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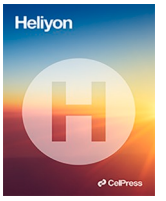

# Cardiac growth and metabolism of the fetal sheep are not vulnerable to a 10 day increase in fetal glucose and insulin concentrations during late gestation☆

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ABSTRACT

*Aims:* To evaluate the effects of fetal glucose infusion in late gestation on the mRNA expression and protein abundance of molecules involved in the regulation of cardiac growth and metabolism.
*Main methods:* Either saline or glucose was infused into fetal sheep from 130 to 140 days (d) gestation (term, 150 d). At 140 d gestation, left ventricle tissue samples were collected. Quantitative real-time RT-PCR and Western blot were used to determine the mRNA expression and protein abundance of key signalling molecules within the left ventricle of the fetal heart.
*Key findings:* Although intra-fetal glucose infusion increased fetal plasma glucose and insulin concentrations, there was no change in the expression of molecules within the signalling pathways that regulate proliferation, hypertrophy, apoptosis or fibrosis in the fetal heart. Cardiac *Solute carrier family 2 member 1* (*SLC2A1*) mRNA expression was decreased by glucose infusion. Glucose infusion increased cardiac mRNA expression of both *Peroxisome proliferator activated receptor alpha (PPARA)* and *peroxisome proliferator activated receptor gamma (PPARG)*. However, there was no change in the mRNA expression of PPAR cofactors or molecules with PPAR response elements. Furthermore, glucose infusion did not impact the protein abundance of the 5 oxidative phosphorylation complexes of the electron transport chain.
*Significance:* Despite a 10-day doubling of fetal plasma glucose and insulin concentrations, the present study suggests that within the fetal left ventricle, the mRNA and protein expression of the signalling molecules involved in cardiac growth, development and metabolism are relatively unaffected.

## 1. Introduction

Cardiomyocytes are the functional contractile units of the heart and are responsible for healthy heart function. Unlike rodents,

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zebrafish and some amphibians, cardiomyocyte endowment in both humans and sheep is set at birth [1]. This is because in late gestation mononucleated cardiomyocytes, which have a proliferative phenotype, become binucleated and then contribute to cardiac growth through hypertrophy [2,3]. Epidemiological studies have attributed a significant portion of adult heart health to regulation and coordination of cardiac growth and functional development during gestation [4,5]. The fetal heart relies predominately on glucose, rather than fatty acids, as its main substrate for ATP production. Thus, perturbations in glucose availability during gestation may have negative implications for cardiac growth and development.

High plasma glucose is associated with hyperinsulinaemia, and although insulin has a higher affinity for the insulin receptor (INSR), insulin can also activate the insulin-like growth factor (IGF) receptor-1 (IGF1R). IGF1R activation acts through the phosphoinsitol-3 kinase (PI3K) and the extracellular signal related (ERK) pathways to up-regulate cyclins and down-regulate cell cycle inhibitors so that the cardiomyocyte can progress through the cell cycle, resulting in increased cardiomyocyte proliferation [6,7] as well as through increasing translational capacity and protein synthesis, resulting in physiological hypertrophy [8,9]. Thus, it is possible that fetal hyperinsulinaemia can activate IGF1R, and that this may result in dysregulated cardiac development. This protocol increased fetal glucose and insulin concentrations and resulted in the expected increase in unilocular fat mass and the expression of genes that regulate appetite in the hypothalamus (Fig. 7) [10]. In contrast, there was a reduction in surfactant protein in the fetal lung [11], which is consistent with findings of late gestation overnutrition in sheep that increase plasma glucose and insulin concentration to 150% for 30 days [12] and increased risk of respiratory distress syndrome in neonates of obese mothers [13].

In the current study, we performed an intrafetal glucose infusion for a period of 10 days in late gestation during a critical window in cardiac development when cardiomyocytes are undergoing a transition from having proliferative to hypertrophic capacity. We hypothesised that a 10-day period of increased fetal plasma glucose and insulin concentrations that impacts fetal fat mass as well as gene expression in the hypothalamus and lung (Fig. 7) would result in an increase in the expression of molecules that regulate cardiac growth and metabolism. Our aims were two-fold. Firstly, to determine whether there is an alteration in the metabolic profile of the fetal heart in response to increased glucose availability. Secondly, to determine whether increased glucose and insulin concentrations, during the critical period when cardiomyocytes are undergoing a change in their growth profiles, have a consequence on the signalling pathways that regulate cardiac growth.

## 2. Materials and Methods

All procedures were approved by the University of Adelaide Animal Ethics Committee and comply with the Australian code of practice for the care and use of animals for scientific purposes. All investigators understood and followed the ethical principles outlined in Grundy et al. [14] and study design was informed by guidelines (ARRIVE [15] and DOHaD research [16,17]).

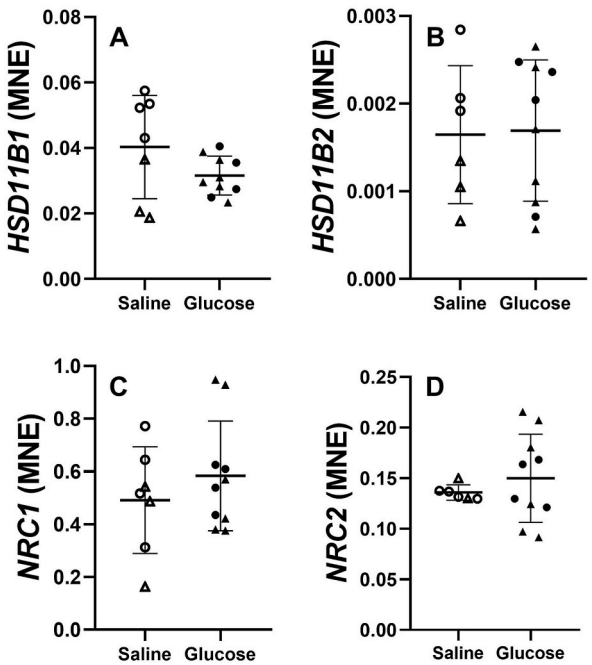


**Fig. 1.** Normalised mRNA expression of molecules that regulate glucocorticoid bioavailability including *HSD11B1* (A), *HSD11B2* (B), glucocorticoid receptor (*NR3C1*, C) and mineralocorticoid receptor (*NR3C2*, D) in males (triangles) and females (circles) after 10 d infusion with saline (open symbols) or glucose (filled symbols). Data expressed as individual data points with mean ± SEM superimposed. Data were analysed by an unpaired Student's t-test; $P < 0.05$ was considered statistically significant; *, treatment; MNE, mean normalised expression.

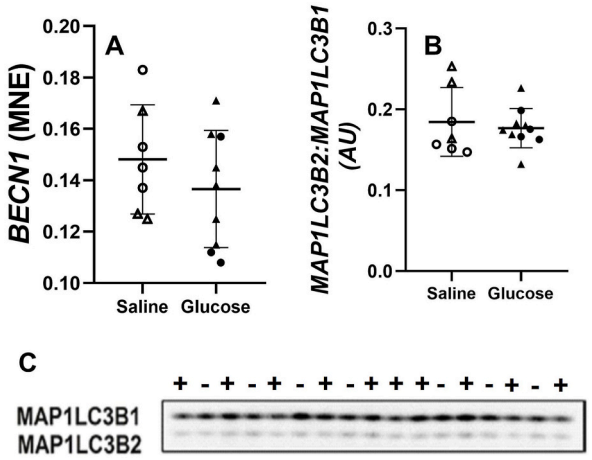


**Fig. 2.** Normalised mRNA expression of *BECN1* (A) and protein abundance of the ratio of MAP1LC3B2: MAP1LC3B1 (B) in male (triangles) and female (circles) fetuses after 10 d infusion with saline (open symbols) or glucose (filled symbols). Data expressed as individual data points with mean ± SEM superimposed. Data were analysed by an unpaired Student's t-test; $P < 0.05$ was considered statistically significant; *, treatment; -, saline infused; +, glucose infused; MNE, mean normalised expression; AU, arbitrary units. Full Western blot images available in supplementary material.

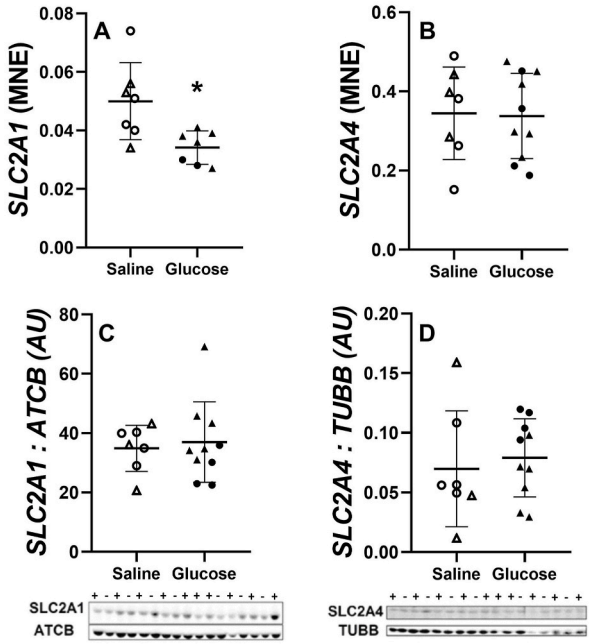


**Fig. 3.** Normalised mRNA expression of *SLC2A1* (A) and *SLC2A4* (B) and protein abundance of SLC2A1 (C) and SLC2A4 (D) in male (triangles) and female (circles) fetuses after 10 d infusion with saline (open symbols) or glucose (filled bars). Data expressed as individual data points with mean ± SEM; Data were analysed by an unpaired Student's t-test; $P < 0.05$ was considered statistically significant; *, treatment: ATCB, β-actin; TUBB, β-tubulin; -, saline infused; +, glucose infused; MNE, mean normalised expression; AU, arbitrary units. Full Western blot images available in supplementary material.

### *2.1. Animals and surgery*

At 118–120 days (d) gestation (term, 150 d), 17 Merino ewes (*Ovis aries*; South Australian Research and Development Industry, Roseworthy, Australia) underwent surgery under aseptic conditions [10]. Sodium thiopentane (1.25 g IV, Pentothal, Rhone Merieux, Pinkenba, Qld, Australia) was administered intravenously to induce general anaesthesia, which was maintained with 2.5–4% halothane in oxygen (Fluothane, ICI, Melbourne, Vic, Australia). Vascular catheters were implanted into the maternal jugular vein, fetal jugular vein and carotid artery as well as the amniotic cavity; as previously described [10]. Ewes received an intramuscular injection of antibiotics (3.5 ml of Norocillin (150 mg/ml procaine penicillin and 112.5 mg/ml benzathine penicillin; Norbrook Laboratories Ltd.,

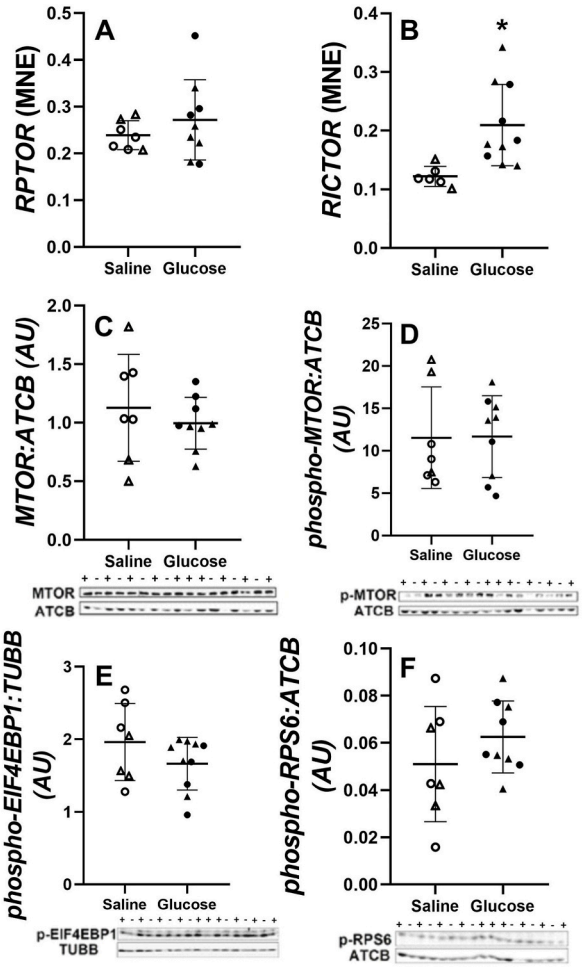


**Fig. 4.** Normalised mRNA expression of *RPTOR* (A) and *RICTOR* (B) and protein abundance of MTOR (C), phospho-MTOR (D) and downstream signalling molecules phospho-EIF4EBP1(E) and phospho-RPS6 (F) in males (triangles) and females (circles) after 10 d infusion with saline (open symbols) or glucose (filled symbols). Data expressed as mean ± SEM; Data were analysed by an unpaired Student's t-test; $P < 0.05$ was considered statistically significant; *, treatment; -, saline infused; +, glucose infused; MNE, mean normalised expression; AU, arbitrary units. Full Western blot images available in supplementary material.

Gisborne, Australia) and 2 ml of 125 mg/ml Dihydrostreptomycin in sterile saline (Sigma, St Louis, MO, USA) for 3 d following surgery. After surgery, all ewes received an analgesic (xylazine, 0.02 mg $kg^{-1}$ IM). Each fetus received antibiotics (500 mg; sodium ampicillin, Commonwealth Serum Laboratories) intraamniotically for 4 d post-surgery.

Ewes were allowed at least 4 d to recover prior to the experimental protocol and were housed in individual pens with a 12 h:12 h light-dark cycle. Ewes were fed a diet compromising of lucerne chaff and concentrated pellets (Ridley Agriproducts, Murray Bridge, Australia), which was calculated to provide the ewe with 100% of the maintenance energy requirements for a pregnant ewe bearing a singleton fetus as specified by the Ministry of Agriculture, Fisheries and Food UK [18].

### 2.2. Glucose infusion

Ewes were randomly assigned to two groups that received either saline (saline, n = 7; male = 3, female = 4) or glucose (n = 10; male = 6, female = 4) via a continuous intravenous infusion into the fetal jugular vein. At 130±2 d gestation, glucose (50% dextrose 500 g $l^{-1}$ in sterile saline) or sterile saline infusion commenced at a rate of 1.9 ml $h^{-1}$. The rate of infusion increased in increments of 1.9 ml $h^{-1}$ per day for 3 days, until the final rate of 7.5 ml $h^{-1}$ was reached on day 4 [10]. This infusion rate was then maintained until post mortem at 138-140 d gestation.

### 2.3. Post mortem and tissue collection

Ewes were humanely killed between 138 and 140 d gestation with an overdose of sodium pentobarbitone (Virbac Pty Ltd,

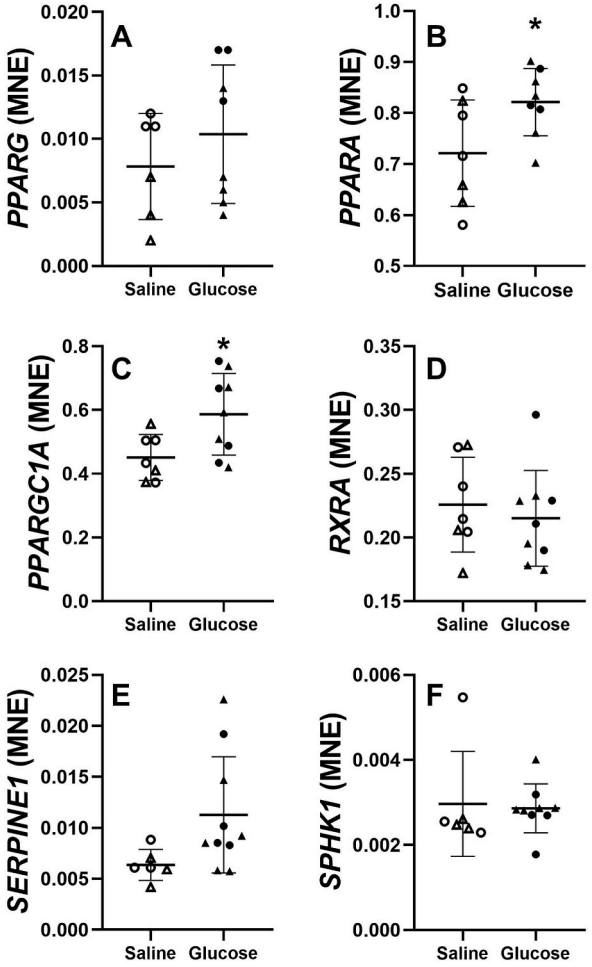


**Fig. 5.** Normalised mRNA expression of *PPARG* (A), PPAR α (B), *PPARGC1A* (C), *RXRA* (D), *SERPINE1* (E) and *SPHK1* (F) in males (triangles) and females (circles) after 10 d infusion with saline (open symbols) or glucose (filled symbols). Data expressed as individual data points with mean ± SEM superimposed. Data were analysed by an unpaired Student's t-test; $P < 0.05$ was considered statistically significant; *, treatment; MNE, mean normalised expression.

Peakhurst, NSW, Australia). Fetuses were delivered by hysterectomy and weighed. The heart was weighed, and samples of the left ventricle were dissected, weighed and snap frozen in liquid nitrogen to be stored at −80 °C for subsequent gene expression and protein abundance analysis. There was no change in fetal weight (Saline = 4.84 ± 0.2 kg; Glucose = 5.12 ± 0.2 kg) and information on adiposity and appetite regulation [10] as well as lung development [11] from this cohort have been reported previously.

### 2.4. Quantification of mRNA transcripts within the left ventricle

***Total RNA extraction:*** Total RNA was extracted from ~50 to 60 mg of left ventricle tissue (n = 17), using Qiagen QIAzol Lysis Reagent and Qiagen RNeasy purification columns (Qiagen Pty Ltd., Doncaster, Vic, Australia) [19,20]. All extracted RNA samples were checked for integrity by running through an agarose gel stained with ethidium bromide. Total RNA was quantified by spectrophotometric measurements at 260 and 280 nm.

***cDNA synthesis:*** The cDNA was synthesized according to manufacturer's guidelines with Superscript III First Strand Synthesis System (Invitrogen), using 1 μg of total diluted RNA, random hexamers, dNTP, DTT and Superscript III in a final volume of 20 μL. A no template control (NTC) containing no RNA transcript and a no amplification control (NAC) containing no Superscript III were used to check for reagent contamination and genomic DNA contamination, respectively.

***Quantitative real-time RT-PCR:*** All essential information regarding our procedure is included as per the MIQE guidelines [21]. The mRNA expression of glucose transporters (*SLC2A1* (U89029.1) and *SLC2A4* (AB005283 [22]), IGF signalling molecules (*IGF1* (DQ152962), *IGF2* (M89789), *IGF1R* (AY162434), *IGF2R* (AF327649 [23]), markers of proliferation (*PTEN* (XM_613125.4), *CDKN1B* (NM_001100346.1 [24]) and *MYC* (NM_001,009,426 [24])), markers of hypertrophy (*NNPA* (NM_001160027.1) [25], *NNPB* (Table 1) and *MYH7* (Table 1)), markers of binucleation (*GATA4* (NM_001192877.1 [24]), genes involved in glucose metabolism (PDK4

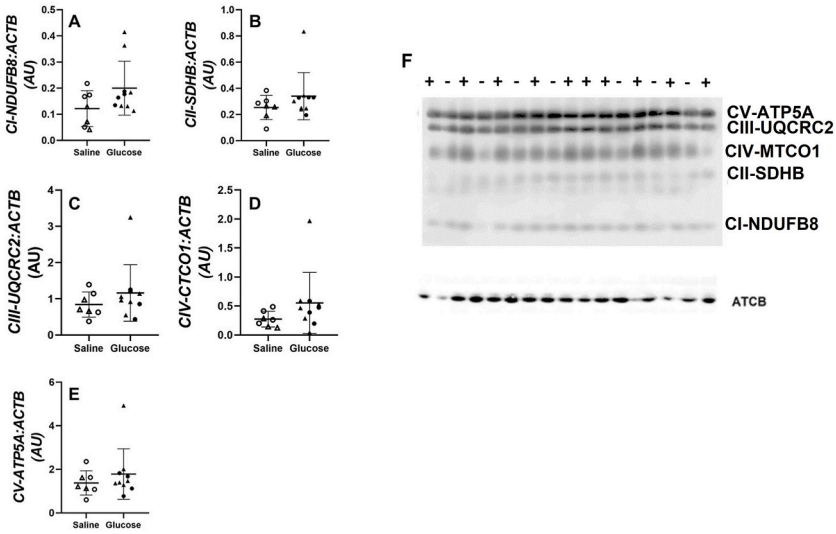


**Fig. 6.** Normalised protein abundance of OXPHOS complexes; Complex 1(A), Complex 2(B), Complex 3(C), Complex 4 (D), Complex 5 (E) in males (triangles) and females (circles) after 10 d infusion with saline (open symbols) or glucose (filled symbols). Western Blot image (F). Data expressed as individual data points with mean ± SEM superimposed; Data were analysed by an unpaired Student's t-test; $P < 0.05$ was considered statistically significant; *, treatment; -, saline infused; +, glucose infused; AU, arbitrary units. Full Western blot images available in supplementary material.

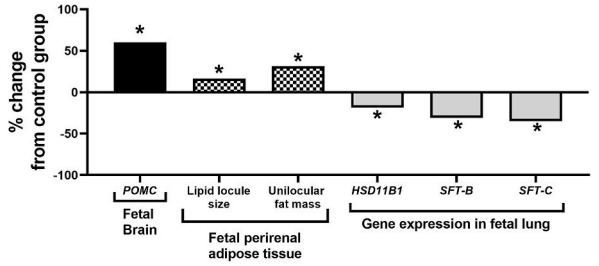


**Fig. 7.** Summary and comparison of the impact of a 10 d glucose infusion during late gestation on the fetal brain, adipose tissue and lung. In comparison to these organs and tissues, the fetal heart is relatively unaffected. Data presented as % change from their respective control groups and obtained from previously published work by our research group [10,11].*, significant difference from respective control group. $P \leq 0.05$. POMC, pro-opiomelanocortin; HSD11B1, Hydroxysteroid 11-Beta Dehydrogenase 1; SFTP, surfactant protein.

(NM_001101883.1) [25]), fatty acid metabolism (*FABP5* (NM_001145180.1 [24]), *PPARG* (NM_001100921.1 [26] and *PPARA* [22])), genes with PPARG response elements (*SERPINE1* (NM_001174114.2) and *SPHK1*(XM_002696204.2)), markers of oxidative stress (*HMOX1* (NM_001014912.1), *SOD1* (FJ546075.1) *SOD2* (NM_001280703.1) and *SOD3* (XM_004009740.1; Table 1)), markers of apoptosis (*BCL2* (HM630309.1), *BAX* (AF163774.1) and *TP53* (NM_001009403.1) [27]), a marker of autophagy (*BECN1* (XM_004012945.1 [27])), markers of myocardial fibrosis (*TGFB1*(NM_001,009,400), *TIMP1* (NM_001009319.2), *TIMP2*(NM_001,166, 186), *TIMP3*(NM_001166187.1), *MMP2* (NM_001166180.1), *MMP9* (FJ185130), *COL1A1* (AF129287) and *COL3A1*(NM_001,076,

**Table 1**
Primer sequences for target genes.

| Primer name | Primer sequence 5′-3′ | Primer Concentration (μM) |
|---|---|---|
| *NNPB* | Fwd: CCTGCTTCTCCTCTTCTTGC | 0.90 |
| | Rev: TAGACGGTCCAACAGCTCCT | 0.90 |
| *SOD1* | Fwd: CTTCGAGGCAAAGGGAGATAAA | 0.45 |
| | Rev: ACTGGTACAGCCTTGTGTATTG | 0.45 |
| *SOD2* | Fwd: AGTAA*ACACA*GTCAGCCTTACAC | 0.45 |
| | Rev: CCACGCTCAGAAACACTACA | 0.45 |
| *SOD3* | Fwd: CATGGCTAGGCTCTAACTGAAA | 0.90 |
| | Rev: CTCAGGTTCATGGGCTTACTT | 0.90 |
| *HMOX1* | Fwd: CTGGTGATGGCGTCTTTGTA | 0.90 |
| | Rev: CAGCTCCTCTGGGAAGTAGA | 0.90 |

Fwd, forward; Rev, reverse.

831)) and the glucocorticoid regulatory genes (*HSD11B1* (NM_001009395.1), *HSD11B2* (NM_001009460.1), *NR3C1* (NM_001114186.1), *NR3C2* (AF349768.1) [11]) were determined using qRT-PCR.

The expression of these target genes was normalised to 3 housekeeper genes; beta-2-microglobulin (B2M; NM_001,009,284), tyrosine 3-monooxygenase (YWAHZ; AY970970) and TATA-binding protein (TBP; XM_004,011,459). These were selected (based on their stability across samples in each treatment group) from a panel of 8 candidate housekeeper genes using the geNorm component of the qBase relative quantification analysis software [28]. Each sample was run in triplicate for each target and housekeeper gene and the mRNA amplification for each sample was determined using Fast SYBR® Green Master Mix (Applied Biosystems) in a final volume of 6 μl on a ViiA 7 Fast Real-time PCR system (Applied Biosystems). Each well on the qRT-PCR plate contained 1 μl of cDNA, 3 μl Fast SYBR Green Master Mix (2×), 2 μl of forward and reverse primer mixed with differing amounts of $H_2O$ depending on the required final primer concentrations. No transcription controls (NTC) for each primer set were included on each plate to check for nonspecific amplification. The threshold was set within the exponential growth phase of the amplification curve and the corresponding $C_t$ values were obtained to quantitate each reaction. The abundance of each mRNA transcript was then normalised to the abundance of the 3 housekeeper genes (TBP, YWAHZ and B2M) using Data Assist 3.0 analysis software (Applied Biosystems) and expressed as mean normalised expression [19,20,29,30].

### 2.5. Protein extraction and western blotting

***Protein extraction:*** Left ventricle tissue (~100 mg; Saline, n = 7; Glucose, n = 10) was sonicated (John Morris Scientific, SA, Australia) in a lysis buffer comprising of 1ml/100 mg tissue of 1 mmol/l Tris HCl (pH = 8, 5 mol/l NaCl, 1% NP-40, 1mmol/LNa Orthovanadate, 30 mmol/l NaF, 10 mmol/l Na Tetrapyrophosphate, 10 mmol/l EDTA) and a protease inhibitor tablet (complete Mini; Roche). Samples were then centrifuged at 14,300*g* and 4 °C for 14 min (Eppendorf Centrifuge 5415, Crown Scientific, Vic, Australia). A Micro bicinchoninic acid (BCA) Protein Assay Kit (PIERCE, Thermo Fisher Scientific Inc., Rockford, USA) was used to determine the protein content of each sample. Bovine serum albumin (BSA; 2 mg/ml stock solution) was used to form a standard curve. Extracted protein samples (50 μg) were subject to SDS page and stained with Coomassie blue [25,27,31].

***Western Blotting:*** Equal volumes of each sample were subjected to SDS page [24,25,29]. Proteins in each sample were then transferred onto a nitrocellulose membrane (Hybond ECL, GE Health Care, NSW, Australia), which was subsequently stained with Ponceau S (0.5% Ponceau in 1% acetic acid) to determine the efficacy of the transfer. The membranes were briefly washed with 7% acetic acid and then subjected to 3 × 5min washes in Tris-Buffered Saline (TBS). The membranes were cut according to the size of the proteins and blocked in 5% BSA in Tris-Buffered Saline with 1% Tween (TBS-T) for 1 h at room temperature. The membranes then underwent 3 × 5min washes in TBS-T and were incubated with their respective primary antibody; phospho-*PRKAA1* (Ser 485, Cell Signalling Technology, Inc), Acetyl CoA Carboxylase (Cell Signalling Technology, Inc), carnitine palmitoyltransferase 1 (CPT1B) (Santa Cruz Biotechnology, Inc.), SLC2A4 (Abcam, Cambridge, UK), Insulin receptor-β (INSR) (Abcam, Cambridge, UK), phospho-insulin receptor substrate 1 (p- IRS1) (Ser 789, Cell Signalling Technology, Inc), AKT1 (Cell Signalling Technology, Inc), phospho-AKT1 (Ser 473, Cell Signalling Technology, Inc), phospho- EIF4EBP (Ser 65, Cell Signalling Technology, Inc), phospho- RPS6 (Ser 235, Cell Signalling Technology, Inc), proliferating cell nuclear antigen (PCNA) (Cell Signalling Technology, Inc), autophagic markers (Light chain 3 $B^1$ and B2 (LC3B1 & LC3B2; Cell Signalling Technology, Inc)) and Total OXPHOS antibody cocktail (Abcam, Cambridge, UK) at 4 °C overnight with agitation [24,25,29]. After incubation with the primary antibody, the blots were washed and incubated with the appropriate Horse Radish Peroxidase labelled secondary IgG antibody for an hour at room temperature. Enhanced chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, IL, USA) was used to detect the blots. The Western blot was imaged using ImageQuant LAS 4000 (GE Healthcare, VIC, Australia) and the protein abundance was quantified by densitometry using Image quant software (GE Healthcare, VIC, Australia). Either β-actin (ATCB; HRP conjugate, Cell Signalling Technology, Inc) or β-tubulin (TUBB; HRP conjugate, Cell Signalling Technology, Inc) was used as a loading control.

### 2.6. Statistical analyses

For gene and protein expression, outliers were defined as values that were >2STD from the mean for the treatment group and were removed. The effect of glucose infusion on heart weight, mRNA and protein expression were determined using an unpaired Student's t-test (GraphPad Prism). Data are presented as mean ± SEM and a probability of 5% ($P < 0.05$) was considered significant for all analyses. Graphical representation of the results is presented in a two-column format separated by treatment group (Saline, Glucose). This study was not powered to investigate fetal sex. However, for clarity and interpretation fetal sex is indicated by different symbols (males, triangles; females, circles) within each treatment column.

## 3. Results

The infusion protocol resulted in a significant increase in both fetal plasma glucose and insulin concentrations with mean values throughout the 10 d infusion period for plasma glucose (Saline, 1.10 ± 0.09 mmol/l; Glucose, 2.20 ± 0.18 mmol/l; $P < 0.05$) and insulin (Saline, 4.93 ± 1.03μU/ml; Glucose, 9.77 ± 1.38μU/ml; $P < 0.05$) concentrations and daily values for these animals have previously been published [10].

### 3.1. Effect of glucose infusion on heart and ventricle weight

To determine whether short term increased glucose availability in late gestation alters fetal cardiac growth, both absolute, relative heart and ventricle weights were determined. There was no effect of glucose infusion on either absolute or relative heart or left or right ventricle weights (Table 2).

### 3.2. Effect of glucose infusion on cardiac mRNA and protein abundance in late gestation

***Factors that regulate heart growth:*** There was no effect of glucose infusion on the cardiac mRNA expression of the major molecules involved in fetal growth (*IGF1*, *IGF2)* and their receptors (*IGF1R* and *IGF2R;* Table 3). There was no effect of glucose infusion on the cardiac mRNA expression of molecules involved in glucocorticoid signalling including receptors and the molecule that converts cortisol to cortisone (*NR3C1 (*Fig. 1C*)*, *NR3C2 (*Fig. 1D*)* or *HSD11B2 (*Fig. 1B)).

***Molecular markers of proliferation:*** There was no effect of glucose infusion on the cardiac mRNA expression of cell cycle inhibitors (*CDKN1B*), the IGF1R–PI3K pathway regulator, *PTEN*, or the translational protein *RPS6* (Table 4). There was no effect of glucose infusion on the protein abundance of PCNA (Table 4), a molecule that is essential for DNA replication.

***Molecular markers of pathological hypertrophy and binucleation:*** There was no effect of glucose infusion on the cardiac mRNA expression of *NNPA*, *NNPB*, *MYH7* or *GATA4* (Table 4).

***Molecular markers of oxidative stress:*** Increased glucose concentrations have previously been linked to increased ROS and thus oxidative stress [32,33]. We report no effect of fetal glucose infusion on the cardiac mRNA expression of *HMOX1* (increased in response to oxidative stress), *SOD1*, *SOD2* or *SOD3* (antioxidants that convert superoxide to hydrogen peroxide; Table 5).

***Molecular markers of apoptosis and autophagy:*** Although, there was no evidence of increased oxidative stress within the left ventricle, both apoptosis and autophagy are associated with increased plasma glucose concentrations [34] and can reduce cardiomyocyte endowment. There was no effect of fetal glucose infusion on the cardiac mRNA expression of pro-apoptotic (*BAX and TP53)* or anti-apoptotic *(BCL2)* molecules (Table 5). Additionally, there was no effect of glucose infusion on the cardiac mRNA expression of the autophagy regulator *BECN1(*Fig. 2A*)* or the ratio of MAP1LC3B1 to MAP1LC3B2 protein abundance (Fig. 2B), the gold standard for measuring autophagy as MAP1LC3B1 is converted to MAP1LC3B2 during autophagy.

***Factors regulating fibrosis in the heart:*** Glucose infusion did not influence the cardiac mRNA expression of molecules regulating myocardial fibrosis such as *TGFB1, TIMP1, TIMP2, TIMP3, MMP2* or *MMP9* (Table 6). There was also no change in the mRNA expression of the main collagen isoforms in the heart; collagen type 1 alpha 1 (*COL1A1)* and collagen type 3 alpha 1 *(COL3A1).*

***Molecules involved in glucose metabolism:*** Glucose is the main cardiac fuel in fetal life and increased availability may result in increased transport and metabolism thus we studied pathways that regulate these processes. There was no effect of glucose infusion on the cardiac mRNA expression of *PDK4* and the insulin dependent glucose transporter *SLC2A4* (Fig. 3B). There was, however, a significant decrease in the mRNA expression of the insulin independent glucose transporter, *SLC2A1* (Fig. 3A). There was no effect of glucose infusion on the cardiac protein expression of insulin signalling molecules; *INSR* and phospho-IRS1 (S789 (Table 7);) or the insulin dependent glucose transporter *SLC2A4* (Fig. 3D). Additionally, there was no effect of glucose infusion on the protein expression of AKT1, phospho-AKT1, the ratio of phospho-AKT1 to AKT1, glycogen synthase (GYS1), phospho-GYS1, the ratio of phospho-GYS1 to GYS1 or glycogen synthase kinase (GSK3B; Table 7).

There was an increase in the mRNA expression of *RICTOR,* a subunit of the MTORC2 complex, in the glucose compared to the saline infused fetuses (Fig. 4A). However, there was no effect of glucose infusion on the mRNA expression of *RPTOR* (Fig. 4B), a subunit of the MTORC1 complex or on the protein abundance of either phospho-MTOR (Fig. 4D) or MTOR (Fig. 4C). Additionally, there was no effect of glucose infusion on the protein abundance of downstream signalling molecules phospho-RPS6 (Fig. 4F) and phospho-EIF4EBP1 (Fig. 4E).

***Molecules involved in fatty acid metabolism:*** Hyperglycaemia is associated with changes in cardiac fatty acid metabolism in adults [35] and thus we analysed the expression of molecules involved in both fatty acid transport and metabolism. There was an increase in cardiac *PPARG* (Fig. 5A) and *PPARA* (Fig. 5B) mRNA expression in the glucose compared to the saline infused fetuses. However, there was no effect of glucose infusion on the cardiac mRNA expression of *SERPINE1*(Fig. 5E), *SPHK1*(Fig. 5F), *RXRA* (Fig. 5D) or *PPARGC1A* (Fig. 5C), all of which have *PPARG* response elements.

There was no effect of glucose infusion on the cardiac mRNA expression of *FABP5* (Table 8), nor was there an effect of glucose infusion on the protein expression of ACACA, CPT1B, PRKAA1or phospho-PRKAA1 (S485; Table 8).

Additionally, efficient production of ATP is dependent on the correct functioning of the electron transport chain (ETC); which is made up of 5 protein complexes (OXPHOS complexes). Complexes I, III and IV act as proton pumps with the goal of creating an electrochemical gradient. Complex V (ATPase) is then powered by these protons to convert ADP to ATP. Alterations to the abundance of these OXPHOS complexes may suggest a dysregulation within the ETC; however, we report that glucose infusion had no effect on the protein abundance of these complexes (Fig. 6A–E).

## 4. Discussion

Cardiac development and metabolism are regulated by insulin and IGF1R signalling pathways and thus any change in insulin or IGF1R signalling molecules may alter fetal cardiac growth and metabolic pathways. However, we found that increased fetal plasma glucose and insulin concentrations for 10 days in the late gestation sheep fetus had no effect on either the insulin or IGF1R signalling pathways within the left ventricle. We found no change in the mRNA expression and protein abundance of molecules within the IGF1R

**Table 2**
Impact of intrafetal glucose infusion on fetal heart weight.

| | Control | Glucose |
|---|---|---|
| | (n = 7) | (n = 10) |
| Heart Weight (g) | 34.11 ± 1.59 | 36.62 ± 1.73 |
| Relative heart weight (g/kg) | 7.15 ± 0.37 | 7.29 ± 0.25 |
| Left ventricle weight (g) | 15.94 ± 1.34 | 17.43 ± 0.75 |
| Left ventricle: heart weight (g/g) | 0.48 ± 0.03 | 0.48 ± 0.02 |
| Left ventricle: body weight (g/kg) | 3.47 ± 0.33 | 3.48 ± 0.12 |

Values are mean ± SEM. $P < 0.05$ was considered significant. Data were analysed by an unpaired Student's t-test.

**Table 3**
Effect of glucose infusion on the mRNA expression of molecules that regulate cardiac growth.

| Gene expression (MNE) | Control | Glucose |
|---|---|---|
| | (n = 7) | (n = 10) |
| *IGF1* | 0.096 ± 0.022 | 0.085 ± 0.012 |
| *IGF2* | 5.648 ± 0.942 | 6.126 ± 0.812 |
| *IGF1*R | 0.534 ± 0.053 | 0.580 ± 0.046 |
| *IGF2*R | 1.280 ± 0.085 | 1.343 ± 0.094 |

Values are mean ± SEM. $P < 0.05$ was considered significant. MNE, mean normalised expression. Data were analysed by an unpaired Student's t-test.

**Table 4**
Effect of glucose infusion on the mRNA or protein expression on markers of proliferation or hypertrophy.

| | Control | Glucose |
|---|---|---|
| | (n = 7) | (n = 10) |
| **Proliferation** | | |
| *CDKN1B* (MNE) | 0.232 ± 0.020 | 0.224 ± 0.016 |
| *MYC* (MNE) | 0.057 ± 0.005 | 0.066 ± 0.003 |
| *PTEN* (MNE) | 1.035 ± 0.051 | 1.069 ± 0.085 |
| PCNA: ATCB (AU) | 0.133 ± 0.027 | 0.138 ± 0.012 |
| **Hypertrophy** | | |
| *NNPA* (MNE) | 0.123 ± 0.042 | 0.176 ± 0.035 |
| *NNPB* (MNE) | 0.780 ± 0.268 | 1.167 ± 0.305 |
| *MYH7* (MNE) | 8.418 ± 1.277 | 7.392 ± 0.594 |
| *GATA4* (MNE) | 0.129 ± 0.012 | 0.148 ± 0.014 |
| *RPS6KB* (MNE) | 0.167 ± 0.004 | 0.152 ± 0.008 |

Values are mean ± SEM. $P < 0.05$ was considered significant. MNE, mean normalised expression; AU, arbitrary units; *, treatment. Data were analysed by an unpaired Student's t-test.

**Table 5**
Effect of glucose infusion on mRNA abundance of molecules that are markers for oxidative stress or regulate apoptosis or autophagy.

| Gene expression (MNE) | Control | Glucose |
|---|---|---|
| | (n = 7) | (n = 10) |
| **Oxidative Stress** | | |
| *SOD1* | 1.065 ± 0.079 | 1.179 ± 0.061 |
| *SOD2* | 1.683 ± 0.130 | 1.694 ± 0.104 |
| *SOD3* | 0.133 ± 0.021 | 0.097 ± 0.010 |
| *HMOX1* | 0.079 ± 0.008 | 0.073 ± 0.002 |
| **Apoptosis** | | |
| *BCL2* | 0.011 ± 0.001 | 0.012 ± 0.001 |
| *BAX* | 0.067 ± 0.006 | 0.065 ± 0.004 |
| *TP53* | 0.137 ± 0.010 | 0.125 ± 0.005 |

Values are mean ± SEM. $P < 0.05$ was considered significant. MNE, mean normalised expression. Data were analysed by an unpaired Student's t-test.

**Table 6**
Effect of glucose infusion on mRNA expression of molecules regulating myocardial fibrosis.

| Gene expression (MNE) | Control (n = 7) | Glucose (n = 10) |
|---|---|---|
| *TIMP1* | 0.712 ± 0.182 | 0.449 ± 0.039 |
| *TIMP2* | 0.392 ± 0.050 | 0.338 ± 0.025 |
| *TIMP3* | 0.778 ± 0.138 | 0.658 ± 0.043 |
| *TGFB1* | 0.099 ± 0.004 | 0.097 ± 0.005 |
| *COL1A1* | 6.248 ± 1.483 | 5.786 ± 0.563 |
| *COL3A1* | 4.873 ± 0.466 | 5.278 ± 0.273 |
| *MMP2* | 0.056 ± 0.014 | 0.031 ± 0.003 |
| *MMP9* | 0.007 ± 0.002 | 0.006 ± 0.001 |

Values are mean ± SEM. P < 0.05 was considered significant. MNE, mean normalised expression. Data were analysed by an unpaired Student's t-test.

**Table 7**
Effect of glucose infusion on mRNA (MNE) or protein expression (AU) of molecules in the glucose metabolic pathway.

| | Control (n = 7) | Glucose (n = 10) |
|---|---|---|
| pIRS1: TUBB (AU) | 3.899 ± 0.192 | 4.214 ± 0.356 |
| AKT1: ATCB (AU) | 5.031 ± 0.643 | 4.603 ± 0.495 |
| pAKT1: ATCB (AU) | 0.964 ± 0.133 | 1.183 ± 0.175 |
| pAKT1: AKT1 (AU) | 4.828 ± 0.565 | 4.809 ± 0.673 |
| GSK3B: ATCB (AU) | 5.986 ± 0.957 | 5.815 ± 0.457 |
| GYS1: ATCB (AU) | 0.764 ± 0.034 | 0.819 ± 0.085 |
| pGYS1: ATCB (AU) | 0.763 ± 0.090 | 1.188 ± 0.192 |
| pGYS1:GYS1 (AU) | 1.011 ± 0.137 | 1.470 ± 0.217 |
| *PDK4* (MNE) | 0.004 ± 0.001 (n = 6) | 0.004 ± 0.001 |

Values are mean ± SEM. $P < 0.05$ was considered significant. p, phospho; MNE, mean normalised expression; AU, arbitrary units. Data were analysed by an unpaired Student's t-test.

**Table 8**
Effect of glucose infusion on mRNA (MNE) or protein (AU) expression of molecules within the fatty acid metabolic pathway.

| | Control (n = 7) | Glucose (n = 10 |
|---|---|---|
| *FABP5* (MNE) | 0.471 ± 0.046 (n = 6) | 0.630 ± 0.066 (n = 8) |
| *PRKAA1: ATCB (AU)* | 0.974 ± 0.092 | 1.016 ± 0.134 |
| pPRKAA1: ATCB (AU) | 0.546 ± 0.106 | 1.234 ± 0.476 |
| ACACA: ATCB (AU) | 0.483 ± 0.142 | 0.808 ± 0.280 |
| CPT1B: ATCB (AU) | 0.557 ± 0.061 | 0.603 ± 0.053 |

Values are mean ± SEM. P < 0.05 was considered significant. p, phospho; MNE, mean normalised expression; AU, arbitrary units. Data were analysed by an unpaired Student's t-test.

signalling pathway. This is consistent with there being no difference in the relative heart weight or relative left ventricle weight as a result of glucose infusion.

Although there was no change in the mRNA expression of *RPTOR* (Raptor), glucose infusion increased the expression of *RICTOR* (Rictor). While the MTORC1 complex is formed by the association of mTOR to RPTOR, MTORC2 is formed by mTOR's association to RICTOR [36]. RPTOR is nutrient sensing and is typically responsive to stress, energy levels, oxygen and growth factors [37,38]. As such it regulates cellular growth, cell cycle progression, autophagy and metabolism by phosphorylating downstream targets that include RPS6 and EIF4EBP1 [38,39]. Glucose infusion had no effect on the protein expression of phospho-RPS6 and phospho-EIF4EBP1, both of which are downstream of mTOR. This suggests that this period of glucose infusion in late gestation did not activate the MTORC1 signalling pathway in the fetal heart. In contrast to RPTOR, RICTOR is not responsive to nutrients [36]. However, it is responsive to growth factors such as insulin through P13K and has the potential to phosphorylate AKT1 at Ser473 [40]. AKT1 regulates metabolism, proliferation, growth and apoptosis through the phosphorylation and inhibition of GSK3B and the phosphorylation of FOXO transcription factors [41,42]. As MTORC2 can phosphorylate AKT1 at Ser473, we examined the protein expression of AKT1 and phospho-AKT1 (Ser473) to determine whether the increase in *RICTOR* mRNA expression was associated with AKT1 signalling. However, intrafetal glucose infusion had no effect on the protein expression or phosphorylation of AKT1.

Diabetic cardiomyopathy is characterised by increased ROS, apoptosis, autophagy and myocardial fibrosis [34]. Hyperglycaemia

increases apoptosis by impairing the antioxidant defence system and allowing the levels of damaging ROS to rise [43]. However, there was no change in the mRNA expression of the antioxidants or molecules responsive to oxidative stress that we measured which suggests that the intrafetal glucose infusion did not stimulate an oxidative stress response in the fetal left ventricle.

Fibrosis in the heart is associated with increases in collagen types 1 and 3, resulting in cardiac stiffness that eventually leads to decreased cardiac contractility [44–46]. We hypothesised that increased fetal plasma glucose and insulin concentrations would increase myocardial fibrosis. However, the lack of change in the mRNA expression of both major collagen isoforms in the heart, *COL1A1 and COL3A*, provided evidence that this was not the case. This is further supported by the lack in change to the enzymes that regulate extracellular matrix remodelling [47]. Myocardial fibrosis in the adult diabetic heart is associated with a more chronic form of hyperglycaemia beginning earlier in gestation. It is well characterised that the gestational timing, duration and severity of *in utero* insults can play a role in outcome [48,49]. For example, the lack of changes to fibrotic markers in the present study could be in part due to the gestational timing and duration of the hyperglycaemic insult as well as the severity of the rise in fetal glucose concentrations experienced by the fetus.

The fetal heart predominantly uses glucose and lactate as its energy source [50] and at birth there is a transition to a greater reliance on fatty acid oxidation [51]. Interestingly, the increase in glucose available for ATP production caused only minor alterations in cardiac metabolism that were restricted to the mRNA transcript level. In this study, we found that glucose infusion in late gestation did not alter the protein abundance or phosphorylation of key insulin transduction molecules or the mRNA and protein abundance of the insulin dependent glucose transporter, *SLC2A4*. There was a significant decrease in the mRNA expression of the insulin independent glucose transporter, *SLC2A1,* although with this did not translate to a concomitant decrease in protein abundance. A previous study reported that there was a time dependant decrease in the protein abundance of both insulin dependent and independent glucose transporters as a result of chronic hyperglycaemia (28 d) in late gestation [52]. In that study, glucose was infused into the mother for either 14 or 28 d and this increased fetal plasma glucose concentrations to $3.5 \pm 0.2$ mmol/l, which is ~30% higher than that observed in the present study ($2.20 \pm 0.18$ mmol/l). It should be noted that this decrease in SLC2A1 and SLC2A4 protein abundance was already evident after 14 days of the glucose infusion. Although, the present study did not find any changes in protein expression, it is possible that this may have been due to the duration of the glucose infusion [48]; with a more protracted period of fetal hyperglycaemia having resulted in a decrease in the protein abundance of cardiac glucose transporters. Although both studies infused 50% dextrose 500 g $l^{-1}$ in sterile saline; the present study made use of a 10 d fetal glucose infusion protocol as opposed to a 14 and 28 d maternal glucose infusion protocol and the previous study obtained much higher fetal arterial plasma glucose plasma concentrations. There is also evidence from other intricate fetal sheep studies that show the degree of fetal hypoxaemia to influence glucose utilization [53]. However, both saline and glucose fetal groups where normoxic which suggests that a greater range of fetal oxygenation status would be required to observe a relationship with molecular markers of glucose metabolism. The down-regulation of *SLC2A1* may be a cardio-protective mechanism to avoid intracellular damage and thus apoptosis either by hyperglycaemia-induced caspase 3 activation [34] or by disruption of the anti-oxidant defence system [54]. However, our data suggest that there is no change in apoptosis or ROS, suggesting that the fetal heart diminishes its expression of glucose transporters in response to increased plasma glucose.

Alterations to fatty acid metabolic signalling molecules have the potential to program an altered metabolic profile in the fetal heart. For example, there is increased reliance on cardiac glucose metabolism in low birth weight lambs (lambs that were exposed to both a deficit in glucose and oxygen availability *in utero*) at 21 d after birth [29]. We have shown that hyperglycaemia in late gestation upregulates the mRNA expression of both *PPARG* and *PPARA*. There were, however, no changes in the mRNA expression of the transcriptional co-factors *PPARGC1A* or *RXRA*. Cardiac overexpression of *PPARA* decreases glucose transport and utilization through the down-regulation of *SLC2A4* and *PDK4* while fatty acid transport and utilization was upregulated [55]. Neither of these changes was apparent in the present study. Although fatty acid metabolism produces more energy, it requires more oxygen [51]. Thus, one explanation for the lack of changes to both glucose and fatty acid metabolic signalling molecules is that as fatty acids do not cross the placenta as readily as glucose and the fetal environment is relatively hypoxaemic; protective mechanisms may be in place to ensure that the fetal heart adheres to a lower oxygen requiring glucose metabolic profile. *PPARG*, which is abundant in adipose tissue and plays a role in lipid storage, had increased mRNA expression in response to glucose infusion. The implications of this remain unclear. Investigation into the expression of genes with PPARG response elements such as *SERPINE1* and *SPHK1* showed that there was no change in their expression in response to glucose infusion. Furthermore, glucose infusion had no impact on the protein abundance of OXPHOS complexes I–V; highlighting that the electron transport chain in the fetal heart was not impacted by the fetal glucose infusion.

There are several limitations to the present study. Although we report gross cardiac morphology, we were unable to determine whether individual cardiomyocyte size or the proportion or mononucleated to binucleated cardiomyocytes was impacted by the increased glucose availability. This would have required cardiomyocytes being dissociated from the fetal hearts [56,57], a process that renders the remaining cardiac tissue unfit for further molecular analysis. That being sad, the expression of the signalling molecules that regulate cardiac growth and maturation were not impacted by the increased glucose availability and thus morphological/maturational alterations at the level of the individual cardiomyocyte are unlikely. Furthermore, a more functional assessment of myocardial glucose delivery, uptake and utilization such as those performed in fetal sheep by Barry et al. would have further elucidated the impact of increased glucose availability on the fetal heart [58,59] as would a molecular assessment of those signalling molecules regulating lactate metabolism. It should also be noted that in this study, molecular assessment was conducted in the left ventricle and it is possible that the right ventricle and/or septum could have been impacted differentially.

## 5. Conclusion

In summary, we have shown that although a 10 d fetal infusion of glucose that significantly increases plasma glucose and insulin

concentrations and induces changes in other organs such as the fetal brain, adipose tissue and the lung (Fig. 7); the fetal left ventricle remains relatively unaffected at the mRNA and protein level. This may in part be due to cardio-protective mechanisms. Furthermore, the glucose infusion was performed in late gestation, a time in the fetal sheep heart where most cardiomyocytes have left their proliferative growth phase and are now binucleated, contributing to heart growth via hypertrophy. We have previously shown that *in utero* insults have differential effects based on their timing, severity and duration [48,49,60]. As such, one possibility is that an increase in fetal glucose and insulin concentrations earlier in gestation during the proliferative phase of cardiac growth may have had a different and potentially detrimental response.

## Author contribution statement

Jack Darby: Performed the experiments; Analysed and interpreted the data; Wrote the paper.
Song Zhang: Performed the experiments; Analysed and interpreted the data.
Stacey L. Holman: Performed the experiments.
Beverly S. Muhlhausler: Performed the experiments; Analysed and interpreted the data.
I Caroline McMillen: Conceived and designed the experiments; Performed the experiments; Analysed and interpreted the data; Contributed reagents, materials, analysis tools or data.
Janna L. Morrison: Conceived and designed the experiments; Performed the experiments; Analysed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

## Data availability statement

Data will be made available on request.

## Ethics statement

All procedures were approved (S-31-2004) by the University of Adelaide Animal Ethics Committee and comply with the Australian code of practice for the care and use of animals for scientific purposes.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18292.

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