using glucagon positive area and α-cell, somatostatin positive area, and δ-cell. The total islet cell area was calculated as a sum of the β-cell, α-cell, and δ-cell area per field of view.

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