Author/s:
Finger, BJ;Harvey, AJ;Green, MP;Gardner, DK

Title:
Combined parental obesity negatively impacts preimplantation mouse embryo development, kinetics, morphology and metabolism

Date:
2015-04-09

Citation:
Finger, B. J., Harvey, A. J., Green, M. P. & Gardner, D. K. (2015). Combined parental obesity negatively impacts preimplantation mouse embryo development, kinetics, morphology and metabolism. Human Reproduction, 30 (9), pp.2084-2096. https://doi.org/10.1093/humrep/dev142.

Combined parental obesity negatively impacts preimplantation mouse embryo development, kinetics, morphology and metabolism

Journal:	*Human Reproduction*
Manuscript ID:	HUMREP-15-0191.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Finger, Bethany; University of Melbourne, BioSciences Harvey, Alexandra; University of Melbourne, BioSciences Green, Mark; University of Melbourne, BioSciences Gardner, David; ; University of Melbourne, BioSciences
Keywords:	obesity, high fat diet, ZONA PELLUCIDA, time-lapse
Specialty:	Embryology

Title: Combined parental obesity negatively impacts preimplantation mouse embryo development, kinetics, morphology and metabolism

Running title: Parental obesity and embryo viability

Bethany J. Finger[1], Alexandra J. Harvey[1], Mark P. Green[1] and David K. Gardner[1*]

[1]School of BioSciences, University of Melbourne, Parkville, Melbourne 3010, Victoria, Australia.

Corresponding author: *Prof David Gardner, School of BioSciences, University of Melbourne, Parkville, Melbourne 3010, Victoria, Australia.

Phone: +61 3 8344 6259, E-mail: david.gardner@unimelb.edu.au

Abstract

Study question: Does combined parental obesity, both an obese mother and father, have a greater effect on mouse preimplantation embryo development and quality than single parent obesity?

Summary answer: Combined parental obesity causes a greater reduction in blastocyst rate, and a greater delay to the timing of key embryonic developmental events than single parental obesity, as well as altering embryonic characteristics, such as zona pellucida width.

What is known already: Maternal or paternal obesity alone are known to have significant and detrimental impacts on preimplantation embryo development. Furthermore, these early embryonic perturbations can have long-term impacts on both offspring health, and further generations. This is one of the first studies to examine the effects of having both an obese mother and obese father.

Study design, size, duration: A cross sectional control versus treatment mouse study of diet-induced obesity was employed, in which 300 embryos per group were generated and studied from reciprocal matings: i) control female and control male (Lean Parented Embryos); ii) control female and obese male (Paternal Obese Parented Embryos); obese female and control male (Maternal Obese Parented Embryos); and iv) obese female and obese male (Combined Obese Parented Embryos). Assessments of embryonic development rate, timing of development, morphological characteristics, metabolic gene expression, metabolism and cell lineage allocation were made at selected time points and analysed in relation to parental obesity status.

Participants/materials, setting, methods: Three-week old C57BL6 male and female mice were fed control (7% kcal fat) or high fat (21% kcal fat) diets for a minimum of eight weeks. Females were superovulated, mated, fertilized zygotes recovered and standard mouse *in vitro* embryo culture performed. Time-lapse monitoring was undertaken to compare developmental timings and morphological characteristics (embryonic area and zona pellucida width) for embryos from all four reciprocal matings. Differential staining identified cell lineage allocation. Real-time quantitative RT-PCR (qRT-PCR) and microfluorescence were used to measure gene expression and metabolism (glucose consumption and lactate production) respectively, in embryos from Lean Parented and Combined Obese Parented matings. This research was completed in a University research laboratory.

Main results and the role of chance: Blastocyst rate was reduced in Combined Obese Parented embryos when compared to both Single Obese (11% decrease for Maternal Obese Parented, $P < 0.05$; 15% for Paternal Obese Parented, $P < 0.05$) and Lean Parented embryos (25% decrease, $P < 0.01$). Time-lapse analysis of developmental kinetics highlighted a delay of 1 h at the 2-3 cell division, extending to 6 h delay by the blastocyst stage for Combined Obese Parented embryos ($P < 0.05$). A reduction in total cell number of Combined Obese Parented blastocysts was a further manifestation of this developmental delay ($P < 0.05$). Zona pellucida width was reduced in Combined Obese Parented embryos ($P < 0.05$). Glucose consumption was increased in Combined Obese Parented embryos ($P < 0.05$), which was associated with the up-regulation of *Glut1* expression ($P < 0.05$).

Limitations and reason for caution: This study was completed in fertile C57BL/6 mice using a well-defined model of diet-induced obesity in which embryos were fertilised *in vivo*. Human obesity is complex, with many causes and co-morbidities, and therefore the impact of combined obesity would require further investigated in human settings.

Wider implications of the findings: This study demonstrates that combined parental obesity has a detrimental impact on mouse embryo development, a finding consistent with previous studies on individual parent obesity. Of note, the effect of combined parental obesity upon embryo development markers was greater than that of individual parental obesity. Plausibly, human embryos will be similarly impacted. The reduction in blastocyst rate and delayed time to developmental events confirms that embryos of obese parents differ from those of lean parents. Allowance for this should therefore be incorporated into clinical practice when selecting the best embryo for transfer of an obese couple.

Study Funding/competing interest(s): Funding was provided by University of Melbourne research monies. M.P.G currently holds the position of Merck Serono Lecturer of Reproductive Biology. D.K.G. received research funds from Vitrolife AB Sweden. The other authors of this manuscript have nothing to declare and no conflicts of interest.

Keywords: obesity, high fat diet, zona pellucida, time-lapse

Introduction

The World Health Organisation recognises obesity as the epidemic of the 21st century, with greater than 30% of adults around the world defined as obese (Body Mass Index ≥ 30 kg/m^2) (WHO, 2013; Ng *et al.,* 2014). The reduction to an individual's reproductive success is one of the many impacts of obesity (Mokdad *et al.,* 2003; Guh *et al.,* 2009). For the non-scientific community, this would be underscored by the lowered ability of obese individuals to conceive and maintain pregnancy (Norman and Clark, 1997; Maheshwari *et al.,* 2007; Feuer *et al.,* 2013). Such reductions in fecundity are consistent with the increased level of aneuploidy, mitochondrial dysfunction, endoplasmic reticulum dysfunction and poor morphology observed in the oocytes and sperm of obese individuals, both human and animal (Jensen *et al.,* 2004; Kort *et al.,* 2006; van der Steeg *et al.,* 2008; Robker *et al.,* 2009; Bakos *et al.,* 2011; Binder *et al.,* 2012a; Luzzo *et al.,* 2012; Dupont *et al.,* 2013; Wu *et al.,* 2015). Of further concern is the ability of parental obesity to act as a "developmental programmer", and thus impact health across multiple generations (Chavatte-Palmer *et al.,* 2012; Lane *et al.,* 2014).

Historically, developmental programming focused upon environmental exposures, typically post-implantation and throughout gestation (Silveira *et al.,* 2007; Wadhwa *et al.,* 2009). Numerous studies in humans and other species ~~however~~ have now established that developmental programming can occur during the peri-conception period, and lead to long-term health impacts upon offspring (Gardner *et al.,* 2004; Watkins *et al.,* 2010; Maloney *et al.,* 2011). Indeed, evidence from rodent studies reveals that maternal obesity results in reduced blastocyst rate, slower embryonic development, down-regulation of key metabolic genes, as well as negative impacts on fetal health (Bermejo-Alvarez *et al.,* 2012; Binder *et al.,* 2012b; Luzzo *et al.,* 2012). Similarly, paternal obesity imparts negative effects on embryonic health including delayed timing of embryonic development, altered glucose metabolism of the blastocyst, perturbed fetal development, as well as impacting fecundity in subsequent generations of mice (Binder *et al.,* 2012a; Binder *et al.,* 2012b; Fullston *et al.,* 2012; McPherson *et al.,* 2014). This is likely mediated through mitochondrial dysfunction (maternal); microRNAs (paternal), as well as genetic and epigenetic mechanisms (Skinner, 2011; Lane *et al.,* 2014). Importantly, human evidence also supports the theory that maternal or paternal obesity alters

embryonic development, as demonstrated by reduced success in IVF cycles (Styne-Gross *et al.,* 2005; van der Steeg *et al.,* 2008; Robker *et al.,* 2009; Bakos *et al.,* 2011a). Of note, the studies above all focussed upon the impacts of individual parental obesity; maternal obesity or paternal obesity alone, with little human data on the effects of combined parental obesity.

Obesity rates are increasing around the world, and couples of reproductive age are likely to share lifestyle choices. Thus, cases of combined parental obesity (both an obese mother and obese father) are becoming more common. The impacts of combined parental obesity upon offspring are however yet to be elucidated. To date, only one study of combined parental obesity has been undertaken, which amongst other findings, noted poor fertilisation rates for obese couples undergoing IVF in Germany (Kupka *et al.,* 2011). However this study included a number of confounding variables, including socioeconomic status and parental age. Hence more clinical studies are required. Based on previous work on maternal and paternal obesity in both humans and animals, it is hypothesised that combined parental obesity has a greater detrimental effect on fertility, as measured by early embryo development and quality, than that of single parental obesity (either maternal or paternal). Therefore, in this study we determined how combined parental obesity impacted ~~pre-implantation~~preimplantation embryo development and quality in a mouse model. The advantage of this model is that it minimises many potential confounders evident in clinical studies. Specifically, the aims of this study were to characterise the effect of combined parental obesity on: i) the developmental potential, morphology, kinetics and cell lineage allocation of the ~~pre-implantation~~preimplantation embryo, and ii) the metabolism and expression of key metabolic genes in the blastocyst. In characterising these effects, this work further highlights the importance of combined parental obesity in the pre-conception period, as well as indicating potential pathways in which these effects can be mitigated and ultimately improve the reproductive success of obese couples.

Materials and Methods

Experimental animals, diets and hormonal stimulation

Three-week-old male and female C57BL/6 mice (WEHI; Melbourne, Australia) were randomly

assigned to either a control diet consisting of 7% total fat and 19.4% protein (AIN93G; Specialty Feeds, Perth, Australia), or a high fat diet (HFD) consisting of 21% total fat and 19% protein (SF00-219; Specialty Feeds, Perth, Australia). Mice were fed for a minimum of eight to a maximum of twelve weeks, at which point they were defined as control or obese, respectively, based on previous characterisation (Bakos *et al.*, 2011b; Mitchell *et al.*, 2011; Binder *et al.*, 2012b; Fullston *et al.*, 2012). Mice were maintained in a 12 h light: 12 h dark photoperiod with food and water supplied *ad libitum*. Female mice were caged in groups of four and male mice were caged individually. Body weight was recorded weekly.

Female mice were superovulated with an intraperitoneal injection of 0.25 IU g^{-1} mare serum gonadotrophin (PMSG; Folligon, Intervet, Bendigo, Australia) followed 48 h later by 0.25 IU g^{-1} human chorionic gonadotrophin (hCG; Chorulon, Intervet). Mice were mated to produce four reciprocal matings from which resultant embryos were studied: i) control female and control male (Lean Parented Embryos); ii) control female and obese male (Paternal Obese Parented Embryos); obese female and control male (Maternal Obese Parented Embryos); and iv) obese female and obese male (Combined Obese Parented Embryos).

Ethical approval

This study was carried out in accordance with the *Australian code of practice for the care and use of animals for scientific purposes*, and all protocols were approved by the Animal Ethics Committee of The University of Melbourne.

Embryo collection

Twenty-two hours post-hCG injection, pronucleate oocytes were collected in G-MOPS handling medium supplemented with 5 mg/mL HSA (GMOPS+; Vitrolife, Göteborg, Sweden). Pronucleate oocytes were denuded of cumulus cells via incubation in GMOPS containing 300 IU/ml hyaluronidase for 20 seconds (bovine testes, type IV; Sigma-Aldrich, Castle Hill, NSW, Australia) followed by washing in GMOPS+. Denuded pronucleate oocytes were immediately washed in

GMOPS+ and cultured as previously detailed (Gardner and Lane, 2014).

Embryo culture: time-lapse analysis

Embryo morphokinetics were assessed by time-lapse analysis using an EmbryoScope multi-gas incubator (Unisense, Aarhus, Denmark). Methods for mouse embryo culture in this incubator were modified from a human protocol (Meseguer *et al.,* 2011), in which, individual pronucleate oocytes were transferred to 25 µl drops of G1 medium (Gardner and Lane, 2014) in EmbryoSlide dishes (Unisense) under 1.2ml paraffin oil (Ovoil, Vitrolife) and cultured until 72 h post-hCG under 6% CO_2, 5% O_2 and 89% N_2 at 37°C. Embryos were then transferred to pre-equilibrated G2 medium (Gardner and Lane, 2014) and cultured for a further 46 h. Images of embryo development were acquired every seven minutes throughout the culture period, at five planes of view. The timings of developmental milestones were calculated post-hCG, as well as from pronuclear envelope breakdown. Resultant embryos were assessed for developmental stage before being subjected to differential staining. Timing of developmental events and specific embryo characteristics (zona pellucida width, perivitelline space and embryo area) were then determined retrospectively from stored images (EmbryoViewer; Unisense). Measurement of individual embryos were standardised to the plane of the maximum width of the polar body, and measured at the pronucleate oocyte and early blastocyst stage. Data were analysed only for embryos that developed ~~on time~~ to the blastocyst stage on time (by day 4.5), as previously defined (Wale and Gardner 2010; Gardner *et al.,* 2004). Only those that developed to the blastocyst stage were used for analysis, to avoid skewing results to groups with lower developmental potential and for relevance to clinical practice in which only data on transferable embryos would be considered.

Embryo culture for metabolic and gene expression analysis

Embryos for metabolic and gene expression analysis were cultured in a Sanyo 19M multi-gas incubator (Sanyo Corporation, Osaka, Japan) under a 6% CO_2, 5% O_2 and 89% N_2 atmosphere at 37°C (Gardner and Lane, 2014). Embryos from Lean Parental matings and Combined Parental Obesity matings were cultured individually in 5 µl pre-equilibrated G1 media under 3.5 ml paraffin

oil. At 72 h post-hCG, embryos were transferred to a) 1 µl pre-equilibrated modified G2 medium (containing glucose as the sole source of carbohydrates) under 3.5 ml paraffin oil for metabolic analysis or b) 5 µl pre-equilibrated G2 medium under 3.5 ml paraffin for a further 48 h before being snap frozen ~~and stored gene expression analysis~~for subsequent gene expression analysis.

RNA extraction and Reverse transcription

Total RNA was isolated from frozen blastocysts from Lean Parental matings and Combined Parental Obesity matings (n =40 blastocysts/group, repeated in triplicate) using an Absolutely RNA Nanoprep Kit (Agilent Technologies; Mulgrave, Australia) according to the manufacturer's instructions (Dupont *et al.,* 2012). Briefly, RNA was bound to a column matrix and a series of salt washes removed contaminants. Isolated RNA was DNase treated to eliminate contaminating DNA according to the manufacturer's specifications (Agilent Technologies). The cDNA was synthesized from RNA using Superscript III Reverse Transcriptase (Life Technologies; Mulgrave, Australia) and Random Primers (Promega; Alexandria, Australia) according to the manufacturer's instructions (Invitrogen; Carlsbad, USA) and as previously described (Harvey *et al.,* 2004).

Quantitative RT-PCR gene expression analysis

Real time qRT-PCR was performed on a ViiA^{TM}7 thermocycler (Applied Biosystems, Mulgrave, Australia) as previously described (Harvey *et al.,* 2004). Primers were designed using Primer Express (Applied Biosystems) and were synthesized by Geneworks (Adelaide, Australia). Primer specificity and efficiency were calculated using dilutions of liver cDNA samples, followed by embryo cDNA samples. The genes investigated were Glucose transporter 1 (*Glut 1*), Pyruvate kinase isoform two (*Pkm2*), Peroxisome proliferator-activated receptor gamma (*Ppar γ*), Insulin growth-like factor 2 receptor (*Igf2r*), and Glucose transporter 3 (*Glut 3*). Primer sequences and product sizes are detailed in Table I. ~~Primer efficiencies were calculated using dilutions of liver and embryo cDNA samples.~~

Real time qRT-PCR was performed in triplicate 10 µl reactions containing 1X SYBR Green Master Mix (Invitrogen) and 500 nM forward and reverse primers. cDNA was diluted in nuclease-free water

(Life Technologies) to a concentration equivalent to cDNA generated from 0.25 embryo per microlitre. Reactions were run according to the following parameters; 50°C for 5 mins, 95°C for 10 mins, then 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 5 mins. The cycle threshold (Ct) was calculated for each sample using the ViiA™7 software. Dissociation curves, to detect non-specific amplification, were generated for all reactions. No template samples containing water substituted in place of cDNA were included in all assays to confirm the absence of non-specific amplification products, as were –minus RT samples to confirm a~~the a~~bsence on DNA contamination. Raw Ct values were analysed using the delta CT method in the Q-Gene software package (Muller *et al.,* 2002; Simon, 2003) normalised to that of 18S~~S~~ rRNA. 18S rRNA was confirmed as an appropriate housekeeper, against a group of candidate housekeeper genes, using NormFinder software package (Andersen *et al.,* 2004). –Data were then expressed as a fold change relative to the Lean Parented embryo group.

Assessment of glucose consumption and lactate production

Individual embryo glucose consumption and lactate production was assessed in a cohort of embryos from Lean Parental matings and Combined Parental Obesity matings. Compacted morula were placed into G2-modified media, containing 0.5 mM glucose as the sole carbohydrate source, and without lactate at 72 h post-hCG for a period of 24 h. Spent medium of resultant early blastocysts (early blastocyst defined as a stage from cavity formation to less than half total embryo volume comprising the blastocoel) was analysed by microfluorescence as previously described (Gardner and Leese, 1990; Lane and Gardner, 1998). Subsequently, total cell number was determined via staining in 0.1 mg/ml Bisbenzimide (Hoechst, 33342; Sigma Aldrich; Castle Hill, Australia) in 10% v/v ethanol for 30 min, washed in GMOPS+ for 5 min then mounted in glycerol on glass slides under coverslips (Thermo Fisher; Scoresby, Australia). Cell numbers were visualised and photographed using a fluorescent microscope (Nikon Eclipse TS100; Olympus, Tokyo, Japan) equipped with a Nikon Digital Sight DS-L2 camera (Nikon; Tokyo, Japan). Cell numbers were retrospectively determined manually using ImageJ Version1.47 (Schneider *et al.,* 2012). Metabolic measurements were expressed on a per

embryo basis, as well as per cell per hour for each individual embryo to account for variation in embryonic cell number.

Assessment of blastocyst cell allocation

Allocation of cells to the inner cell mass (ICM) and the trophectoderm (TE) of blastocysts was determined via differential nuclear staining, as previously described (Hardy *et al.,* 1989). Briefly, blastocysts were placed in 0.5% pronase (Sigma-Aldrich) until the zona pellucida disbanded, followed by washing in GMOPS+ for 5 mins. Embryos were then incubated in 10 mM 2,4,6-trinitrobenzene sulfonic acid (TNBS; Sigma-Aldrich) for 10 min then washed in GMOPS+ for 5 min, before a 10 min incubation in 0.1 mg/ml anti-dinitrophenol (Sigma-Aldrich). Blastocysts were subsequently washed for 5 min in GMOPS+, then incubated in 10% v/v guinea pig serum with 25 mg/ml propidium iodide (IMVS, Adelaide, Australia) for 5 min. Blastocysts were transferred to 0.1 mg/ml Bisbenzimide (Sigma-Aldrich; Hoechst, 33342) in 10% v/v ethanol for 15 min, washed in in GMOPS+ and finally mounted in glycerol on glass slides under coverslips (Thermo Fisher). Cell were visualised and photographed using a fluorescent microscope (Nikon Eclipse TS100) equipped with a Nikon Digital Sight DS-L2 camera (Nikon). Cell numbers were then counted manually using ImageJ Version1.47 (Schneider *et al.,* 2012).

Statistical analyses

All data were assessed for normal distribution via the Shapiro–Wilk test. Animal body weight gain was analysed using a two-tailed t-test. Day 5 blastocyst rate was arc-sine transformed prior to analysis. Metabolic data were measured as pmol/embryo/h, and also normalised for cell number prior to analysis. Gene expression levels were normalised to that of the Lean Parented embryo group. Time-lapse data were analysed relative to hours post-hCG, and also normalised to individual embryo pronuclear envelope breakdown to mitigate possible effects of the timing of mating. The effect of parental obesity on embryo measurements (zona pellucida width, perivitelline space and embryo area), timing of developmental events and cell allocation were analysed by ANOVA using a PROC MIXED procedure employing a Tukey's post hoc analysis to identify differences between groups.

Culture replicate was included in the model as a random factor. Metabolic data, total cell counts and gene expression data were analysed using two tailed t-tests. All analyses were performed in SPSS Version 20 (IBM, Armonk, USA). Data are presented as a mean ± SEM unless otherwise stated. Significance was determined at the level of $P < 0.05$.

Results

Impact of dietary fat on parental weight gain

Female mice fed a HFD gained significantly more weight after an eight week feeding period compared with control fed mice, as well as having significantly increased peritoneal fat deposits ($P < 0.001$, Table II). Similarly, male mice fed a high fat diet for eight weeks gained significantly more weight than mice on control diets and had increased peritoneal fat deposit weight ($P < 0.01$, Table II).

Impact of parental obesity upon mating and fertilisation success

The presence of a copulatory plug (indicative of mating success) was significantly lower in the Combined Obesity Parental matings (33.56% ± 5.87) compared with Lean Parental matings (68.05% ± 5.76, $P < 0.001$). There was no significant difference between Paternal Obese mating success or Maternal Obese mating success compared with Lean Parental matings ($P > 0.05$). The number of oocytes ovulated per female (including fertilised, unfertilised and degenerate) was significantly reduced in the females mated in Combined Obese Parental matings (11.74 ± 0.73, $P < 0.01$). The number of fertilised eggs per female was also significantly reduced in the females of Maternal Obese matings (9.21 ± 1.56, $P < 0.05$), and of Combined Obese Parental matings (8.36 ± 0.66, $P < 0.001$) compared with females of Lean Parental matings (12.65 ± 0.71).

~~The presence of a copulatory plug (indicative of mating success) was significantly lower in the Combined Obesity Parental matings (33.56%±5.87) compared with Lean Parental matings (68.05%±5.76) ($P < 0.001$). There was no significant difference between Paternal Obese matings or Maternal Obese matings compared with Lean Parental matings ($P > 0.05$). The number of oocytes ovulated per female (including fertilised, unfertilised and degenerate) was significantly reduced ($P < 0.01$) in the females mated in Combined Obese Parental matings (11.74±0.73). The number of~~

~~fertilised eggs per female was also significantly reduced in the females of Maternal Obese matings (9.21±1.56) ($P < 0.05$), and of Combined Obese Parental matings (8.36±0.66) ($P < 0.001$) compared with females of Lean Parental matings (12.65±0.71).~~

~~The presence of a copulatory plug (indicative of mating success) was significantly lower in the Combined Obesity Parental matings compared with Lean Parental matings ($P < 0.001$). The number of oocytes ovulated per female (including fertilised, unfertilised and degenerate) was significantly reduced ($P < 0.01$) in the females mated in Combined Obese Parental matings. The number of fertilised eggs per female was also significantly reduced in the females of Maternal Obese matings ($P < 0.05$), and of Combined Obese Parental matings ($P < 0.001$).~~

Obesity effects on developmental potential

Compared with Lean Parented embryo development rates, the percentage of fertilised zygotes reaching the blastocyst stage was significantly reduced in groups in which one parent was obese (Paternal Obese Parented 15% decrease, Maternal Obese Parented 11% decrease, $P < 0.05$; Figure 1). Similarly, compared with the Lean Parented embryo development rates blastocyst development from Combined Obese Parented embryos was further decreased (27% decrease, $P < 0.01$). Development rates of Combined Obese Parented embryos did not differ from embryos that had one obese parent ($P > 0.1$). This reduction in blastocyst rate did not correlate to a block at a specific developmental stage, rather development failure occurred throughout the culture period.

Obesity effects on developmental kinetics

Analysis of developmental timings relative to hours post-hCG administration demonstrated a delay of 1 h by pronuclear envelope breakdown (PNB, $P <$ 0.05) ~~in~~ in Combined Obese Parented compared with Lean Parented embryos. Timing of the pronuclear envelope breakdown in Combined Obese Parented embryos did not differ from either Maternal Obese Parented or Paternal Obese Parented embryos ($P > 0.1$). Paternal Obese Parented and Maternal Obese Parented embryos did not show significant delays in developmental timing compared to Lean Parented embryos (P -> 0.1). The delay in developmental timings evident in Combined Parental Obese Parented embryos extended to ~~7~~ 7 h at

the time of blastocoel formation (tSB, $P < 0.01$; Figure 2a) relative to those of Lean Parented embryos. ~~, and equated to an additional 2 h delay when compared with embryos from Maternal Obese Parented ($P < 0.05$) or Paternal Obese Parented embryos ($P < 0.05$).~~

When developmental timings were expressed relative to individual embryo pronuclear envelope breakdown, rather than hours post-hCG to remove any potential cofounding effects in the time of mating, delays in development to the 2-cell stage were not evident (t2, $P > 0.1$). ~~However, delays of 1 and 3 h ($P < 0.05$) were evident at the blastocyst stage in both the Maternal Obese Parented and Paternal Obese Parented embryos, respectively, relative to Lean Parented embryo developmental timing.~~ Combined Obese Parented embryos displayed ~~no delay at the first cleavage, but~~ a 1 h delay at the 4-cell division (t4, $P < 0.05$), which extended to a 6 h delay at the time of blastocoel formation (tSB, $P < 0.01$; Figure 2b) relative to Lean Parented embryos. Timing of the first cleavage division did not differ significantly from Maternal Obese Parented or Paternal Obese Parented embryos ($P > 0.1$). Significant differences between Combined Obese Parented Embryos and Maternal Obese Parented or Paternal Obese Parented embryos appeared from the 5-cell division onwards and persisted until the time of blastocoel formation (tSB, $P < 0.05$). ~~and blastocyst formation in Combined Obese Parented embryos did not differ significantly from Maternal Obese Parented or Paternal Obese Parented embryos ($P > 0.1$).~~

Obesity effects on blastocyst cell lineage allocation

No differences in inner cell mass (ICM) cell number or ICM:TE ratio were observed between any of the groups (Figure 3a and c). ~~Total and~~ Trophectoderm (TE) and Total cell numbers were reduced in both Maternal Obese Parented and Combined Obese Parented embryos relative to Lean Parented embryos ($P < 0.05$; Figure 3b~~a~~ and d~~b~~).

Metabolic analysis

Glucose consumption and lactate production when expressed per embryo were not different between Lean Parented and Combined Obese Parented embryos (Figure 4a). Total cell number was ~~higher~~

lower in Combined Obese Parented than Lean Parented embryos ($P < 0.01$, Figure 4b). Glucose consumption, when normalised for total embryo cell number, was higher ($P < 0.01$) in embryos from Combined Obese Parented embryos compared with Lean Parented embryos (Figure 4c). Lactate production did not differ between the two groups ($P > 0.1$). Glycolytic rate was higher in Combined Obese Parented embryos (52%) compared with Lean Parented embryos (42%), however this difference was not significant (Figure 4d, $P > 0.1$).

Quantitative gene expression analysis in response to combined parental obesity

Glucose transporter 1 (*Glut1*, also known as *Slc2a1*) expression was increased in Combined Obese Parented relative to Lean Parented embryos ($P < 0.05$~~1~~, Figure 5a). Pyruvate kinase isoform 2 (*Pkm2*~~)~~) expression was significantly increased in Combined Obese Parented relative to Lean Parented embryos ($P < 0.01$, Figure 5b). ~~and p~~Peroxisome proliferator-activated receptor gamma (*Pparγ*) showed a trend towards increased expression in the Combined Obese Parented compared with Lean Parented embryos ($P = 0.08$~~, and $P = 0.07$ respectively ,~~ –Figure ~~5b and~~ 5c). No differences between groups were evident in insulin-like growth factor two receptor (*Igf2r*) or glucose transporter three (*Glut3*) expression (Figure 5d and 5e), respectively.

Obesity alters zona pellucida characteristics

At the pronucleate oocyte stage, zona pellucida (ZP) width was reduced in Combined Obese Parented embryos compared to Lean Parented embryos (Figure 6, $P < 0.05$). Paternal Obese Parented embryos showed no difference in ZP width from Lean Parented embryos. Maternal Obese Parented embryos did not demonstrate a difference in ZP width from Lean Parented or Combined Obese Parented embryos ($P > 0.1$). There was no difference in measurements of total embryo area and width of the peri-vituline space between any groups ($P < 0.1$). For measurements taken at the blastocyst stage, there was a reduction in ZP width for all groups compared to the pronucleate oocyte stage ($P < 0.05$). ~~By the blastocyst stage, the ZP of Combined Obese Parented embryos remained thinner, although not significantly, from those of embryos in all other groups. Similarly to the pronucleate oocyte stage, there was no difference across groups regarding total embryo area or width of the peri-vituline space.~~

373

374

375 **Discussion**

376 ***Developmental rate and kinetics are reduced by the obese state of parents***

377 The number of two-cell embryos that developed on ~~to~~ time to the blastocyst stage was significantly
378 reduced across all groups with an obese parent compared to the Lean Parented embryos, and this
379 reduction was more pronounced in Combined Obese Parented embryos. A reduction in developmental
380 competence of embryos produced when both parents are obese is consistent with previous studies on
381 individual parent obesity in both animals and humans (Bakos *et al.,* 2011a; Bakos *et al.,* 2011b;
382 Binder *et al.,* 2012b; Luzzo *et al.,* 2012) and is likely caused by a number of factors, contributed from
383 both the mother and the father, as reviewed by Lane (2014). One of the principal issues appears to be
384 that oocytes from obese female mice and women show increased levels of aneuploidy, mitochondrial
385 and endoplasmic reticulum stress and apoptosis, hence these conditions may lead to embryonic
386 developmental arrest and may not be permissible to blastocyst formation (Hardy *et al.,* 2001; Acton *et*
387 *al.,* 2004; Igosheva *et al.,* 2010; Luzzo *et al.,* 2012; Wu *et al.,* 2015). Human ~~Human~~ embryos of
388 obese couples ~~are likely to~~may show a similar reduction in blastocyst rate, as observed in human
389 paternal obese parented embryos (Bakos *et al.,* 2011a). There may also be a ~~if not~~ greater reduction
390 in human blastocyst rates based on additional confounding factors, such as subfertility and other
391 comorbidities, which would translate to a decreased potential for implantation and successful
392 pregnancy.

393

394 Notably, in the current study for those embryos that did develop to the blastocyst stage, development
395 was at a slower rate than that of Lean Parented embryos, with an initial delay of 1 h at the 3-cell
396 division, which accumulated to 6 h by the blastocyst stage. This delay is greater than previously seen
397 in individual parental obesity studies, which reported differences of one hour or less throughout
398 development in IVF and mated models of embryonic development (Binder *et al.,* 2012a; Binder *et al.,*
399 2012b). Human studies of the impacts of parental obesity and timing of embryonic development
400 require further investigation, as these studies remain limited, with conflicting reports (Bellver *et al.,*

2013; Leary *et al.*, 2015), potentially due to small sample sizes. The combined contributions of oocytes and sperm from obese parents may lead to the increase in developmental times and/or greater variation in the time at which embryos reach expected developmental time points. This may therefore have clinical implications when standardising embryo developmental timings across patients with a range of BMIs, as well as for selecting the best embryo to transfer. The present study employed a mated model, thus variation in mating time may also contribute to the delay in embryonic development. However, in the present study, timings were also calculated from pronuclei disappearance to account for any differences in time of mating. Irrespective of this correction, delays in embryonic ~~the~~ developmental timing~~s~~ were still evident. Furthermore, the length of time between 2- and 3-cell divisions, which is independent of mating time, was increased in the Combined Obese Parented embryos. Importantly in ART clinics, this length of time has previously been determined as a marker for embryo quality in two independent human studies, with increased time giving embryos a 'negative score' in the algorithm regarding transferrable quality embryos (Wong *et al.,* 2010; Meseguer *et al.,* 2011). Therefore, the innate ability of the resulting zygote to develop appears compromised independent of differences in time of mating.

The exposure to reproductive tract fluids in this model may also contribute to the alteration in embryonic developmental timing. Seminal fluid is known to have increased triglyceride concentrations in obese animals and humans, and it is likely female reproductive fluids would show similar changes in response to diet (Martini *et al.,* 2010; Binder *et al.,* 2014; Bromfield *et al.,* 2014). The increase in fat content may alter the viscosity and density of the various reproductive fluids, impacting the ability of sperm to swim towards the ampulla thereby extending the time to fertilisation and extending the timing of development seen in the present study (De Celis *et al.,* 2000; Gulaya *et al.,* 2001). Individual parental obesity embryos show a slight delay in their developmental timings, although this does not differ significantly from Lean Parented embryos. Importantly, this demonstrates that an effect on embryonic development is evident when either maternal or paternal obesity is present. The increased, and significant, delay in Combined Obese Parental embryos may

reflect the suboptimal interaction between high fat male and female reproductive fluids, as well as with the embryo.

On time development of embryos has been ~~is~~associated with increased pregnancy rates in humans (Meseguer *et al.,* 2012; Wong *et al.,* 2013; Rubio *et al.,* 2014). However, this theory remains contentious, highlighting the needs for further prospective studies (Kaser and Racowsky, 2014; Kirkegaard *et al.,* 2015). Delayed embryonic development may have an impact on implantation, considering that~~.~~ ~~i~~Implantation is dependent on an intricate dialogue between the embryo and the uterus ~~within the limited window of implantation~~, and involves a number of factors from both the uterus and embryo (Wang and Dey, 2006). As the Combined Obese Parented embryos are delayed on day 4 (earlier developmental stage than required for implantation), they may not be developmentally competent for implantation, and this may translate in a reduced ability for implantation to occur (Paria *et al.,* 1993). Furthermore, Combined Obese Parented embryos have reduced TE cells, which can translate to poor implantation and placentation (Hardy *et al.,* 1989; Cross *et al.,* 1994). This is consistent with previous mouse studies that demonstrate slower developmental timing, caused by parental obesity or other means, and has been associated with decreased implantation rates (Mitchell *et al.,* 2010; Binder *et al.,* 2012a). It would be of interest to investigate the chemical composition, such as triglyceride levels and how these interact with key hormones, as potentially this would alter the physiology of the tract and receptivity to embryonic implantation. ~~Therefore, this knowledge and~~ T~~t~~he understanding of how delayed embryonic development interacts with an altered maternal uterine environment~~,~~ is ~~fundamental~~ an area that could potentially improve~~to potentially improving~~ reproductive outcomes in obese couples.

Combined parental obesity alters glucose metabolism and metabolic gene expression

Obese and lean individuals are considered to have vastly different metabolic states and employ different metabolic pathways, and this concept should be extended to embryos. Combined Obese Parented blastocysts exhibited an increased uptake of glucose from media compared to Lean Parented blastocysts, when expressed per cell. ~~–~~Previous studies have demonstrated that maternal obesity in the

mouse leads to increased glucose uptake per cell (Binder *et al.,* 2012b). ~~(Binder *et al.*, 2012)(Binder *et al.*, 2014)~~Increased glucose uptake may suggest increased glycolysis, a common and essential metabolic pathway for the blastocyst (Leese and Barton, 1984; Gardner and Wale, 2013). However, lactate, the final product of glycolysis, output remained unchanged from Lean Parented embryos, suggesting the excess glucose is being metabolised via an alternative pathway, such as the Pentose Phosphate Pathway (PPP). Potentially, the PPP is used as the concomitant production of NADPH can mitigate the effects of oxidative stress seen in the gametes and embryos of obese parents (McCord, 2000; Igosheva *et al.,* 2010; Bakos *et al.,* 2011b; Binder *et al.,* 2012b). Alternatively, glucose could be being metabolised through the hexosamine biosynthesis pathway, which supports amine sugar synthesis while also having key roles in growth and the development of insulin resistance (Sutton-McDowall *et al.,* 2010). ~~In contrast to the data presented, human studies on maternal obesity reported a decrease in blastocyst glucose consumption with obesity. However, the blastocysts derived from patients with a BMI>25 kg/m^2 were smaller in diameter and had significantly fewer cells (Leary *et al.*, 2015).~~ In contrast to the data presented, a recent human study on maternal obesity reported a decrease in total blastocyst glucose consumption with increasing BMI. However, these findings, based on seven patients, determined blastocysts from women with a BMI>25 kg/m^2 were smaller in diameter and had significantly fewer cells (Leary *et al.,* 2015). Unlike the current study, it is also postulated that metabolic manifestations associated with obesity, in terms of perturbed glucose and insulin homeostasis, would also be evident in these obese patients further impacting embryo physiology. The data presented in this study indicate that more detailed metabolomic analyses of Combined Obese Parented embryos are warranted.

Assessment of metabolic gene expression levels in blastocysts of Combined Obese Parented embryos revealed a significant increase in glucose transporter 1 (*Glut1*) and Peroxisome proliferator-activated receptor gamma (*Ppar γ*), and a trend for increased pyruvate kinase 2 (*Pkm2*~~) and Peroxisome proliferator-activated receptor gamma (*Ppar γ*)~~ expression. In the blastocyst, *Glut1* is localised to apical membranes of the trophectoderm, and has been shown to have a key role in detecting and responding to the surrounding glucose environment (Pantaleon and Kaye, 1998). The observed

increase in *Glut1* expression and increased glucose uptake in this study may indicate metabolic stress in the Combined Obese Parented embryos. While increased glucose uptake has been linked to increased developmental competence in blastocysts, glucose uptake must be regulated within a narrow range to ensure viability (Gardner and Wale, 2013). Up-regulation of *Glut1* may also drive the increased expression of *Pkm2* evident in the Combined Obese Parented blastocyst. *Pkm*2 is essential to the aerobic conversion of phosphenolpyruvate to pyruvate and is essential for biosynthesis in proliferative cell types, such as cancer embryonic cells (Gupta and Bamezai, 2010; Krisher and Prather, 2012; Gardner and Wale, 2013; Gardner and Harvey, 2015). Increased *Pkm2* expression does not result in an increase in glycolysis, even in the presence of high glucose uptake, to allow for increased biosynthesis for key embryonic components such as nucleic acids, which occurs via the alternate Pentose Phosphate Pathway (Krisher and Prather, 2012; Gardner and Wale, 2013). The trend towards increased *Pparγ* expression, which is activated by fatty acids, may also indicate metabolic stress, as it plays a key role in glucose homeostasis (Minge *et al.,* 2008).

The model of diet-induced obesity employed in the present study is free from the many metabolic complications evident in humans. The short feeding period (8 weeks) does not affect fasting plasma glucose levels (Bakos *et al.,* 2011; Mitchell *et al.,* 2011; Palmer *et al.,* 2012), or glucose or insulin tolerance (Fullston *et al.,* 2013). Similar models of diet-induced obesity, involving higher fat diet, generational feeding, and additional environmental stressors, do demonstrate pre-diabetic symptoms in mice (Winzell and Ahrén, 2004; Sun *et al.,* 2009; Groover *et al.,* 2013; Hillian *et al.,* 2013; Ge *et al.,* 2014). Indeed, the obese state is associated with hyperglycaemia and increased free fatty acids (Boden, 2008; Martyn *et al.,* 2008), which have negative impacts on early mammalian embryo development (Van Hoeck *et al.,* 2011; Cagnone *et al.,* 2012). Therefore, in the more complex metabolic state of obese humans, there is likely to be a greater impact upon early embryonic development. ~~such as insulin resistance, due to the strain of mouse and short feeding period (Lin *et al.*, 2000; Mitchell *et al.*, 2011). However, obesity in humans leads to a hyperglycaemic environment for many individuals (Martyn *et al.*, 2008). Hyperglycaemic embryo culture environments have been shown to negatively affect blastocyst development and attachment, while also up-regulating metabolic~~

genes in bovine embryos (Cagnone *et al.*, 2012). The exposure of oocytes, sperm and the embryo to the more complex metabolic environment in humans may thus lead to a greater up-regulation of these metabolic genes in the embryo than found in this study. This could have great impacts on gene, and protein function, resulting in further perturbed embryonic phenotypes and potentially adult disease.

Embryo characteristics are altered by obesity

This is the first study to demonstrate that obesity has an impact on zona pellucida width. Combined Obese Parented embryos exhibited a significant reduction in zona pellucida width. Zona pellucida width is further reduced by the contribution of parental obesity in Combined Obese Parented embryos, suggesting there is a further paternal contribution. Previous studies have associated higher zona integrity with increased implantation (Montag *et al.,* 2008). Further studies are required to determine how obesity impacts zona pellucida width with ZP gene expression and glycoprotein cross-linking potential candidates. The role of the zona pellucida and perturbations to its structure during embryonic development is interesting and warrants further investigation, especially as more individuals seek ART (Ferraretti *et al.,* 2013).

Conclusion

The present study demonstrates that combined parental obesity prior to and at the time of conception, results in a detrimental impact upon blastocyst developmental potential, consistent with previous studies of individual parent obesity.

This study demonstrates that combined parental obesity results in a detrimental impact upon blastocyst developmental potential, consistent with previous studies of individual parent obesity. Zona pellucida width was shown, for the first time, to be thinner in Combined Obese Parented embryos and may play a critical role in modulating embryonic development and the embryos interaction with its surrounding environment. Glucose metabolism was perturbed in Combined Obese Parented embryos, with significantly more glucose being taken up per cell than that of Lean Parented embryos. Furthermore, key genes in the regulation of metabolism were up-regulated in Combined Obese Parented embryos, indicative of oxidative stress. Furthermore, pPersistent changes to this gene

expression could confer increased adult disease risk. While long-term programming cannot be confirmed in this current study, as future studies with embryo transfers are required, it is clear that combined parental obesity negatively impacts ~~pre-implantation~~preimplantation embryonic development. Previous studies indicate early embryonic changes do program long-term health of the individual (Sinclair and Singh, 2007; Lane *et al.,* 2014). This study is limited to mouse embryos, however human embryos may be similarly impacted by combined parental obesity. As more individuals become obese, issues surrounding early embryonic development will become more pertinent to ART protocols. Therefore, promoting early intervention for parents, as well as identifying and incorporating variability in embryonic parameters as a result of parental obesity will aid the development of appropriate, customised ART protocols.

Author's roles

All authors contributed to the design of the research study; B.J.F. performed the experiments; all authors contributed to the analysis of the data ~~writing of the manuscript analysis of the data~~; D.K.G. provided funding; all authors contributed to the writing of the manuscript.

Acknowledgements

The authors would like to thank Tania Long and Darren Cipolla for their assistance in mice handling and care. We would also like to acknowledge Unisense Fertilitech for the use of the Embryoscope.

Funding

This study was funded from University of Melbourne research monies and did not receive any funding from a commercial company.

Conflict of interest

M.P.G currently holds the position of Merck Serono Lecturer of Reproductive Biology. D.K.G. received research funds from Vitrolife AB Sweden. The other authors of this manuscript have nothing to declare and no conflicts of interest.

References

Acton BM, Jurisicova A, Jurisica I, Casper RF. Alterations in mitochondrial membrane potential during preimplantation stages of mouse and human embryo development. *Mol Hum Reprod* 2004;**10**: 23-32.

Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* 2004;**64**: 5245-5250

Bakos HW, Henshaw RC, Mitchell M, Lane M. Paternal body mass index is associated with decreased blastocyst development and reduced live birth rates following assisted reproductive technology. *Fertil Steril* 2011a;**95**: 1700-1704.

Bakos HW, Mitchell M, Setchell BP, Lane M. The effect of paternal diet-induced obesity on sperm function and fertilization in a mouse model. *Int J Androl* 2011b;**34**: 402-410.

Bellver J, Mifsud A, Grau N, Privitera L, Meseguer M. Similar morphokinetic patterns in embryos derived from obese and normoweight infertile women: a time-lapse study. *Hum Reprod* 2013;**28**: 794-800.

Bermejo-Alvarez P, Rosenfeld CS, Roberts RM. Effect of maternal obesity on estrous cyclicity, embryo development and blastocyst gene expression in a mouse model. *Hum Reprod* 2012;**27**: 3513-3522.

Binder NK, Hannan NJ, Gardner DK. Paternal diet-induced obesity retards early mouse embryo development, mitochondrial activity and pregnancy health. *PloS One* 2012a;**7**: e52304.

Binder NK, Hannan NJ, Sheedy JR, Gardner DK. Male obesity is associated with changed spermatozoa Cox4i1 mRNA level and altered seminal vesicle fluid composition in a mouse model. *Mol. Hum. Reprod.* 2015 doi:10.1093/molehr/gav010.

Binder NK, Mitchell M, Gardner DK. Parental diet-induced obesity leads to retarded early mouse embryo development and altered carbohydrate utilisation by the blastocyst. *Reprod Fertil Dev 2012*b;**24**: 804-812.

Boden G. Obesity and Free Fatty Acids (FFA). *Endocrinol Metab Clin North Am* 2008;**37**: 635.

Bromfield JJ, Schjenken JE, Chin PY, Care AS, Jasper MJ, Robertson SA. Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proc Natl Acad Sci U S A* 2014;**111**: 2200-2205.

Cagnone GL, Dufort I, Vigneault C, Sirard MA. Differential gene expression profile in bovine blastocysts resulting from hyperglycemia exposure during early cleavage stages. *Biol Reprod* 2012;**86**: 2, 1-12.

Chang K-J, Huang D, Cuatrecasas P. The defect in insulin receptors in obese-hyperglycemic mice: A probable accompaniment of more generalized alterations in membrane glycoproteins. *Biochem Biophysical Research Commun* 1975;**64**: 566-573.

Chavatte-Palmer P, Tarrade A, Levy R. [Developmental origins of health and disease in adults: role of maternal environment]. *Gynecol Obstet Fertil* 2012;**40**: 517-519.

Formatted: Space After: 0 pt

Cross JC, Werb Z, Fisher SJ. Implantation and the placenta: key pieces of the development puzzle. *Science* 1994;**266**: 1508-1518.

De Celis R, Feria-Velasco A, González-Unzaga M, Torres-Calleja J, Pedrón-Nuevo N. Semen quality of workers occupationally exposed to hydrocarbons. *Fertil Steril* 2000;**73**: 221-228.

Dupont C, Harvey AJ, Armant DR, Zelinski MB, Brenner CA. Expression profiles of cohesins, shugoshins and spindle assembly checkpoint genes in rhesus macaque oocytes predict their susceptibility for aneuploidy during embryonic development. *Cell Cycle* 2012;**11**: 740-748.

Dupont C, Faure C, Sermondade N, Boubaya M, Eustache F, Clement P, Briot P, Berthaut I, Levy V, Cedrin-Durnerin I, Benzacken B, Chavatte-Palmer P, Levy R. Obesity leads to higher risk of sperm DNA damage in infertile patients. *Asian J Androl* 2013;**15**: 622-625.

Ferraretti AP, Goossens M, Kupka S, Bhattacharya J, de Mouzon J, Castilla A, Erb K et al. Assisted reproductive technology in Europe, 2009: results generated from European registers by ESHRE. *Hum Reprod* 2009;**28**: 2318-2331

Feuer SK, Camarano L, Rinaudo PF. ART and health: clinical outcomes and insights on molecular mechanisms from rodent studies. *Mol Human Reprod* 2013;**19**: 189-204.

Fullston T, Palmer NO, Owens JA, Mitchell M, Bakos HW, Lane M. Diet-induced paternal obesity in the absence of diabetes diminishes the reproductive health of two subsequent generations of mice. *Hum Reprod* 2012;**27**: 1391-1400.

Fullston T, Ohlsson Teague EMC, Palmer NO, DeBlasio MJ, Mitchell M, Corbett M, Print CG, Owens JA, Lane M. Paternal obesity initiates metabolic disturbances in two generations of mice with incomplete penetrance to the F2 generation and alters the transcriptional profile of testis and sperm microRNA content. *FASEB J* 2013;**27**: 4226-4243.

Gardner DK, Harvey AJ. Blastocyst metabolism. *Reprod Fert Dev* 2015 doi: 10.1071/RD14421.

Gardner DK, Lane M. Mammalian preimplantation embryo culture. *Methods Mol Biol* 2014;**1092**: 167-182.

Gardner DK, Lane M, Watson AJ. *A laboratory guide to the mammalian embryo*, 2004. Oxford University Press.

Gardner DK, Leese HJ. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. *Reproduction* 1990;**88**: 361-368.

Gardner DK, Wale PL. Analysis of metabolism to select viable human embryos for transfer. *Fertil Steril* 2013;**99**: 1062-1072.

Gardner DS, Pearce S, Dandrea J, Walker R, Ramsay M, Stephenson T, Symonds ME. Peri-implantation undernutrition programs blunted angiotensin II evoked baroreflex responses in young adult sheep. *Hypertension* 2004;**43**: 1290-1296.

Ge Z-J, Liang Q-X, Hou Y, Han Z-M, Schatten H, Sun Q-Y, Zhang C-L. Maternal obesity and diabetes may cause DNA methylation alteration in the spermatozoa of offspring in mice. *Reprod Biol Endocrinol* 2014;**12**: 29-29.

645 Goto Y, Noda Y, Narimoto K, Umaoka Y, Mori T. Oxidative stress on mouse embryo development in
646 vitro. *Free Radic Biol Med* 1992;**13**: 47-53.

647 Guh DP, Zhang W, Bansback N, Amarsi Z, Birmingham CL, Anis AH. The incidence of co-
648 morbidities related to obesity and overweight: a systematic review and meta-analysis. *BMC Public*
649 *Health* 2009;**9**: 88.

650 Gulaya NM, Margitich VM, Govseeva NM, Klimashevsky VM, Gorpynchenko, II, Boyko MI.
651 Phospholipid composition of human sperm and seminal plasma in relation to sperm fertility. *Arch*
652 *Androl* 2001;**46**: 169-175.

653 Gupta V, Bamezai RN. Human pyruvate kinase M2: a multifunctional protein. *Protein Sci* 2010;**19**:
654 2031-2044.

655 Groover AL, Ryals JM, Guilford BL, Wilson NM, Christianson JA, Wright DE. Exercise-mediated
656 improvements in painful neuropathy associated with prediabetes in mice. *Pain* 2013;**154**: 2658-2667.

657 Hardy K, Handyside AH, Winston RM. The human blastocyst: cell number, death and allocation
658 during late preimplantation development in vitro. *Development* 989;**107**: 597-604.

659 Hardy K, Spanos S, Becker D, Iannelli P, Winston RML, Stark J. From cell death to embryo arrest:
660 Mathematical models of human preimplantation embryo development. *Proc Natl Acad Sci U S A*
661 2001;**98**: 1655-1660.

662 Harvey AJ, Kind KL, Pantaleon M, Armstrong DT, Thompson JG. Oxygen-regulated gene expression
663 in bovine blastocysts. *Biol Reprod* 2004;**71**: 1108-1119.

664 Hillian AD, McMullen MR, Sebastian BM, Rowchowdhury S, Kashyap SR, Schauer PR, Kirwan JP,
665 Feldstein AE, Nagy LE. Mice lacking C1q are protected from high fat diet-induced hepatic insulin
666 resistance and impaired glucose homeostasis. *J Biol Chem* 2013;**288**: 22565-22575.

667 Igosheva N, Abramov AY, Poston L, Eckert JJ, Fleming TP, Duchen MR, McConnell J. Maternal
668 diet-induced obesity alters mitochondrial activity and redox status in mouse oocytes and zygotes. *PloS*
669 *One* 2010;**5**: 0010074.

670 Jensen TK, Andersson A-M, Jørgensen N, Andersen A-G, Carlsen E, Petersen JøH, Skakkebæk NE.
671 Body mass index in relation to semen quality and reproductive hormonesamong 1,558 Danish men.
672 *Fertil Steril* 2004;**82**: 863-870.

673 Kaser DJ, Racowsky C. Clinical outcomes following selection of human preimplantation embryos
674 with time-lapse monitoring: a systematic review. *Hum Reprod Update* 2014;**20**: 617-631.

675 Kind KL, Collett RA, Harvey AJ, Thompson JG. Oxygen-regulated expression of GLUT-1, GLUT-3,
676 and VEGF in the mouse blastocyst. *Mol Reprod Dev* 2005;**70**: 37-44.

677 Kirkegaard K, Ahlström A, Ingerslev HJ, Hardarson T. Choosing the best embryo by time lapse
678 versus standard morphology. *Fertili and Steril* 2015;**103**: 323-332.

679 Kort HI, Massey JB, Elsner CW, Mitchell-Leef D, Shapiro DB, Witt MA, Roudebush WE. Impact of
680 body mass index values on sperm quantity and quality. *J Androl* 2006;**27**: 450-452.

681 Krisher RL, Prather RS. A role for the Warburg effect in preimplantation embryo development:
682 metabolic modification to support rapid cell proliferation. *Mol Reprod Dev* 2012;**79**: 311-320.

683 Kupka MS, Gnoth C, Buehler K, Dahncke W, Kruessel J-S. Impact of female and male obesity on
684 IVF/ICSI: results of 700,000 ART-cycles in Germany. *Gynecol Endocrinol* 2011;**27**: 144-149.

685 Lane M, Gardner DK. Amino acids and vitamins prevent culture-induced metabolic perturbations and
686 associated loss of viability of mouse blastocysts. *Hum Reprod* 1998;**13**: 991-997.

687 Lane M, Robker RL, Robertson SA. Parenting from before conception. *Science* 2014;**345**: 756-760.

688 Leary C, Leese HJ, Sturmey RG. Human embryos from overweight and obese women display
689 phenotypic and metabolic abnormalities. *Hum Reprod* 2015;**30**: 122-132.

690 Leese HJ, Barton AM. Pyruvate and glucose uptake by mouse ova and preimplantation embryos.
691 *Reproduction* 1984;**72**: 9-13.

692 Lin S, Thomas T, Storlien L, Huang X. Development of high fat diet-induced obesity and leptin
693 resistance in C57Bl/6J mice. *Int J Obes Relat Metab Disord* 2000;**24**: 639-646.

694 Luo W, Semenza GL. Emerging roles of PKM2 in cell metabolism and cancer progression. *Trends*
695 *Endocrinol Metab* 2012;**23**: 560-566.

696 Luzzo KM, Wang Q, Purcell SH, Chi M, Jimenez PT, Grindler N, Schedl T, Moley KH. High fat diet
697 induced developmental defects in the mouse: oocyte meiotic aneuploidy and fetal growth
698 retardation/brain defects. *PloS One* 2012;**7**: 12.

699 Maheshwari A, Stofberg L, Bhattacharya S. Effect of overweight and obesity on assisted reproductive
700 technology: a systematic review. *Hum Reprod Update* 2007;**13**: 433-444.

701 Maloney C, Hay S, Young L, Sinclair K, Reese, W. A methyl-deficient diet fed to rat dams during the
702 peri-conception period programs glucose homeostasis in adult male but not female offspring. *J Nutr*
703 2011;**14**: 95-100

704 Martini AC, Tissera A, Estofán D, Molina RI, Mangeaud A, de Cuneo MF, Ruiz RD. Overweight and
705 seminal quality: a study of 794 patients. *Fertil Steril* 2010;**94**: 1739-1743.

706 Martyn JA, Kaneki M, Yasuhara S. Obesity-induced insulin resistance and hyperglycemia: etiologic
707 factors and molecular mechanisms. *Anesthesiology* 2008;**109**: 137-148.

708 McCord JM. The evolution of free radicals and oxidative stress. *Am J Med* 2000;**108**: 652-659.

709 McPherson NO, Fullston T, Bakos HW, Setchell BP, Lane M. Obese father's metabolic state,
710 adiposity, and reproductive capacity indicate son's reproductive health. *Fertil Steril* 2014;**101**: 865-
711 873.e861.

712 Meseguer M, Herrero J, Tejera A, Hilligsøe KM, Ramsing NB, Remohí J. The use of morphokinetics
713 as a predictor of embryo implantation. *Hum Reprod* 2011;**26**: 2658-2671.

714 Meseguer M, Rubio I, Cruz M, Basile N, Marcos J, Requena A. Embryo incubation and selection in a
715 time-lapse monitoring system improves pregnancy outcome compared with a standard incubator: a
716 retrospective cohort study. *Fertil and Steril* 2012;**98**: 1481-1489 e1410.

717 Minge C, Robker R, Norman R. PPAR Gamma: Coordinating metabolic and immune contributions to
718 female fertility. *PPAR Res* 2008;**2008**.

719 Mitchell M, Bakos HW, Lane M. Paternal diet-induced obesity impairs embryo development and
720 implantation in the mouse. *Fertil Steril* 2011;**95**: 1349-1353.

721 Mokdad AH, Ford ES, Bowman BA, Dietz WH, Vinicor F, Bales VS, Marks JS. Prevalence of
722 obesity, diabetes, and obesity-related health risk factors. *JAMA* 2003;**289**: 76-79.

723 Montag M, Schimming T, Köster M, Zhou C, Dorn C, Rösing B, van der Ven H, van der Ven K.
724 Oocyte zona birefringence intensity is associated with embryonic implantation potential in ICSI
725 cycles. *Reprod Biomed Online* 2008;**16**: 239-244.

726 Muller PY, Janovjak H, Miserez AR, Dobbie Z. Processing of gene expression data generated by
727 quantitative real-time RT-PCR. *Biotechniques* 2002;**32**: 1372-1380.

728 Ng M, Fleming T, Robinson M, Thomson B, Graetz N, Margono C, Mullany EC, Biryukov S,
729 Abbafati C, Abera SF., et al. Global, regional, and national prevalence of overweight and obesity in
730 children and adults during 1980?: a systematic analysis for the Global Burden of Disease Study 2013.
731 *Lancet* 2014;**384**: 766-781.

732 Norman RJ, Clark AM. Obesity and reproductive disorders: a review. *Reprod Fertility Dev* 1997;**10**:
733 55-63.

734 Palmer NO, Bakos HW, Owens JA, Setchell BP, Lane M. Diet and exercise in an obese mouse fed a
735 high-fat diet improve metabolic health and reverse perturbed sperm function. *Am J Physiol*
736 *Endocrinol Metab* 2012;**302**: e768-e780.

737 Pantaleon M, Kaye PL. Glucose transporters in preimplantation development. *Reproduction* 1998;**3**:
738 77-81.

739 Paria BC, Huet-Hudson YM, Dey SK. Blastocyst's state of activity determines the "window" of
740 implantation in the receptive mouse uterus. *Proc Natl Acad U S A* 1993;**90**: 10159-10162.

741 Robker RL, Akison LK, Bennett BD, Thrupp PN, Chura LR, Russell DL, Lane M, Norman RJ. Obese
742 women exhibit differences in ovarian metabolites, hormones, and gene expression compared with
743 moderate-weight women. *J Clin Endocrinol Metab* 2009;**94**: 1533-1540.

744 Rubio I, Galan A, Larreategui Z, Ayerdi F, Bellver J, Herrero J, Meseguer M. Clinical validation of
745 embryo culture and selection by morphokinetic analysis: a randomized, controlled trial of the
746 EmbryoScope. *Fertil and Steril* 2014;**102**: 1287-1294 e1285.

747 Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat*
748 *Meth* 2012;**9**: 671-675.

749 Silveira PP, Portella AK, Goldani MZ, Barbieri MA. Developmental origins of health and disease
750 (DOHaD). *Journal Pediatr* 2007;**83**: 494-504.

751 Simon P. Q-Gene: processing quantitative real-time RT–PCR data. *Bioinformatics* 2003;**19**: 1439-
752 1440.

753 Sinclair KD, Singh R. Modelling the developmental origins of health and disease in the early embryo.
754 *Theriogenology* 2007;**67**: 43-53.

755 Skinner MK. Role of epigenetics in developmental biology and transgenerational inheritance. *Birth*
756 *Defects Res, Part C* 2011;**93**: 51-55.

757 Styne-Gross A, Elkind-Hirsch K, Scott Jr RT. Obesity does not impact implantation rates or
758 pregnancy outcome in women attempting conception through oocyte donation. *Fertil Steril* 2005;**83**:
759 1629-1634.

760 Sun Q, Yue P, Deiuliis JA, Lumeng CN, Kampfrath T, Mikolaj MB, Cai Y, Ostrowski MC, Lu B,
761 Parthasarathy S., et al. Ambient air pollution exaggerates adipose inflammation and insulin resistance
762 in a mouse model of diet-induced obesity. *Circulation* 2009;**119**: 538-546.

763 Sutton-McDowall ML, Gilchrist RB, Thompson JG. The pivotal role of glucose metabolism in
764 determining oocyte developmental competence. *Reproduction* 2010;**139**: 685-695.

765 Takahashi M, Keicho K, Takahashi H, Ogawa H, Schulte RM, Okano A. Effect of oxidative stress on
766 development and DNA damage in in-vitro cultured bovine embryos by comet assay. *Theriogenology*
767 2000;**54**: 137-145.

768 Talior I, Yarkoni M, Bashan N, Eldar-Finkelman H. Increased glucose uptake promotes oxidative
769 stress and PKC-δ activation in adipocytes of obese, insulin-resistant mice. *Am J Physiol Endocrinol*
770 *Metab* 2003;**285**: E295-E302.

771 Van Hoeck V, Sturmey RG, Bermejo-Alvarez P, Rizos D, Gutierrez-Adan A, Leese HJ, Bols PEJ,
772 Leroy JLMR. Elevated Non-Esterified Fatty Acid Concentrations during Bovine Oocyte Maturation
773 Compromise Early Embryo Physiology. *PloS One* 2011;**6**: e23183.

774 van der Steeg JW, Steures P, Eijkemans MJ, Habbema JD, Hompes PG, Burggraaff JM, Oosterhuis
775 GJ, Bossuyt PM, van der Veen F, Mol BW. Obesity affects spontaneous pregnancy chances in
776 subfertile, ovulatory women. *Hum Reprod* 2008;**23**: 324-328.

777 Wadhwa PD, Buss C, Entringer S, Swanson JM. Developmental origins of health and disease: brief
778 history of the approach and current focus on epigenetic mechanisms *Semin Reprod Med* 2009;**27**:
779 358-368.

780 Wahli W, Braissant O, Desvergne B. Peroxisome proliferator activated receptors: transcriptional
781 regulators of adipogenesis, lipid metabolism and more. *Chem Biol* 1995;**2**: 261-266.

782 Wale PL, Gardner DK. Time-lapse analysis of mouse embryo development in oxygen gradients.
783 *Reprod Biomed Online* 2010;**21**: 402-410.

784 Wang H, Dey SK. Roadmap to embryo implantation: clues from mouse models. *Nat Rev Genet*
785 2006;**7**: 185-199.

786 Watkins A, Lucas E, Fleming T. Impact of the periconceptional environment on the programming of
787 adult disease. *J DOHaD* 2010;**1**: 97-95.

788 Winzell MS, Ahrén B. The High-Fat Diet–Fed Mouse: A Model for Studying Mechanisms and
789 Treatment of Impaired Glucose Tolerance and Type 2 Diabetes. *Diabetes* 2004;**53**: S215-S219.

790 Wong C, Chen AA, Behr B, Shen S. Time-lapse microscopy and image analysis in basic and clinical
791 embryo development research. *Reprod Biomed Online* 2013;**26**: 120-129.

792 Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM, Pera RAR. Non-invasive
793 imaging of human embryos before embryonic genome activation predicts development to the
794 blastocyst stage. *Nat Biotech* 2010;**28**: 1115-1121.

795 World Health Organisation. Obesity and Overweight [Fact Sheet] 2015. Retrieved 20/01/2015 from
796 http://www.who.int/mediacentre/factsheets/fs311/en

797 Wu LL, Russell DL, Wong SL, Chen M, Tsai T-S, St John JC, Norman RJ, Febbraio MA, Carroll J,
798 Robker RL. Mitochondrial dysfunction in oocytes of obese mothers: transmission to offspring and

799 reversal by pharmacological endoplasmic reticulum stress inhibitors. *Development* 2015;**142**: 681-
800 691.

FIGURE LEGENDS

Figure 1. The percentage of fertilised zygotes that reach the blastocyst stage for each of the four reciprocal mating groups; Lean Parented embryos = LP (n = 188), Paternal Obese Parented embryos = POP (n = 96), Maternal Obese Parented embryos = MOP (n = 96) and Combined Obese Parented embryos = COP (n = 157). ~~Lean Parental (LP, open bar) (n = 188), Paternal Obese Parented (POP, diagonal bar) (n = 96), Maternal Obese Parented (MOP, horizontal bar) (n = 96), Combined Obese Parents (COP, black bar) (n = 157).~~ Different superscript letters show significant differences ($P < 0.05$) between groups. Data expressed as mean ± SEM.

Figure 2. Timing of major developmental events, expressed in hours post-hCG injection (Figure 2a), and hours post pronuclear envelope breakdown (Figure 2b). ~~tPF~~ tPNB = timing of pronucle~~ar envelope~~ breakdown, t2 = timing of 2-cell division, t4 = timing of 4-cell division, t6 = timing of 6 cell division, t8 = timing of 8-cell division, tSB = time blastocoel starts formation. Lean Parented embryos = ~~(~~LP~~, , open bars),~~ Paternal Obese Parented embryos = ~~(~~POP~~, , diagonal hashed bars),~~ Maternal Obese Parented embryos = ~~(~~MOP~~, horizontal hashed bars),~~ and Combined Obese Parented embryos = ~~(~~COP~~, shaded bars)~~. Different superscript letters indicate a significant difference ($P < 0.05$) between groups. n > 60 embryos per group. Data expressed as mean ± SEM.

Figure 3. Mean cell counts and ratios to show lineage specification for all embryos that developed to the blastocyst stage (Day 5) at the expected time for each of the four groups. a) inner cell mass (ICM) cell counts, b) trophectoderm (TE) cell counts, c) ICM to TE cell ratio, and d) Total cell number. Lean Parented embryos (LP, open bars), Paternal Obese Parented embryos (POP, diagonal hashed bars), Maternal Obese Parented embryos (MOP, horizontal hashed bars), and Combined Obese Parented embryos (COP, shaded bars). * denotes significantly ($P < 0.05$) different from Lean Parented (LP) embryos, n > 60 embryos per group. Data expressed as mean ± SEM.

Figure 4. a) Glucose and lactate concentration in spent culture media expressed ~~by~~ h/embryo, b) Total cell number (Day 4 early blastocysts (< half the embryo comprising of the blastocoel)), c) Glucose

and lactate concentration in spent culture media expressed ~~by~~ h/cell, d) Glycolytic rate (%glucose/lactate) of Lean Parented embryos (LP, open bars) and Combined Obese Parented (COP, shaded bars) blastocysts. $^{**}P < 0.01$. n > 20 embryos per group. Data expressed as mean ± SEM.

Figure 5. Mean expression of a) Glucose transporter 1 *(Glut 1),* b) Pyruvate kinase isoform two *(Pkm2),* c) Peroxisome proliferator-activated receptor gamma (*Ppar γ*), *d)* Insulin growth-like factor 2 receptor *(Igf2r),* and e) Glucose transporter 3 *(Glut 3)* relative to 18S rRNA. Expression from Combined Obese Parented (COP) embryos is reflected by ~~in filled~~ shaded bars. Values for COP embryos are normalised to the expression of embryos from Lean Parented (LP) embryo group (open bars) set at 1. * $P < 0.05$ $^{**}P < 0.01$, ^ $P < 0.08$, n=3 replicates of > 40 embryos per group. Data are expressed as mean ± SEM.

Figure 6. Zona pellucida width (μm) at the two-pronucleate stage. Lean Parented embryos = LP, Paternal Obese Parented embryos = POP, Maternal Obese Parented embryos = MOP and Combined Obese Parented embryos = COP. ~~Lean Parented embryos (LP, open bars), Paternal Obese Parented embryos (POP, diagonal hashed bars), Maternal Obese Parented embryos (MOP, horizontal hashed bars) and Combined Obese Parented embryos (COP, shaded bars).~~ Different superscript letters denote a significant difference ($P < 0.05$) between groups in zona pellucida width n > 30 embryos per group. Data expressed as mean ± SEM.

Title: Combined parental obesity negatively impacts preimplantation mouse embryo development, kinetics, morphology and metabolism

Running title: Parental obesity and embryo viability

Bethany J. Finger[1], Alexandra J. Harvey[1], Mark P. Green[1] and David K. Gardner[1*]

[1]School of BioSciences, University of Melbourne, Parkville, Melbourne 3010, Victoria, Australia.

Corresponding author: *Prof David Gardner, School of BioSciences, University of Melbourne, Parkville, Melbourne 3010, Victoria, Australia.

Phone: +61 3 8344 6259, E-mail: david.gardner@unimelb.edu.au

Abstract

Study question: Does combined parental obesity, both an obese mother and father, have a greater effect on mouse preimplantation embryo development and quality than single parent obesity?

Summary answer: Combined parental obesity causes a greater reduction in blastocyst rate, and a greater delay to the timing of key embryonic developmental events than single parental obesity, as well as altering embryonic characteristics, such as zona pellucida width.

What is known already: Maternal or paternal obesity alone are known to have significant and detrimental impacts on preimplantation embryo development. Furthermore, these early embryonic perturbations can have long-term impacts on both offspring health, and further generations. This is one of the first studies to examine the effects of having both an obese mother and obese father.

Study design, size, duration: A cross sectional control versus treatment mouse study of diet-induced obesity was employed, in which 300 embryos per group were generated and studied from reciprocal matings: i) control female and control male (Lean Parented Embryos); ii) control female and obese male (Paternal Obese Parented Embryos); obese female and control male (Maternal Obese Parented Embryos); and iv) obese female and obese male (Combined Obese Parented Embryos). Assessments of embryonic development rate, timing of development, morphological characteristics, metabolic gene expression, metabolism and cell lineage allocation were made at selected time points and analysed in relation to parental obesity status.

Participants/materials, setting, methods: Three-week old C57BL6 male and female mice were fed control (7% kcal fat) or high fat (21% kcal fat) diets for a minimum of eight weeks. Females were superovulated, mated, fertilized zygotes recovered and standard mouse *in vitro* embryo culture performed. Time-lapse monitoring was undertaken to compare developmental timings and morphological characteristics (embryonic area and zona pellucida width) for embryos from all four reciprocal matings. Differential staining identified cell lineage allocation. Real-time quantitative RT-PCR (qRT-PCR) and microfluorescence were used to measure gene expression and metabolism (glucose consumption and lactate production) respectively, in embryos from Lean Parented and Combined Obese Parented matings. This research was completed in a University research laboratory.

Main results and the role of chance: Blastocyst rate was reduced in Combined Obese Parented embryos when compared to both Single Obese (11% decrease for Maternal Obese Parented, $P < 0.05$; 15% for Paternal Obese Parented, $P < 0.05$) and Lean Parented embryos (25% decrease, $P < 0.01$). Time-lapse analysis of developmental kinetics highlighted a delay of 1 h at the 2-3 cell division, extending to 6 h delay by the blastocyst stage for Combined Obese Parented embryos ($P < 0.05$). A reduction in total cell number of Combined Obese Parented blastocysts was a further manifestation of this developmental delay ($P < 0.05$). Zona pellucida width was reduced in Combined Obese Parented embryos ($P < 0.05$). Glucose consumption was increased in Combined Obese Parented embryos ($P < 0.05$), which was associated with the up-regulation of *Glut1* expression ($P < 0.05$).

Limitations and reason for caution: This study was completed in fertile C57BL/6 mice using a well-defined model of diet-induced obesity in which embryos were fertilised *in vivo*. Human obesity is complex, with many causes and co-morbidities, and therefore the impact of combined obesity would require further investigated in human settings.

Wider implications of the findings: This study demonstrates that combined parental obesity has a detrimental impact on mouse embryo development, a finding consistent with previous studies on individual parent obesity. Of note, the effect of combined parental obesity upon embryo development markers was greater than that of individual parental obesity. Plausibly, human embryos will be similarly impacted. The reduction in blastocyst rate and delayed time to developmental events confirms that embryos of obese parents differ from those of lean parents. Allowance for this should therefore be incorporated into clinical practice when selecting the best embryo for transfer of an obese couple.

Study Funding/competing interest(s): Funding was provided by University of Melbourne research monies. M.P.G currently holds the position of Merck Serono Lecturer of Reproductive Biology. D.K.G. received research funds from Vitrolife AB Sweden. The other authors of this manuscript have nothing to declare and no conflicts of interest.

Keywords: obesity, high fat diet, zona pellucida, time-lapse

Introduction

The World Health Organisation recognises obesity as the epidemic of the 21st century, with greater than 30% of adults around the world defined as obese (Body Mass Index ≥ 30 kg/m^2) (WHO, 2013; Ng *et al.,* 2014). The reduction to an individual's reproductive success is one of the many impacts of obesity (Mokdad *et al.,* 2003; Guh *et al.,* 2009). For the non-scientific community, this would be underscored by the lowered ability of obese individuals to conceive and maintain pregnancy (Norman and Clark, 1997; Maheshwari *et al.,* 2007; Feuer *et al.,* 2013). Such reductions in fecundity are consistent with the increased level of aneuploidy, mitochondrial dysfunction, endoplasmic reticulum dysfunction and poor morphology observed in the oocytes and sperm of obese individuals, both human and animal (Jensen *et al.,* 2004; Kort *et al.,* 2006; van der Steeg *et al.,* 2008; Robker *et al.,* 2009; Bakos *et al.,* 2011; Binder *et al.,* 2012a; Luzzo *et al.,* 2012; Dupont *et al.,* 2013; Wu *et al.,* 2015). Of further concern is the ability of parental obesity to act as a "developmental programmer", and thus impact health across multiple generations (Chavatte-Palmer *et al.,* 2012; Lane *et al.,* 2014).

Historically, developmental programming focused upon environmental exposures, typically post-implantation and throughout gestation (Silveira *et al.,* 2007; Wadhwa *et al.,* 2009). Numerous studies in humans and other species have now established that developmental programming can occur during the peri-conception period, and lead to long-term health impacts upon offspring (Gardner *et al.,* 2004; Watkins *et al.,* 2010; Maloney *et al.,* 2011). Indeed, evidence from rodent studies reveals that maternal obesity results in reduced blastocyst rate, slower embryonic development, down-regulation of key metabolic genes, as well as negative impacts on fetal health (Bermejo-Alvarez *et al.,* 2012; Binder *et al.,* 2012b; Luzzo *et al.,* 2012). Similarly, paternal obesity imparts negative effects on embryonic health including delayed timing of embryonic development, altered glucose metabolism of the blastocyst, perturbed fetal development, as well as impacting fecundity in subsequent generations of mice (Binder *et al.,* 2012a; Binder *et al.,* 2012b; Fullston *et al.,* 2012; McPherson *et al.,* 2014). This is likely mediated through mitochondrial dysfunction (maternal); microRNAs (paternal), as well as genetic and epigenetic mechanisms (Skinner, 2011; Lane *et al.,* 2014). Importantly, human evidence also supports the theory that maternal or paternal obesity alters embryonic development, as

demonstrated by reduced success in IVF cycles (Styne-Gross *et al.,* 2005; van der Steeg *et al.,* 2008; Robker *et al.,* 2009; Bakos *et al.,* 2011a). Of note, the studies above all focussed upon the impacts of individual parental obesity; maternal obesity or paternal obesity alone, with little human data on the effects of combined parental obesity.

Obesity rates are increasing around the world, and couples of reproductive age are likely to share lifestyle choices. Thus, cases of combined parental obesity (both an obese mother and obese father) are becoming more common. The impacts of combined parental obesity upon offspring are however yet to be elucidated. To date, only one study of combined parental obesity has been undertaken, which amongst other findings, noted poor fertilisation rates for obese couples undergoing IVF in Germany (Kupka *et al.,* 2011). However this study included a number of confounding variables, including socioeconomic status and parental age. Hence more clinical studies are required. Based on previous work on maternal and paternal obesity in both humans and animals, it is hypothesised that combined parental obesity has a greater detrimental effect on fertility, as measured by early embryo development and quality, than that of single parental obesity (either maternal or paternal). Therefore, in this study we determined how combined parental obesity impacted preimplantation embryo development and quality in a mouse model. The advantage of this model is that it minimises many potential confounders evident in clinical studies. Specifically, the aims of this study were to characterise the effect of combined parental obesity on: i) the developmental potential, morphology, kinetics and cell lineage allocation of the preimplantation embryo, and ii) the metabolism and expression of key metabolic genes in the blastocyst. In characterising these effects, this work further highlights the importance of combined parental obesity in the pre-conception period, as well as indicating potential pathways in which these effects can be mitigated and ultimately improve the reproductive success of obese couples.

Materials and Methods

Experimental animals, diets and hormonal stimulation

Three-week-old male and female C57BL/6 mice (WEHI; Melbourne, Australia) were randomly

assigned to either a control diet consisting of 7% total fat and 19.4% protein (AIN93G; Specialty Feeds, Perth, Australia), or a high fat diet (HFD) consisting of 21% total fat and 19% protein (SF00-219; Specialty Feeds, Perth, Australia). Mice were fed for a minimum of eight to a maximum of twelve weeks, at which point they were defined as control or obese, respectively, based on previous characterisation (Bakos *et al.,* 2011b; Mitchell *et al.,* 2011; Binder *et al.,* 2012b; Fullston *et al.,* 2012). Mice were maintained in a 12 h light: 12 h dark photoperiod with food and water supplied *ad libitum*. Female mice were caged in groups of four and male mice were caged individually. Body weight was recorded weekly.

Female mice were superovulated with an intraperitoneal injection of 0.25 IU g^{-1} mare serum gonadotrophin (PMSG; Folligon, Intervet, Bendigo, Australia) followed 48 h later by 0.25 IU g^{-1} human chorionic gonadotrophin (hCG; Chorulon, Intervet). Mice were mated to produce four reciprocal matings from which resultant embryos were studied: i) control female and control male (Lean Parented Embryos); ii) control female and obese male (Paternal Obese Parented Embryos); obese female and control male (Maternal Obese Parented Embryos); and iv) obese female and obese male (Combined Obese Parented Embryos).

Ethical approval

This study was carried out in accordance with the *Australian code of practice for the care and use of animals for scientific purposes*, and all protocols were approved by the Animal Ethics Committee of The University of Melbourne.

Embryo collection

Twenty-two hours post-hCG injection, pronucleate oocytes were collected in G-MOPS handling medium supplemented with 5 mg/mL HSA (GMOPS+; Vitrolife, Göteborg, Sweden). Pronucleate oocytes were denuded of cumulus cells via incubation in GMOPS containing 300 IU/ml hyaluronidase for 20 seconds (bovine testes, type IV; Sigma-Aldrich, Castle Hill, NSW, Australia) followed by washing in GMOPS+. Denuded pronucleate oocytes were immediately washed in

GMOPS+ and cultured as previously detailed (Gardner and Lane, 2014).

Embryo culture: time-lapse analysis

Embryo morphokinetics were assessed by time-lapse analysis using an EmbryoScope multi-gas incubator (Unisense, Aarhus, Denmark). Methods for mouse embryo culture in this incubator were modified from a human protocol (Meseguer *et al.,* 2011), in which, individual pronucleate oocytes were transferred to 25 µl drops of G1 medium (Gardner and Lane, 2014) in EmbryoSlide dishes (Unisense) under 1.2ml paraffin oil (Ovoil, Vitrolife) and cultured until 72 h post-hCG under 6% CO_2, 5% O_2 and 89% N_2 at 37°C. Embryos were then transferred to pre-equilibrated G2 medium (Gardner and Lane, 2014) and cultured for a further 46 h. Images of embryo development were acquired every seven minutes throughout the culture period, at five planes of view. The timings of developmental milestones were calculated post-hCG, as well as from pronuclear envelope breakdown. Resultant embryos were assessed for developmental stage before being subjected to differential staining. Timing of developmental events and specific embryo characteristics (zona pellucida width, perivitelline space and embryo area) were then determined retrospectively from stored images (EmbryoViewer; Unisense). Measurement of individual embryos were standardised to the plane of the maximum width of the polar body, and measured at the pronucleate oocyte and early blastocyst stage. Data were analysed only for embryos that developed to the blastocyst stage on time (by day 4.5), as previously defined (Wale and Gardner 2010; Gardner *et al.,* 2004). Only those that developed to the blastocyst stage were used for analysis, to avoid skewing results to groups with lower developmental potential and for relevance to clinical practice in which only data on transferable embryos would be considered.

Embryo culture for metabolic and gene expression analysis

Embryos for metabolic and gene expression analysis were cultured in a Sanyo 19M multi-gas incubator (Sanyo Corporation, Osaka, Japan) under a 6% CO_2, 5% O_2 and 89% N_2 atmosphere at 37°C (Gardner and Lane, 2014). Embryos from Lean Parental matings and Combined Parental Obesity matings were cultured individually in 5 µl pre-equilibrated G1 media under 3.5 ml paraffin

oil. At 72 h post-hCG, embryos were transferred to a) 1 µl pre-equilibrated modified G2 medium (containing glucose as the sole source of carbohydrates) under 3.5 ml paraffin oil for metabolic analysis or b) 5 µl pre-equilibrated G2 medium under 3.5 ml paraffin for a further 48 h before being snap frozen for subsequent gene expression analysis.

RNA extraction and Reverse transcription

Total RNA was isolated from frozen blastocysts from Lean Parental matings and Combined Parental Obesity matings (n =40 blastocysts/group, repeated in triplicate) using an Absolutely RNA Nanoprep Kit (Agilent Technologies; Mulgrave, Australia) according to the manufacturer's instructions (Dupont *et al.,* 2012). Briefly, RNA was bound to a column matrix and a series of salt washes removed contaminants. Isolated RNA was DNase treated to eliminate contaminating DNA according to the manufacturer's specifications (Agilent Technologies). The cDNA was synthesized from RNA using Superscript III Reverse Transcriptase (Life Technologies; Mulgrave, Australia) and Random Primers (Promega; Alexandria, Australia) according to the manufacturer's instructions (Invitrogen; Carlsbad, USA) and as previously described (Harvey *et al.,* 2004).

Quantitative RT-PCR gene expression analysis

Real time qRT-PCR was performed on a ViiA^{TM}7 thermocycler (Applied Biosystems, Mulgrave, Australia) as previously described (Harvey *et al.,* 2004). Primers were designed using Primer Express (Applied Biosystems) and were synthesized by Geneworks (Adelaide, Australia). Primer specificity and efficiency were calculated using dilutions of liver cDNA samples, followed by embryo cDNA samples. The genes investigated were Glucose transporter 1 (*Glut 1*), Pyruvate kinase isoform two (*Pkm2*), Peroxisome proliferator-activated receptor gamma (*Ppar γ*), Insulin growth-like factor 2 receptor (*Igf2r*), and Glucose transporter 3 (*Glut 3*). Primer sequences and product sizes are detailed in Table I.

Real time qRT-PCR was performed in triplicate 10 µl reactions containing 1X SYBR Green Master Mix (Invitrogen) and 500 nM forward and reverse primers. cDNA was diluted in nuclease-free water

(Life Technologies) to a concentration equivalent to cDNA generated from 0.25 embryo per microlitre. Reactions were run according to the following parameters; 50°C for 5 mins, 95°C for 10 mins, then 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 5 mins. The cycle threshold (Ct) was calculated for each sample using the ViiA^{TM}7 software. Dissociation curves, to detect non-specific amplification, were generated for all reactions. No template samples containing water substituted in place of cDNA were included in all assays to confirm the absence of non-specific amplification products, as were minus RT samples to confirm absence on DNA contamination. Raw Ct values were analysed using the delta CT method in the Q-Gene software package (Muller *et al.,* 2002; Simon, 2003) normalised to that of 18S rRNA. 18S rRNA was confirmed as an appropriate housekeeper, against a group of candidate housekeeper genes, using NormFinder software package (Andersen *et al.,* 2004). Data were then expressed as a fold change relative to the Lean Parented embryo group.

Assessment of glucose consumption and lactate production

Individual embryo glucose consumption and lactate production was assessed in a cohort of embryos from Lean Parental matings and Combined Parental Obesity matings. Compacted morula were placed into G2-modified media, containing 0.5 mM glucose as the sole carbohydrate source, and without lactate at 72 h post-hCG for a period of 24 h. Spent medium of resultant early blastocysts (early blastocyst defined as a stage from cavity formation to less than half total embryo volume comprising the blastocoel) was analysed by microfluorescence as previously described (Gardner and Leese, 1990; Lane and Gardner, 1998). Subsequently, total cell number was determined via staining in 0.1 mg/ml Bisbenzimide (Hoechst, 33342; Sigma Aldrich; Castle Hill, Australia) in 10% v/v ethanol for 30 min, washed in GMOPS+ for 5 min then mounted in glycerol on glass slides under coverslips (Thermo Fisher; Scoresby, Australia). Cell numbers were visualised and photographed using a fluorescent microscope (Nikon Eclipse TS100; Olympus, Tokyo, Japan) equipped with a Nikon Digital Sight DS-L2 camera (Nikon; Tokyo, Japan). Cell numbers were retrospectively determined manually using ImageJ Version1.47 (Schneider *et al.,* 2012). Metabolic measurements were expressed on a per

embryo basis, as well as per cell per hour for each individual embryo to account for variation in embryonic cell number.

Assessment of blastocyst cell allocation

Allocation of cells to the inner cell mass (ICM) and the trophectoderm (TE) of blastocysts was determined via differential nuclear staining, as previously described (Hardy *et al.,* 1989). Briefly, blastocysts were placed in 0.5% pronase (Sigma-Aldrich) until the zona pellucida disbanded, followed by washing in GMOPS+ for 5 mins. Embryos were then incubated in 10 mM 2,4,6-trinitrobenzene sulfonic acid (TNBS; Sigma-Aldrich) for 10 min then washed in GMOPS+ for 5 min, before a 10 min incubation in 0.1 mg/ml anti-dinitrophenol (Sigma-Aldrich). Blastocysts were subsequently washed for 5 min in GMOPS+, then incubated in 10% v/v guinea pig serum with 25 mg/ml propidium iodide (IMVS, Adelaide, Australia) for 5 min. Blastocysts were transferred to 0.1 mg/ml Bisbenzimide (Sigma-Aldrich; Hoechst, 33342) in 10% v/v ethanol for 15 min, washed in in GMOPS+ and finally mounted in glycerol on glass slides under coverslips (Thermo Fisher). Cell were visualised and photographed using a fluorescent microscope (Nikon Eclipse TS100) equipped with a Nikon Digital Sight DS-L2 camera (Nikon). Cell numbers were then counted manually using ImageJ Version1.47 (Schneider *et al.,* 2012).

Statistical analyses

All data were assessed for normal distribution via the Shapiro–Wilk test. Animal body weight gain was analysed using a two-tailed t-test. Day 5 blastocyst rate was arc-sine transformed prior to analysis. Metabolic data were measured as pmol/embryo/h, and also normalised for cell number prior to analysis. Gene expression levels were normalised to that of the Lean Parented embryo group. Time-lapse data were analysed relative to hours post-hCG, and also normalised to individual embryo pronuclear envelope breakdown to mitigate possible effects of the timing of mating. The effect of parental obesity on embryo measurements (zona pellucida width, perivitelline space and embryo area), timing of developmental events and cell allocation were analysed by ANOVA using a PROC MIXED procedure employing a Tukey's post hoc analysis to identify differences between groups.

Culture replicate was included in the model as a random factor. Metabolic data, total cell counts and gene expression data were analysed using two tailed t-tests. All analyses were performed in SPSS Version 20 (IBM, Armonk, USA). Data are presented as a mean ± SEM unless otherwise stated. Significance was determined at the level of $P < 0.05$.

Results

Impact of dietary fat on parental weight gain

Female mice fed a HFD gained significantly more weight after an eight week feeding period compared with control fed mice, as well as having significantly increased peritoneal fat deposits ($P < 0.001$, Table II). Similarly, male mice fed a high fat diet for eight weeks gained significantly more weight than mice on control diets and had increased peritoneal fat deposit weight ($P < 0.01$, Table II).

Impact of parental obesity upon mating and fertilisation success

The presence of a copulatory plug (indicative of mating success) was significantly lower in the Combined Obesity Parental matings (33.56% ± 5.87) compared with Lean Parental matings (68.05% ± 5.76, $P < 0.001$). There was no significant difference between Paternal Obese mating success or Maternal Obese mating success compared with Lean Parental matings ($P > 0.05$). The number of oocytes ovulated per female (including fertilised, unfertilised and degenerate) was significantly reduced in the females mated in Combined Obese Parental matings (11.74 ± 0.73, $P < 0.01$). The number of fertilised eggs per female was also significantly reduced in the females of Maternal Obese matings (9.21 ± 1.56, $P < 0.05$), and of Combined Obese Parental matings (8.36 ± 0.66, $P < 0.001$) compared with females of Lean Parental matings (12.65 ± 0.71).

Obesity effects on developmental potential

Compared with Lean Parented embryo development rates, the percentage of fertilised zygotes reaching the blastocyst stage was significantly reduced in groups in which one parent was obese (Paternal Obese Parented 15% decrease, Maternal Obese Parented 11% decrease, $P < 0.05$; Figure 1). Similarly, compared with the Lean Parented embryo development rates blastocyst development from

Combined Obese Parented embryos was further decreased (27% decrease, $P < 0.01$). Development rates of Combined Obese Parented embryos did not differ from embryos that had one obese parent ($P > 0.1$). This reduction in blastocyst rate did not correlate to a block at a specific developmental stage, rather development failure occurred throughout the culture period.

Obesity effects on developmental kinetics

Analysis of developmental timings relative to hours post-hCG administration demonstrated a delay of 1 h by pronuclear envelope breakdown (PNB, $P < 0.05$) in Combined Obese Parented compared with Lean Parented embryos. Timing of the pronuclear envelope breakdown in Combined Obese Parented embryos did not differ from either Maternal Obese Parented or Paternal Obese Parented embryos ($P > 0.1$). Paternal Obese Parented and Maternal Obese Parented embryos did not show significant delays in developmental timing compared to Lean Parented embryos ($P > 0.1$). The delay in developmental timings evident in Combined Parental Obese Parented embryos extended to 7 h at the time of blastocoel formation (tSB, $P < 0.01$; Figure 2a) relative to those of Lean Parented embryos.

When developmental timings were expressed relative to individual embryo pronuclear envelope breakdown, rather than hours post-hCG to remove any potential cofounding effects in the time of mating, delays in development to the 2-cell stage were not evident (t2, $P > 0.1$). Combined Obese Parented embryos displayed a 1 h delay at the 4-cell division (t4, $P < 0.05$), which extended to a 6 h delay at the time of blastocoel formation (tSB, $P < 0.01$; Figure 2b) relative to Lean Parented embryos. Timing of the first cleavage division did not differ significantly from Maternal Obese Parented or Paternal Obese Parented embryos ($P > 0.1$). Significant differences between Combined Obese Parented Embryos and Maternal Obese Parented or Paternal Obese Parented embryos appeared from the 5-cell division onwards and persisted until the time of blastocoel formation (tSB, $P < 0.05$).

Obesity effects on blastocyst cell lineage allocation

No differences in inner cell mass (ICM) cell number or ICM:TE ratio were observed between any of the groups (Figure 3a and c). Trophectoderm (TE) and Total cell numbers were reduced in both

Maternal Obese Parented and Combined Obese Parented embryos relative to Lean Parented embryos ($P < 0.05$; Figure 3b and d).

Metabolic analysis

Glucose consumption and lactate production when expressed per embryo were not different between Lean Parented and Combined Obese Parented embryos (Figure 4a). Total cell number was lower in Combined Obese Parented than Lean Parented embryos ($P < 0.01$, Figure 4b). Glucose consumption, when normalised for total embryo cell number, was higher ($P < 0.01$) in embryos from Combined Obese Parented embryos compared with Lean Parented embryos (Figure 4c). Lactate production did not differ between the two groups ($P > 0.1$). Glycolytic rate was higher in Combined Obese Parented embryos (52%) compared with Lean Parented embryos (42%), however this difference was not significant (Figure 4d, $P > 0.1$).

Quantitative gene expression analysis in response to combined parental obesity

Glucose transporter 1 (*Glut1*, also known as *Slc2a1*) expression was increased in Combined Obese Parented relative to Lean Parented embryos ($P < 0.05$, Figure 5a). Pyruvate kinase isoform 2 (*Pkm2*) expression was significantly increased in Combined Obese Parented relative to Lean Parented embryos ($P < 0.01$, Figure 5b). Peroxisome proliferator-activated receptor gamma (*Pparγ*) showed a trend towards increased expression in the Combined Obese Parented compared with Lean Parented embryos ($P = 0.08$, Figure 5c). No differences between groups were evident in insulin-like growth factor two receptor (*Igf2r*) or glucose transporter three (*Glut3*) expression (Figure 5d and 5e), respectively.

Obesity alters zona pellucida characteristics

At the pronucleate oocyte stage, zona pellucida (ZP) width was reduced in Combined Obese Parented embryos compared to Lean Parented embryos (Figure 6, $P < 0.05$). Paternal Obese Parented embryos showed no difference in ZP width from Lean Parented embryos. Maternal Obese Parented embryos did not demonstrate a difference in ZP width from Lean Parented or Combined Obese Parented

embryos ($P > 0.1$). There was no difference in measurements of total embryo area and width of the peri-vituline space between any groups ($P < 0.1$). For measurements taken at the blastocyst stage, there was a reduction in ZP width for all groups compared to the pronucleate oocyte stage ($P < 0.05$).

Discussion

Developmental rate and kinetics are reduced by the obese state of parents

The number of two-cell embryos that developed on time to the blastocyst stage was significantly reduced across all groups with an obese parent compared to the Lean Parented embryos, and this reduction was more pronounced in Combined Obese Parented embryos. A reduction in developmental competence of embryos produced when both parents are obese is consistent with previous studies on individual parent obesity in both animals and humans (Bakos *et al.,* 2011a; Bakos *et al.,* 2011b; Binder *et al.,* 2012b; Luzzo *et al.,* 2012) and is likely caused by a number of factors, contributed from both the mother and the father, as reviewed by Lane (2014). One of the principal issues appears to be that oocytes from obese female mice and women show increased levels of aneuploidy, mitochondrial and endoplasmic reticulum stress and apoptosis, hence these conditions may lead to embryonic developmental arrest and may not be permissible to blastocyst formation (Hardy *et al.,* 2001; Acton *et al.,* 2004; Igosheva *et al.,* 2010; Luzzo *et al.,* 2012; Wu *et al.,* 2015). Human embryos of obese couples may show a similar reduction in blastocyst rate, as observed in human paternal obese parented embryos (Bakos *et al.,* 2011a). There may also be a greater reduction in human blastocyst rates based on additional confounding factors, such as subfertility and other comorbidities, which would translate to a decreased potential for implantation and successful pregnancy.

Notably, in the current study for those embryos that did develop to the blastocyst stage, development was at a slower rate than that of Lean Parented embryos, with an initial delay of 1 h at the 3-cell division, which accumulated to 6 h by the blastocyst stage. This delay is greater than previously seen in individual parental obesity studies, which reported differences of one hour or less throughout development in IVF and mated models of embryonic development (Binder *et al.,* 2012a; Binder *et al.,* 2012b). Human studies of the impacts of parental obesity and timing of embryonic development

require further investigation, as these studies remain limited, with conflicting reports (Bellver *et al.*, 2013; Leary *et al.*, 2015), potentially due to small sample sizes. The combined contributions of oocytes and sperm from obese parents may lead to the increase in developmental times and/or greater variation in the time at which embryos reach expected developmental time points. This may therefore have clinical implications when standardising embryo developmental timings across patients with a range of BMIs, as well as for selecting the best embryo to transfer. The present study employed a mated model, thus variation in mating time may also contribute to the delay in embryonic development. However, in the present study, timings were also calculated from pronuclei disappearance to account for any differences in time of mating. Irrespective of this correction, delays in embryonic developmental timing were still evident. Furthermore, the length of time between 2- and 3-cell divisions, which is independent of mating time, was increased in the Combined Obese Parented embryos. Importantly in ART clinics, this length of time has previously been determined as a marker for embryo quality in two independent human studies, with increased time giving embryos a 'negative score' in the algorithm regarding transferrable quality embryos (Wong *et al.,* 2010; Meseguer *et al.,* 2011). Therefore, the innate ability of the resulting zygote to develop appears compromised independent of differences in time of mating.

The exposure to reproductive tract fluids in this model may also contribute to the alteration in embryonic developmental timing. Seminal fluid is known to have increased triglyceride concentrations in obese animals and humans, and it is likely female reproductive fluids would show similar changes in response to diet (Martini *et al.,* 2010; Binder *et al.,* 2014; Bromfield *et al.,* 2014). The increase in fat content may alter the viscosity and density of the various reproductive fluids, impacting the ability of sperm to swim towards the ampulla thereby extending the time to fertilisation and extending the timing of development seen in the present study (De Celis *et al.,* 2000; Gulaya *et al.,* 2001). Individual parental obesity embryos show a slight delay in their developmental timings, although this does not differ significantly from Lean Parented embryos. Importantly, this demonstrates that an effect on embryonic development is evident when either maternal or paternal obesity is present. The increased, and significant, delay in Combined Obese Parental embryos may

reflect the suboptimal interaction between high fat male and female reproductive fluids, as well as with the embryo.

On time development of embryos has been associated with increased pregnancy rates in humans (Meseguer *et al.,* 2012; Wong *et al.,* 2013; Rubio *et al.,* 2014). However, this theory remains contentious, highlighting the needs for further prospective studies (Kaser and Racowsky, 2014; Kirkegaard *et al.,* 2015). Delayed embryonic development may have an impact on implantation, considering that implantation is dependent on an intricate dialogue between the embryo and the uterus, and involves a number of factors from both the uterus and embryo (Wang and Dey, 2006). As the Combined Obese Parented embryos are delayed on day 4 (earlier developmental stage than required for implantation), they may not be developmentally competent for implantation, and this may translate in a reduced ability for implantation to occur (Paria *et al.,* 1993). Furthermore, Combined Obese Parented embryos have reduced TE cells, which can translate to poor implantation and placentation (Hardy *et al.,* 1989; Cross *et al.,* 1994). This is consistent with previous mouse studies that demonstrate slower developmental timing, caused by parental obesity or other means, and has been associated with decreased implantation rates (Mitchell *et al.,* 2010; Binder *et al.,* 2012a). It would be of interest to investigate the chemical composition, such as triglyceride levels and how these interact with key hormones, as potentially this would alter the physiology of the tract and receptivity to embryonic implantation. The understanding of how delayed embryonic development interacts with an altered maternal uterine environment is an area that could potentially improve reproductive outcomes in obese couples.

Combined parental obesity alters glucose metabolism and metabolic gene expression

Obese and lean individuals are considered to have vastly different metabolic states and employ different metabolic pathways, and this concept should be extended to embryos. Combined Obese Parented blastocysts exhibited an increased uptake of glucose from media compared to Lean Parented blastocysts, when expressed per cell. Previous studies have demonstrated that maternal obesity in the mouse leads to increased glucose uptake per cell (Binder *et al.,* 2012b). Increased glucose uptake may

suggest increased glycolysis, a common and essential metabolic pathway for the blastocyst (Leese and Barton, 1984; Gardner and Wale, 2013). However, lactate, the final product of glycolysis, output remained unchanged from Lean Parented embryos, suggesting the excess glucose is being metabolised via an alternative pathway, such as the Pentose Phosphate Pathway (PPP). Potentially, the PPP is used as the concomitant production of NADPH can mitigate the effects of oxidative stress seen in the gametes and embryos of obese parents (McCord, 2000; Igosheva *et al.,* 2010; Bakos *et al.,* 2011b; Binder *et al.,* 2012b). Alternatively, glucose could be being metabolised through the hexosamine biosynthesis pathway, which supports amine sugar synthesis while also having key roles in growth and the development of insulin resistance (Sutton-McDowall *et al.,* 2010). In contrast to the data presented, a recent human study on maternal obesity reported a decrease in total blastocyst glucose consumption with increasing BMI. However, these findings, based on seven patients, determined blastocysts from women with a BMI>25 kg/m^2 were smaller in diameter and had significantly fewer cells (Leary *et al.,* 2015). Unlike the current study, it is also postulated that metabolic manifestations associated with obesity, in terms of perturbed glucose and insulin homeostasis, would also be evident in these obese patients further impacting embryo physiology. The data presented in this study indicate that more detailed metabolomic analyses of Combined Obese Parented embryos are warranted.

Assessment of metabolic gene expression levels in blastocysts of Combined Obese Parented embryos revealed a significant increase in glucose transporter 1 (*Glut1*) and Peroxisome proliferator-activated receptor gamma (*Ppar γ),* and a trend for increased pyruvate kinase 2 (*Pkm2)* expression. In the blastocyst, *Glut1* is localised to apical membranes of the trophectoderm, and has been shown to have a key role in detecting and responding to the surrounding glucose environment (Pantaleon and Kaye, 1998). The observed increase in *Glut1* expression and increased glucose uptake in this study may indicate metabolic stress in the Combined Obese Parented embryos. While increased glucose uptake has been linked to increased developmental competence in blastocysts, glucose uptake must be regulated within a narrow range to ensure viability (Gardner and Wale, 2013). Up-regulation of *Glut1* may also drive the increased expression of *Pkm2* evident in the Combined Obese Parented blastocyst.

457 *Pkm*2 is essential to the aerobic conversion of phosphenolpyruvate to pyruvate and is essential for
458 biosynthesis in proliferative cell types, such as cancer embryonic cells (Gupta and Bamezai, 2010;
459 Krisher and Prather, 2012; Gardner and Wale, 2013; Gardner and Harvey, 2015). Increased *Pkm2*
460 expression does not result in an increase in glycolysis, even in the presence of high glucose uptake, to
461 allow for increased biosynthesis for key embryonic components such as nucleic acids, which occurs
462 via the alternate Pentose Phosphate Pathway (Krisher and Prather, 2012; Gardner and Wale, 2013).
463 The trend towards increased *Pparγ* expression, which is activated by fatty acids, may also indicate
464 metabolic stress, as it plays a key role in glucose homeostasis (Minge *et al.,* 2008).
465
466 The model of diet-induced obesity employed in the present study is free from the many metabolic
467 complications evident in humans. The short feeding period (8 weeks) does not affect fasting plasma
468 glucose levels (Bakos *et al.,* 2011; Mitchell *et al.,* 2011; Palmer *et al.,* 2012), or glucose or insulin
469 tolerance (Fullston *et al.,* 2013). Similar models of diet-induced obesity, involving higher fat diet,
470 generational feeding, and additional environmental stressors, do demonstrate pre-diabetic symptoms
471 in mice (Winzell and Ahrén, 2004; Sun *et al.,* 2009; Groover *et al.,* 2013; Hillian *et al.,* 2013; Ge *et*
472 *al.,* 2014). Indeed, the obese state is associated with hyperglycaemia and increased free fatty acids
473 (Boden, 2008; Martyn *et al.,* 2008), which have negative impacts on early mammalian embryo
474 development (Van Hoeck *et al.,* 2011; Cagnone *et al.,* 2012). Therefore, in the more complex
475 metabolic state of obese humans, there is likely to be a greater impact upon early embryonic
476 development. The exposure of oocytes, sperm and the embryo to the more complex metabolic
477 environment in humans may thus lead to a greater up-regulation of these metabolic genes in the
478 embryo than found in this study. This could have great impacts on gene, and protein function,
479 resulting in further perturbed embryonic phenotypes and potentially adult disease.
480
481 ***Embryo characteristics are altered by obesity***
482 This is the first study to demonstrate that obesity has an impact on zona pellucida width. Combined
483 Obese Parented embryos exhibited a significant reduction in zona pellucida width. Zona pellucida
484 width is further reduced by the contribution of parental obesity in Combined Obese Parented

485 embryos, suggesting there is a further paternal contribution. Previous studies have associated higher
486 zona integrity with increased implantation (Montag *et al.,* 2008). Further studies are required to
487 determine how obesity impacts zona pellucida width with ZP gene expression and glycoprotein cross-
488 linking potential candidates. The role of the zona pellucida and perturbations to its structure during
489 embryonic development is interesting and warrants further investigation, especially as more
490 individuals seek ART (Ferraretti *et al.,* 2013).
491
492 ***Conclusion***
493 The present study demonstrates that combined parental obesity prior to and at the time of conception,
494 results in a detrimental impact upon blastocyst developmental potential, consistent with previous
495 studies of individual parent obesity. Zona pellucida width was shown, for the first time, to be thinner
496 in Combined Obese Parented embryos and may play a critical role in modulating embryonic
497 development and the embryos interaction with its surrounding environment. Glucose metabolism was
498 perturbed in Combined Obese Parented embryos, with significantly more glucose being taken up per
499 cell than that of Lean Parented embryos. Furthermore, key genes in the regulation of metabolism were
500 up-regulated in Combined Obese Parented embryos, indicative of oxidative stress. Persistent changes
501 to this gene expression could confer increased adult disease risk. While long-term programming
502 cannot be confirmed in this current study, as future studies with embryo transfers are required, it is
503 clear that combined parental obesity negatively impacts preimplantation embryonic development.
504 Previous studies indicate early embryonic changes do program long-term health of the individual
505 (Sinclair and Singh, 2007; Lane *et al.,* 2014). This study is limited to mouse embryos, however
506 human embryos may be similarly impacted by combined parental obesity. As more individuals
507 become obese, issues surrounding early embryonic development will become more pertinent to ART
508 protocols. Therefore, promoting early intervention for parents, as well as identifying and
509 incorporating variability in embryonic parameters as a result of parental obesity will aid the
510 development of appropriate, customised ART protocols.
511

Author's roles

All authors contributed to the design of the research study; B.J.F. performed the experiments; all authors contributed to the analysis of the data; D.K.G. provided funding; all authors contributed to the writing of the manuscript.

Acknowledgements

The authors would like to thank Tania Long and Darren Cipolla for their assistance in mice handling and care. We would also like to acknowledge Unisense Fertilitech for the use of the Embryoscope.

Funding

This study was funded from University of Melbourne research monies and did not receive any funding from a commercial company.

Conflict of interest

M.P.G currently holds the position of Merck Serono Lecturer of Reproductive Biology. D.K.G. received research funds from Vitrolife AB Sweden. The other authors of this manuscript have nothing to declare and no conflicts of interest.

References

Acton BM, Jurisicova A, Jurisica I, Casper RF. Alterations in mitochondrial membrane potential during preimplantation stages of mouse and human embryo development. *Mol Hum Reprod* 2004;**10**: 23-32.

Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* 2004;**64**: 5245-5250

Bakos HW, Henshaw RC, Mitchell M, Lane M. Paternal body mass index is associated with decreased blastocyst development and reduced live birth rates following assisted reproductive technology. *Fertil Steril* 2011a;**95**: 1700-1704.

Bakos HW, Mitchell M, Setchell BP, Lane M. The effect of paternal diet-induced obesity on sperm function and fertilization in a mouse model. *Int J Androl* 2011b;**34**: 402-410.

Bellver J, Mifsud A, Grau N, Privitera L, Meseguer M. Similar morphokinetic patterns in embryos derived from obese and normoweight infertile women: a time-lapse study. *Hum Reprod* 2013;**28**: 794-800.

Bermejo-Alvarez P, Rosenfeld CS, Roberts RM. Effect of maternal obesity on estrous cyclicity, embryo development and blastocyst gene expression in a mouse model. *Hum Reprod* 2012;**27**: 3513-3522.

Binder NK, Hannan NJ, Gardner DK. Paternal diet-induced obesity retards early mouse embryo development, mitochondrial activity and pregnancy health. *PloS One* 2012a;**7**: e52304.

Binder NK, Hannan NJ, Sheedy JR, Gardner DK. Male obesity is associated with changed spermatozoa Cox4i1 mRNA level and altered seminal vesicle fluid composition in a mouse model. *Mol. Hum. Reprod.* 2015 doi:10.1093/molehr/gav010.

Binder NK, Mitchell M, Gardner DK. Parental diet-induced obesity leads to retarded early mouse embryo development and altered carbohydrate utilisation by the blastocyst. *Reprod Fertil Dev 2012*b;**24**: 804-812.

Boden G. Obesity and Free Fatty Acids (FFA). *Endocrinol Metab Clin North Am* 2008;**37**: 635.

Bromfield JJ, Schjenken JE, Chin PY, Care AS, Jasper MJ, Robertson SA. Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proc Natl Acad Sci U S A* 2014;**111**: 2200-2205.

Cagnone GL, Dufort I, Vigneault C, Sirard MA. Differential gene expression profile in bovine blastocysts resulting from hyperglycemia exposure during early cleavage stages. *Biol Reprod* 2012;**86**: 2, 1-12.

Chang K-J, Huang D, Cuatrecasas P. The defect in insulin receptors in obese-hyperglycemic mice: A probable accompaniment of more generalized alterations in membrane glycoproteins. *Biochem Biophysical Research Commun* 1975;**64**: 566-573.

Chavatte-Palmer P, Tarrade A, Levy R. [Developmental origins of health and disease in adults: role of maternal environment]. *Gynecol Obstet Fertil* 2012;**40**: 517-519.

Cross JC, Werb Z, Fisher SJ. Implantation and the placenta: key pieces of the development puzzle. *Science* 1994;**266**: 1508-1518.

De Celis R, Feria-Velasco A, González-Unzaga M, Torres-Calleja J, Pedrón-Nuevo N. Semen quality of workers occupationally exposed to hydrocarbons. *Fertil Steril* 2000;**73**: 221-228.

Dupont C, Harvey AJ, Armant DR, Zelinski MB, Brenner CA. Expression profiles of cohesins, shugoshins and spindle assembly checkpoint genes in rhesus macaque oocytes predict their susceptibility for aneuploidy during embryonic development. *Cell Cycle* 2012;**11**: 740-748.

Dupont C, Faure C, Sermondade N, Boubaya M, Eustache F, Clement P, Briot P, Berthaut I, Levy V, Cedrin-Durnerin I, Benzacken B, Chavatte-Palmer P, Levy R. Obesity leads to higher risk of sperm DNA damage in infertile patients. *Asian J Androl* 2013;**15**: 622-625.

Ferraretti AP, Goossens M, Kupka S, Bhattacharya J, de Mouzon J, Castilla A, Erb K et al. Assisted reproductive technology in Europe, 2009: results generated from European registers by ESHRE. *Hum Reprod* 2009;**28**: 2318-2331

Feuer SK, Camarano L, Rinaudo PF. ART and health: clinical outcomes and insights on molecular mechanisms from rodent studies. *Mol Human Reprod* 2013;**19**: 189-204.

Fullston T, Palmer NO, Owens JA, Mitchell M, Bakos HW, Lane M. Diet-induced paternal obesity in the absence of diabetes diminishes the reproductive health of two subsequent generations of mice. *Hum Reprod* 2012;**27**: 1391-1400.

Fullston T, Ohlsson Teague EMC, Palmer NO, DeBlasio MJ, Mitchell M, Corbett M, Print CG, Owens JA, Lane M. Paternal obesity initiates metabolic disturbances in two generations of mice with incomplete penetrance to the F2 generation and alters the transcriptional profile of testis and sperm microRNA content. *FASEB J* 2013;**27**: 4226-4243.

Gardner DK, Harvey AJ. Blastocyst metabolism. *Reprod Fert Dev* 2015 doi: 10.1071/RD14421.

Gardner DK, Lane M. Mammalian preimplantation embryo culture. *Methods Mol Biol* 2014;**1092**: 167-182.

Gardner DK, Lane M, Watson AJ. *A laboratory guide to the mammalian embryo*, 2004. Oxford University Press.

Gardner DK, Leese HJ. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. *Reproduction* 1990;**88**: 361-368.

Gardner DK, Wale PL. Analysis of metabolism to select viable human embryos for transfer. *Fertil Steril* 2013;**99**: 1062-1072.

Gardner DS, Pearce S, Dandrea J, Walker R, Ramsay M, Stephenson T, Symonds ME. Peri-implantation undernutrition programs blunted angiotensin II evoked baroreflex responses in young adult sheep. *Hypertension* 2004;**43**: 1290-1296.

Ge Z-J, Liang Q-X, Hou Y, Han Z-M, Schatten H, Sun Q-Y, Zhang C-L. Maternal obesity and diabetes may cause DNA methylation alteration in the spermatozoa of offspring in mice. *Reprod Biol Endocrinol* 2014;**12**: 29-29.

Goto Y, Noda Y, Narimoto K, Umaoka Y, Mori T. Oxidative stress on mouse embryo development in vitro. *Free Radic Biol Med* 1992;**13**: 47-53.

Guh DP, Zhang W, Bansback N, Amarsi Z, Birmingham CL, Anis AH. The incidence of co-morbidities related to obesity and overweight: a systematic review and meta-analysis. *BMC Public Health* 2009;**9**: 88.

Gulaya NM, Margitich VM, Govseeva NM, Klimashevsky VM, Gorpynchenko, II, Boyko MI. Phospholipid composition of human sperm and seminal plasma in relation to sperm fertility. *Arch Androl* 2001;**46**: 169-175.

Gupta V, Bamezai RN. Human pyruvate kinase M2: a multifunctional protein. *Protein Sci* 2010;**19**: 2031-2044.

Groover AL, Ryals JM, Guilford BL, Wilson NM, Christianson JA, Wright DE. Exercise-mediated improvements in painful neuropathy associated with prediabetes in mice. *Pain* 2013;**154**: 2658-2667.

Hardy K, Handyside AH, Winston RM. The human blastocyst: cell number, death and allocation during late preimplantation development in vitro. *Development* 989;**107**: 597-604.

Hardy K, Spanos S, Becker D, Iannelli P, Winston RML, Stark J. From cell death to embryo arrest: Mathematical models of human preimplantation embryo development. *Proc Natl Acad Sci U S A* 2001;**98**: 1655-1660.

Harvey AJ, Kind KL, Pantaleon M, Armstrong DT, Thompson JG. Oxygen-regulated gene expression in bovine blastocysts. *Biol Reprod* 2004;**71**: 1108-1119.

Hillian AD, McMullen MR, Sebastian BM, Rowchowdhury S, Kashyap SR, Schauer PR, Kirwan JP, Feldstein AE, Nagy LE. Mice lacking C1q are protected from high fat diet-induced hepatic insulin resistance and impaired glucose homeostasis. *J Biol Chem* 2013;**288**: 22565-22575.

Igosheva N, Abramov AY, Poston L, Eckert JJ, Fleming TP, Duchen MR, McConnell J. Maternal diet-induced obesity alters mitochondrial activity and redox status in mouse oocytes and zygotes. *PloS One* 2010;**5**: 0010074.

Jensen TK, Andersson A-M, Jørgensen N, Andersen A-G, Carlsen E, Petersen JøH, Skakkebæk NE. Body mass index in relation to semen quality and reproductive hormonesamong 1,558 Danish men. *Fertil Steril* 2004;**82**: 863-870.

Kaser DJ, Racowsky C. Clinical outcomes following selection of human preimplantation embryos with time-lapse monitoring: a systematic review. *Hum Reprod Update* 2014;**20**: 617-631.

Kind KL, Collett RA, Harvey AJ, Thompson JG. Oxygen-regulated expression of GLUT-1, GLUT-3, and VEGF in the mouse blastocyst. *Mol Reprod Dev* 2005;**70**: 37-44.

Kirkegaard K, Ahlström A, Ingerslev HJ, Hardarson T. Choosing the best embryo by time lapse versus standard morphology. *Fertili and Steril* 2015;**103**: 323-332.

Kort HI, Massey JB, Elsner CW, Mitchell-Leef D, Shapiro DB, Witt MA, Roudebush WE. Impact of body mass index values on sperm quantity and quality. *J Androl* 2006;**27**: 450-452.

Krisher RL, Prather RS. A role for the Warburg effect in preimplantation embryo development: metabolic modification to support rapid cell proliferation. *Mol Reprod Dev* 2012;**79**: 311-320.

Kupka MS, Gnoth C, Buehler K, Dahncke W, Kruessel J-S. Impact of female and male obesity on IVF/ICSI: results of 700,000 ART-cycles in Germany. *Gynecol Endocrinol* 2011;**27**: 144-149.

Lane M, Gardner DK. Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts. *Hum Reprod* 1998;**13**: 991-997.

Lane M, Robker RL, Robertson SA. Parenting from before conception. *Science* 2014;**345**: 756-760.

Leary C, Leese HJ, Sturmey RG. Human embryos from overweight and obese women display phenotypic and metabolic abnormalities. *Hum Reprod* 2015;**30**: 122-132.

Leese HJ, Barton AM. Pyruvate and glucose uptake by mouse ova and preimplantation embryos. *Reproduction* 1984;**72**: 9-13.

Lin S, Thomas T, Storlien L, Huang X. Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice. *Int J Obes Relat Metab Disord* 2000;**24**: 639-646.

Luo W, Semenza GL. Emerging roles of PKM2 in cell metabolism and cancer progression. *Trends Endocrinol Metab* 2012;**23**: 560-566.

655 Luzzo KM, Wang Q, Purcell SH, Chi M, Jimenez PT, Grindler N, Schedl T, Moley KH. High fat diet
656 induced developmental defects in the mouse: oocyte meiotic aneuploidy and fetal growth
657 retardation/brain defects. *PloS One* 2012;**7**: 12.

658 Maheshwari A, Stofberg L, Bhattacharya S. Effect of overweight and obesity on assisted reproductive
659 technology: a systematic review. *Hum Reprod Update* 2007;**13**: 433-444.

660 Maloney C, Hay S, Young L, Sinclair K, Reese, W. A methyl-deficient diet fed to rat dams during the
661 peri-conception period programs glucose homeostasis in adult male but not female offspring. *J Nutr*
662 2011;**14**: 95-100

663 Martini AC, Tissera A, Estofán D, Molina RI, Mangeaud A, de Cuneo MF, Ruiz RD. Overweight and
664 seminal quality: a study of 794 patients. *Fertil Steril* 2010;**94**: 1739-1743.

665 Martyn JA, Kaneki M, Yasuhara S. Obesity-induced insulin resistance and hyperglycemia: etiologic
666 factors and molecular mechanisms. *Anesthesiology* 2008;**109**: 137-148.

667 McCord JM. The evolution of free radicals and oxidative stress. *Am J Med* 2000;**108**: 652-659.

668 McPherson NO, Fullston T, Bakos HW, Setchell BP, Lane M. Obese father's metabolic state,
669 adiposity, and reproductive capacity indicate son's reproductive health. *Fertil Steril* 2014;**101**: 865-
670 873.e861.

671 Meseguer M, Herrero J, Tejera A, Hilligsøe KM, Ramsing NB, Remohí J. The use of morphokinetics
672 as a predictor of embryo implantation. *Hum Reprod* 2011;**26**: 2658-2671.

673 Meseguer M, Rubio I, Cruz M, Basile N, Marcos J, Requena A. Embryo incubation and selection in a
674 time-lapse monitoring system improves pregnancy outcome compared with a standard incubator: a
675 retrospective cohort study. *Fertil and Steril* 2012;**98**: 1481-1489 e1410.

676 Minge C, Robker R, Norman R. PPAR Gamma: Coordinating metabolic and immune contributions to
677 female fertility. *PPAR Res* 2008;**2008**.

678 Mitchell M, Bakos HW, Lane M. Paternal diet-induced obesity impairs embryo development and
679 implantation in the mouse. *Fertil Steril* 2011;**95**: 1349-1353.

680 Mokdad AH, Ford ES, Bowman BA, Dietz WH, Vinicor F, Bales VS, Marks JS. Prevalence of
681 obesity, diabetes, and obesity-related health risk factors. *JAMA* 2003;**289**: 76-79.

682 Montag M, Schimming T, Köster M, Zhou C, Dorn C, Rösing B, van der Ven H, van der Ven K.
683 Oocyte zona birefringence intensity is associated with embryonic implantation potential in ICSI
684 cycles. *Reprod Biomed Online* 2008;**16**: 239-244.

685 Muller PY, Janovjak H, Miserez AR, Dobbie Z. Processing of gene expression data generated by
686 quantitative real-time RT-PCR. *Biotechniques* 2002;**32**: 1372-1380.

687 Ng M, Fleming T, Robinson M, Thomson B, Graetz N, Margono C, Mullany EC, Biryukov S,
688 Abbafati C, Abera SF., et al. Global, regional, and national prevalence of overweight and obesity in
689 children and adults during 1980?: a systematic analysis for the Global Burden of Disease Study 2013.
690 *Lancet* 2014;**384**: 766-781.

691 Norman RJ, Clark AM. Obesity and reproductive disorders: a review. *Reprod Fertility Dev* 1997;**10**:
692 55-63.

693 Palmer NO, Bakos HW, Owens JA, Setchell BP, Lane M. Diet and exercise in an obese mouse fed a
694 high-fat diet improve metabolic health and reverse perturbed sperm function. *Am J Physiol*
695 *Endocrinol Metab* 2012;**302**: e768-e780.

696 Pantaleon M, Kaye PL. Glucose transporters in preimplantation development. *Reproduction* 1998;**3**:
697 77-81.

698 Paria BC, Huet-Hudson YM, Dey SK. Blastocyst's state of activity determines the "window" of
699 implantation in the receptive mouse uterus. *Proc Natl Acad U S A* 1993;**90**: 10159-10162.

700 Robker RL, Akison LK, Bennett BD, Thrupp PN, Chura LR, Russell DL, Lane M, Norman RJ. Obese
701 women exhibit differences in ovarian metabolites, hormones, and gene expression compared with
702 moderate-weight women. *J Clin Endocrinol Metab* 2009;**94**: 1533-1540.

703 Rubio I, Galan A, Larreategui Z, Ayerdi F, Bellver J, Herrero J, Meseguer M. Clinical validation of
704 embryo culture and selection by morphokinetic analysis: a randomized, controlled trial of the
705 EmbryoScope. *Fertil and Steril* 2014;**102**: 1287-1294 e1285.

706 Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat*
707 *Meth* 2012;**9**: 671-675.

708 Silveira PP, Portella AK, Goldani MZ, Barbieri MA. Developmental origins of health and disease
709 (DOHaD). *Journal Pediatr* 2007;**83**: 494-504.

710 Simon P. Q-Gene: processing quantitative real-time RT–PCR data. *Bioinformatics* 2003;**19**: 1439-
711 1440.

712 Sinclair KD, Singh R. Modelling the developmental origins of health and disease in the early embryo.
713 *Theriogenology* 2007;**67**: 43-53.

714 Skinner MK. Role of epigenetics in developmental biology and transgenerational inheritance. *Birth*
715 *Defects Res, Part C* 2011;**93**: 51-55.

716 Styne-Gross A, Elkind-Hirsch K, Scott Jr RT. Obesity does not impact implantation rates or
717 pregnancy outcome in women attempting conception through oocyte donation. *Fertil Steril* 2005;**83**:
718 1629-1634.

719 Sun Q, Yue P, Deiuliis JA, Lumeng CN, Kampfrath T, Mikolaj MB, Cai Y, Ostrowski MC, Lu B,
720 Parthasarathy S., et al. Ambient air pollution exaggerates adipose inflammation and insulin resistance
721 in a mouse model of diet-induced obesity. *Circulation* 2009;**119**: 538-546.

722 Sutton-McDowall ML, Gilchrist RB, Thompson JG. The pivotal role of glucose metabolism in
723 determining oocyte developmental competence. *Reproduction* 2010;**139**: 685-695.

724 Takahashi M, Keicho K, Takahashi H, Ogawa H, Schulte RM, Okano A. Effect of oxidative stress on
725 development and DNA damage in in-vitro cultured bovine embryos by comet assay. *Theriogenology*
726 2000;**54**: 137-145.

727 Talior I, Yarkoni M, Bashan N, Eldar-Finkelman H. Increased glucose uptake promotes oxidative
728 stress and PKC-δ activation in adipocytes of obese, insulin-resistant mice. *Am J Physiol Endocrinol*
729 *Metab* 2003;**285**: E295-E302.

730 Van Hoeck V, Sturmey RG, Bermejo-Alvarez P, Rizos D, Gutierrez-Adan A, Leese HJ, Bols PEJ,
731 Leroy JLMR. Elevated Non-Esterified Fatty Acid Concentrations during Bovine Oocyte Maturation
732 Compromise Early Embryo Physiology. *PloS One* 2011;**6**: e23183.

van der Steeg JW, Steures P, Eijkemans MJ, Habbema JD, Hompes PG, Burggraaff JM, Oosterhuis GJ, Bossuyt PM, van der Veen F, Mol BW. Obesity affects spontaneous pregnancy chances in subfertile, ovulatory women. *Hum Reprod* 2008;**23**: 324-328.

Wadhwa PD, Buss C, Entringer S, Swanson JM. Developmental origins of health and disease: brief history of the approach and current focus on epigenetic mechanisms *Semin Reprod Med* 2009;**27**: 358-368.

Wahli W, Braissant O, Desvergne B. Peroxisome proliferator activated receptors: transcriptional regulators of adipogenesis, lipid metabolism and more. *Chem Biol* 1995;**2**: 261-266.

Wale PL, Gardner DK. Time-lapse analysis of mouse embryo development in oxygen gradients. *Reprod Biomed Online* 2010;**21**: 402-410.

Wang H, Dey SK. Roadmap to embryo implantation: clues from mouse models. *Nat Rev Genet* 2006;**7**: 185-199.

Watkins A, Lucas E, Fleming T. Impact of the periconceptional environment on the programming of adult disease. *J DOHaD* 2010;**1**: 97-95.

Winzell MS, Ahrén B. The High-Fat Diet–Fed Mouse: A Model for Studying Mechanisms and Treatment of Impaired Glucose Tolerance and Type 2 Diabetes. *Diabetes* 2004;**53**: S215-S219.

Wong C, Chen AA, Behr B, Shen S. Time-lapse microscopy and image analysis in basic and clinical embryo development research. *Reprod Biomed Online* 2013;**26**: 120-129.

Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM, Pera RAR. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nat Biotech* 2010;**28**: 1115-1121.

World Health Organisation. Obesity and Overweight [Fact Sheet] 2015. Retrieved 20/01/2015 from http://www.who.int/mediacentre/factsheets/fs311/en

Wu LL, Russell DL, Wong SL, Chen M, Tsai T-S, St John JC, Norman RJ, Febbraio MA, Carroll J, Robker RL. Mitochondrial dysfunction in oocytes of obese mothers: transmission to offspring and reversal by pharmacological endoplasmic reticulum stress inhibitors. *Development* 2015;**142**: 681-691.

FIGURE LEGENDS

Figure 1. The percentage of fertilised zygotes that reach the blastocyst stage for each of the four reciprocal mating groups; Lean Parented embryos = LP (n = 188), Paternal Obese Parented embryos = POP (n = 96), Maternal Obese Parented embryos = MOP (n = 96) and Combined Obese Parented embryos = COP (n = 157). Different superscript letters show significant differences ($P < 0.05$) between groups. Data expressed as mean ± SEM.

Figure 2. Timing of major developmental events, expressed in hours post-hCG injection (Figure 2a), and hours post pronuclear envelope breakdown (Figure 2b). tPNB = timing of pronuclei breakdown, t2 = timing of 2-cell division, t4 = timing of 4-cell division, t6 = timing of 6 cell division, t8 = timing of 8-cell division, tSB = time blastocoel starts formation. Lean Parented embryos = LP, Paternal Obese Parented embryos = POP, Maternal Obese Parented embryos = MOP and Combined Obese Parented embryos = COP. Different superscript letters indicate a significant difference ($P < 0.05$) between groups. n > 60 embryos per group. Data expressed as mean ± SEM.

Figure 3. Mean cell counts and ratios to show lineage specification for all embryos that developed to the blastocyst stage (Day 5) at the expected time for each of the four groups. a) inner cell mass (ICM) cell counts, b) trophectoderm (TE) cell counts, c) ICM to TE cell ratio, and d) Total cell number. Lean Parented embryos (LP, open bars), Paternal Obese Parented embryos (POP, diagonal hashed bars), Maternal Obese Parented embryos (MOP, horizontal hashed bars), and Combined Obese Parented embryos (COP, shaded bars). * denotes significantly ($P < 0.05$) different from Lean Parented (LP) embryos, n > 60 embryos per group. Data expressed as mean ± SEM.

Figure 4. a) Glucose and lactate concentration in spent culture media expressed h/embryo, b) Total cell number (Day 4 early blastocysts (< half the embryo comprising of the blastocoel)), c) Glucose and lactate concentration in spent culture media expressed h/cell, d) Glycolytic rate (%glucose/lactate) of Lean Parented embryos (LP, open bars) and Combined Obese Parented (COP, shaded bars) blastocysts. **$P < 0.01$. n > 20 embryos per group. Data expressed as mean ± SEM.

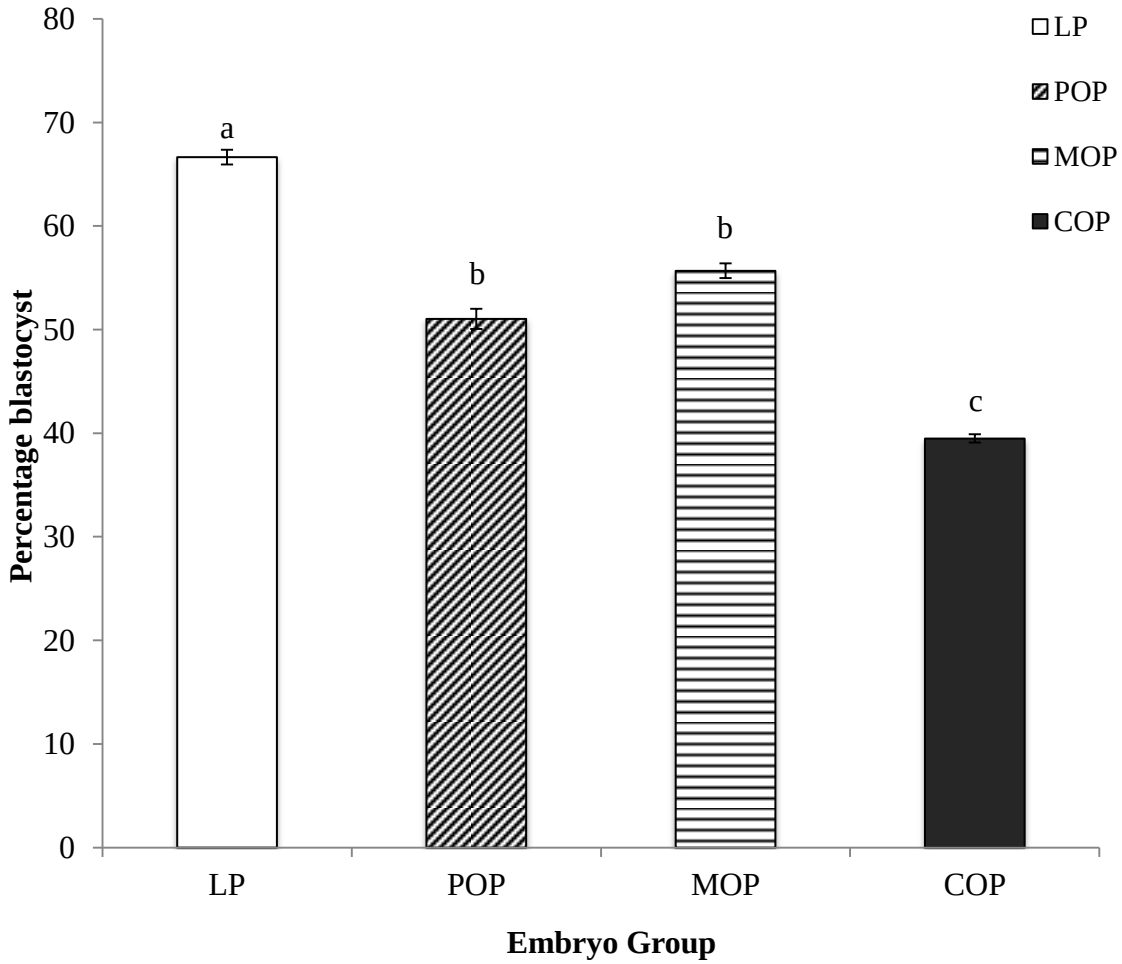
788

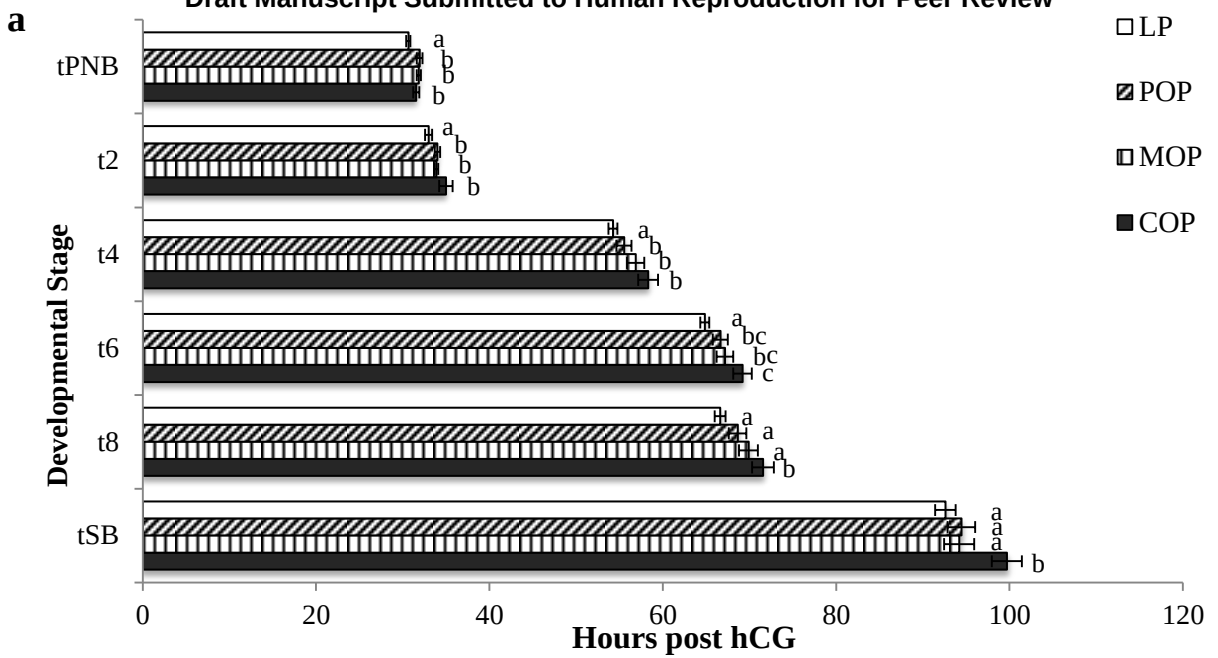
789 **Figure 5.** Mean expression of a) Glucose transporter 1 *(Glut 1),* b) Pyruvate kinase isoform two
790 *(Pkm2),* c) Peroxisome proliferator-activated receptor gamma (*Ppar γ*), *d)* Insulin growth-like factor 2
791 receptor *(Igf2r),* and e) Glucose transporter 3 *(Glut 3)* relative to 18S rRNA. Expression from
792 Combined Obese Parented (COP) embryos is reflected by shaded bars. Values for COP embryos are
793 normalised to the expression of embryos from Lean Parented (LP) embryo group (open bars) set at 1.
794 * $P < 0.05$ **$P < 0.01$, ^ $P < 0.08$, n=3 replicates of > 40 embryos per group. Data are expressed as
795 mean ± SEM.

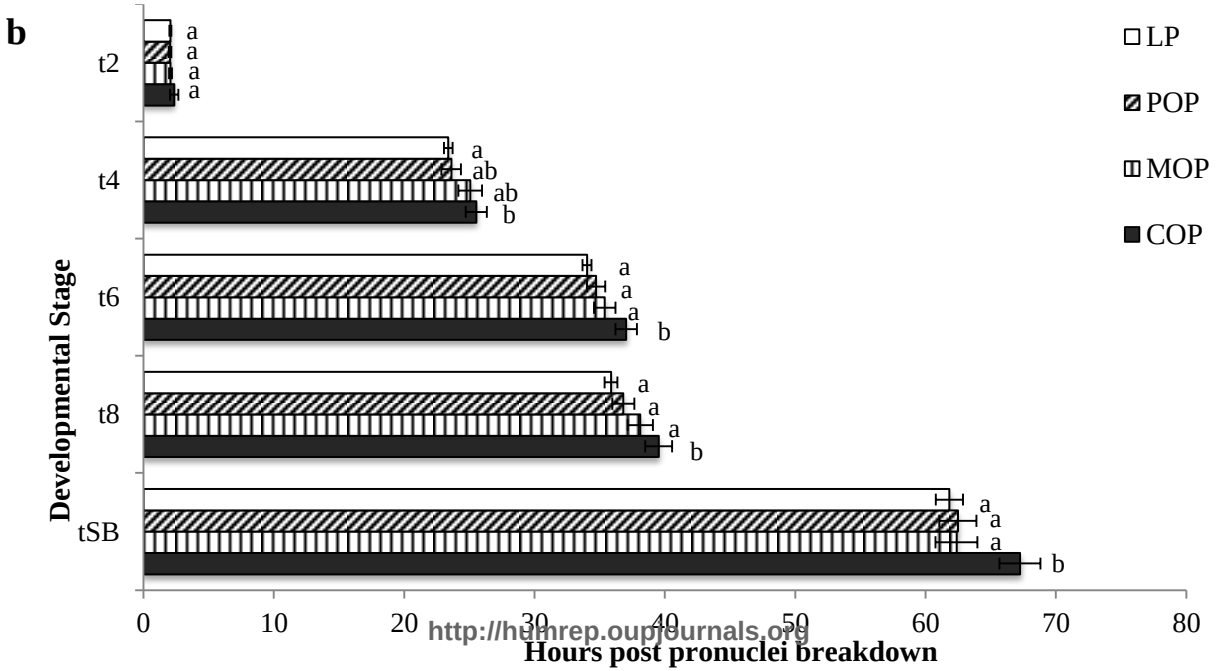
796

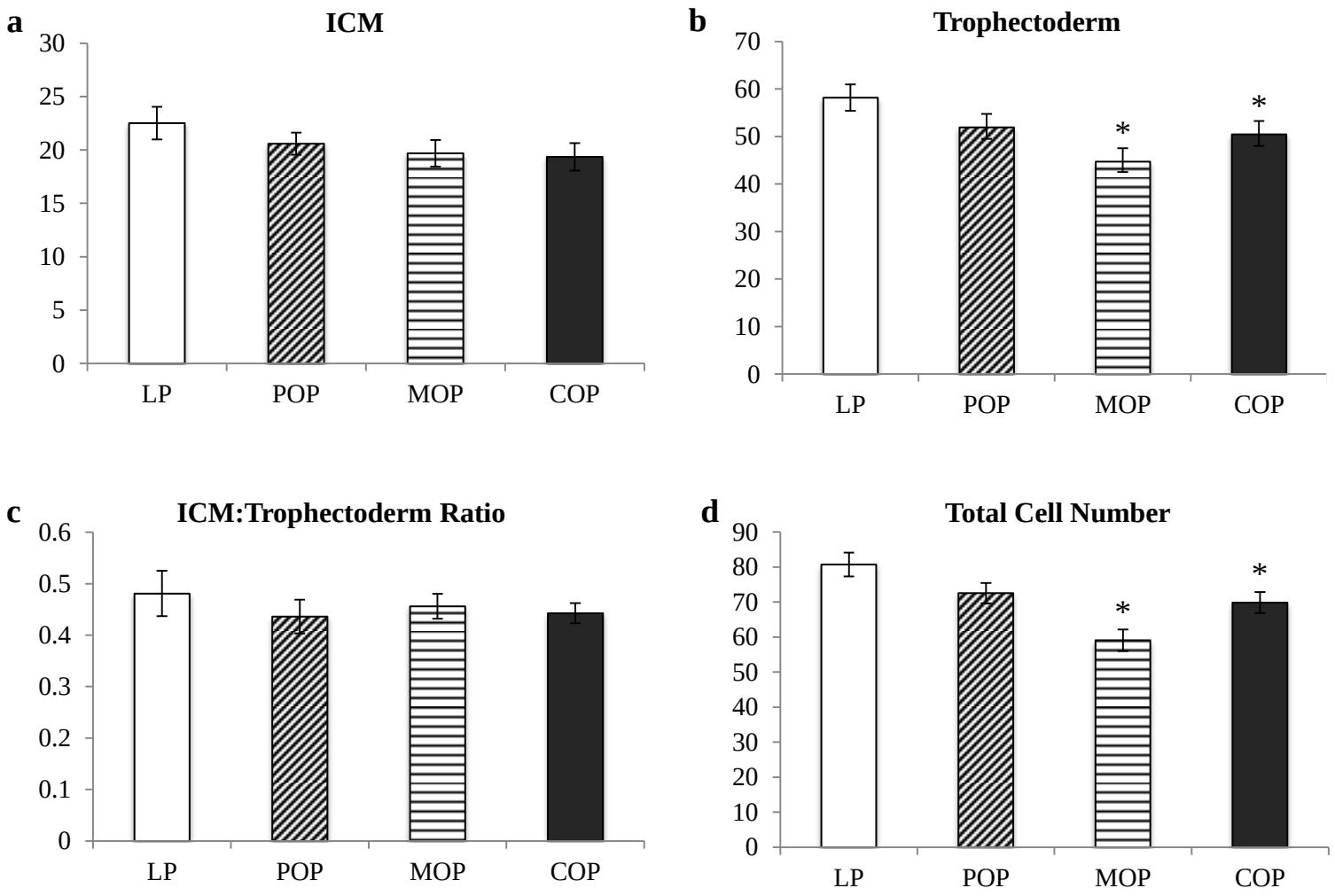
797 **Figure 6.** Zona pellucida width (μm) at the two-pronucleate stage. Lean Parented embryos = LP,
798 Paternal Obese Parented embryos = POP, Maternal Obese Parented embryos = MOP and Combined
799 Obese Parented embryos = COP. Different superscript letters denote a significant difference ($P <$
800 0.05) between groups in zona pellucida width n > 30 embryos per group. Data expressed as mean ±
801 SEM.

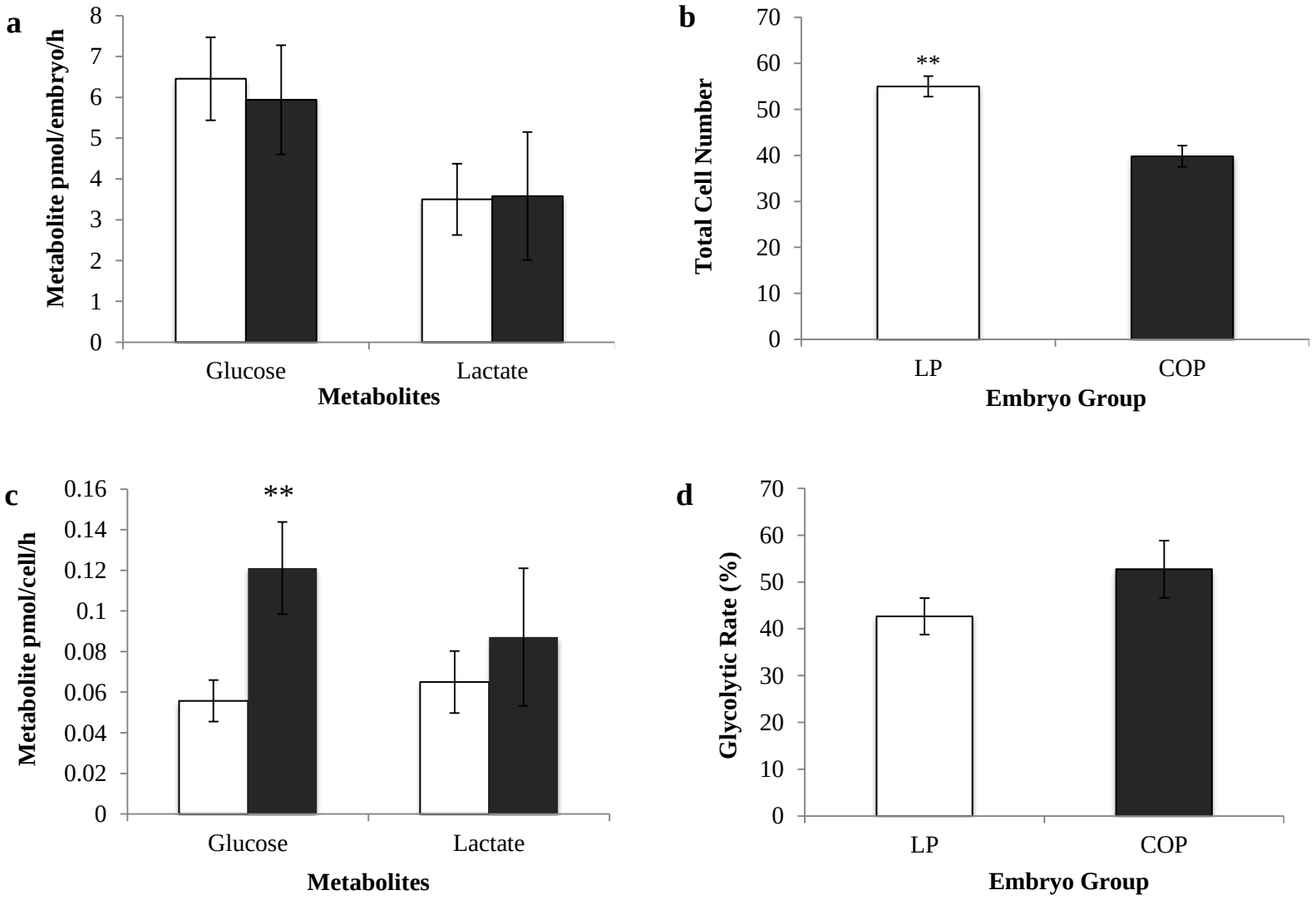
Figure 1

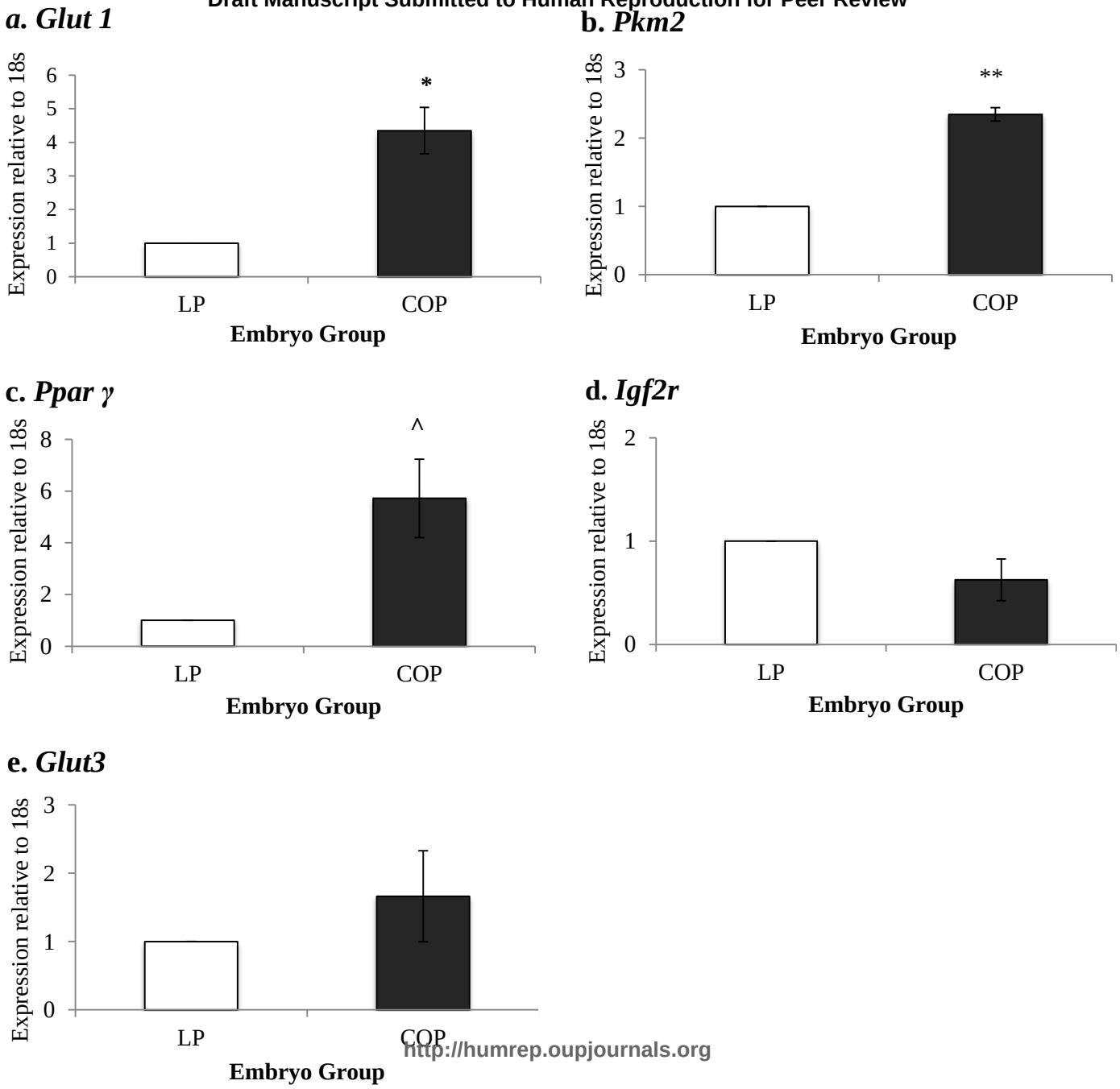

80
70
60
50
40
30
20
10
0
Percentage blastocyst
a
b
b
c
LP
POP
MOP
COP
Embryo Group






a
ICM
30
25
20
15
10
5
0
LP
POP
MOP
COP
b
Trophectoderm
70
60
50
40
30
20
10
0
*
*
LP
POP
MOP
COP
c
ICM:Trophectoderm Ratio
0.6
0.5
0.4
0.3
0.2
0.1
0
LP
POP
MOP
COP
d
Total Cell Number
90
80
70
60
50
40
30
20
10
0
*
*
LP
POP
MOP
COP


a
Metabolite pmol/embryo/h
8
7
6
5
4
3
2
1
0
Glucose
Lactate
Metabolites
b
Total Cell Number
70
60
50
40
30
20
10
0
**
LP
COP
Embryo Group
c
Metabolite pmol/cell/h
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
**
Glucose
Lactate
Metabolites
d
Glycolytic Rate (%)
70
60
50
40
30
20
10
0
LP
COP
Embryo Group


a. Glut 1
Expression relative to 18s
0
1
2
3
4
5
6
*
LP
COP
Embryo Group
b. Pkm2
Expression relative to 18s
0
1
2
3
**
LP
COP
Embryo Group
c. Ppar γ
Expression relative to 18s
0
2
4
6
8
^
LP
COP
Embryo Group
d. Igf2r
Expression relative to 18s
0
1
2
LP
COP
Embryo Group
e. Glut3
Expression relative to 18s
0
1
2
3
LP
COP
Embryo Group

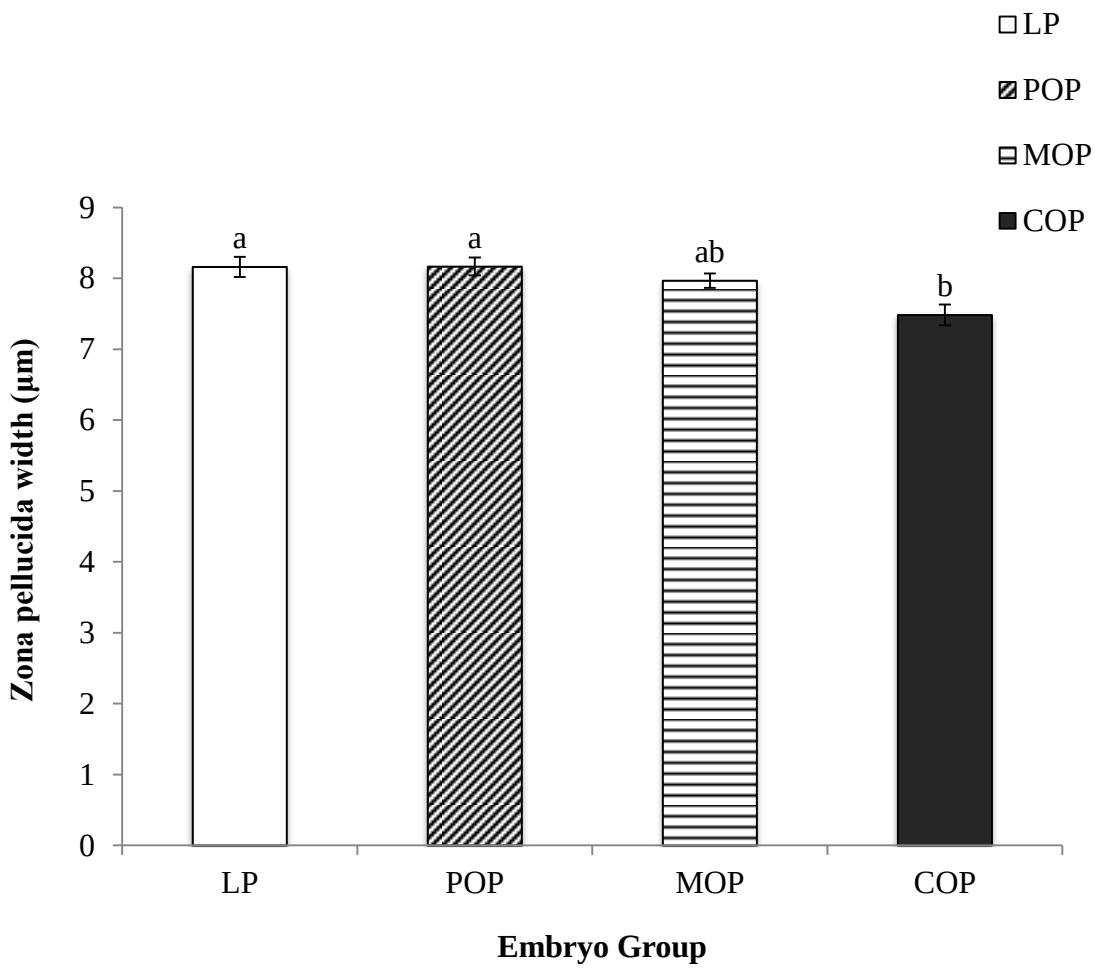

LP
POP
MOP
COP
Zona pellucida width (µm)
0
1
2
3
4
5
6
7
8
9
a
a
ab
b
LP
POP
MOP
COP
Embryo Group

Table I. Primer sequences and associated details used for Real time qRT-PCR studies.

Gene	Accession Number	Forward Primer (5'→3')	Reverse Primer (3'→5')	Product Length	Reference (if applicable)
***18s* rRNA**	NR_003278.3	GAACGGCTACCACATCCAA	CCTGTATTGTTATTTTTCGTCACTACCT	91	Kind *et al.*, 2005
Pkm2	NM_011099.3	TCTTCCCTGTGCTGTGTAA	CCACCCGGTCAGCACAAT	140	Not applicable
Igf2r	NM_010515.2	CTTGCCCTCCAGAAACGGAT	TGCTACACCACAGTTTCGCT	111	Not applicable
Glut1	NM_011400.3	CCAGCTGGGAATCGTCGTT	CAAGTCTGCATTGCCCATGAT	76	Not applicable
Glut3	NM_011400.3	CGGTGATAGTCCTTAAGCCTTCT	ATGGGGTCACCTTGCTTGTC	146	Not applicable
Pparγ	NM_008904.2	TATGGAGTGACATAGAGTGTGCT	GTCGCTACACCACTTCAATCC	143	Not applicable

Table II. Body weight, weight change and body fat of three-week old male (n = 15) and female (n = 48) mice were fed a control (7% kcal fat) or high fat (21% kcal fat) diet for a minimum period of eight weeks.

Measure	Control Diet Male	High Fat Diet Males	*P* value	Control Diet Females	High Fat Diet Females	*P* value
Initial weight (g)	10.74 ± 0.49	10.1 ± 0.51	NS	12.3 ± 0.25	12.1 ± 0.24	NS
Final weight (g)	33.1 ± 1.28	37.9 ± 1.48	< 0.05	21.3 ± 0.24	25.1 ± 0.35	< 0.001
Weight gain (g)	22.4 ± 1.01	27.7 ± 1.05	< 0.01	9.17 ± 0.30	12.9 ± 0.42	< 0.001
Proportion fat (%b.w.)	2.58 ± 0.45	4.37 ± 0.51	< 0.05	2.26 ± 0.21	3.50 ± 0.14	< 0.001

Response to reviewers' comments: HUMREP-15-0191 Finger et al.

The authors would like to thank the reviewers for their time in reviewing the manuscript. We greatly appreciate their comments and suggestions in order to improve the manuscript. Please see below our responses to the specific comments and questions. All line numbers refer to the document "markups".

Reviewer: 1

Comments to the Author

The study looks at the effect of single parent and combined parent obesity on embryo development and quality in a mouse model. The work has the potential to be of very high impact across a number of reproductive health and social health disciplines. The manuscript is very well written and concise but there are some dubious interpretations of data that needs to be addressed before publication can be considered.

1. In the introduction, emphasis is placed on parental obesity and yet in the last sentence you refer to the importance of pre-conception diet. Whilst obesity is correlated with high fat diet, it is not always the case that poor diet will lead to obesity. What is not obvious from the claims is whether the effects observed is due to obesity AND high fat diet, high fat diet alone, or obesity alone.

In the present study, a high fat diet was used to induce a state of obesity in the mouse. The feeding of this diet for a short period of time has been previously verified as a model of obesity, free from metabolic complications, particularly in regards to embryonic development studies (Bakos et al., 2011; Mitchell et al., 2011; Binder et al., 2012; Fullston et al., 2012). These studies are referenced in the methods (Line 126). There is a significant increase in body weight and body fat in high fat diet fed mice compared to control mice, noted in these previous studies, and in this present study (Table II) which is also consistent with other models of obesity (Dubuc, 1976; Dong et al., 2006). To clarify that the data generated are due to obesity alone and not attributed to the feeding of a high fat diet, the authors have revised the final sentence in the introduction. This should hopefully make the aim clearer to the reader.

2. Please explain the rationale for not using single obese parent matings in the study for embryo culture for metabolic and gene expression analysis.

We choose to look at the two extreme groups (LP and COP), especially as some previous studies have already noted changes in metabolism (Binder et al., 2012; Binder et al., 2012) and gene expression of single obese parent embryos (Bermejo-Alvarez et al., 2012). Hence, the importance was placed on investigating the effects of combined parental obesity. In addition, logistic, duration of student candidature and financial constraints meant we were limited in the number of embryo groups that could be investigated.

3. Obesity effects on developmental kinetics section. This section is confusing and does not marry up with parts of the data shown in Figure 2. The authors need to be clear with what they are claiming as blastocyst formation. Are authors referring to the start of blastocoel formation (as indicated in the figure) for blastocyst development or actual blastocysts? In Figure 2b, there is no statistical difference between MOP and POP blastocyst development (I assume this is the tSB as referred to in the figure) relative to LP and yet the authors claim there is a difference. If the authors are referring to fully formed blastocysts rather than the

early blastocyst (i.e. formation of the cavity) then delays of 1 hr and 3 h in MOP and POP may be evident, but this is not presented in the figure. In addition, the authors claim that the timing of blastocyst formation in COP embryos did not differ significantly from MOP or POP embryos whereas there appears to be a very significant change shown in Figure 2b (and indeed this is one of the fundamental points of the manuscript?) I would like to advise the authors to look at the data analysis for figure 2 in its entirety as the annotations do not make sense in terms of significance values.

When discussing blastocyst formation the authors are referring to time of formation of the cavity (time blastocoel starts formation = tSB), and this is reflected in the figure legend. All data analyses have been thoroughly reviewed and amendments made to the superscripts on Figure 2b, as well as updated in the manuscript text (lines 299-312). In order to simplify this section, the authors have rewritten the section and removed the description of many of the non-significant results and differences between Figure 2a and 2b. This should allow the reader to focus only on the main findings. Abbreviations to the different timings have also been inserted in the text to facilitate easier cross-referencing to Figure 2a and b.

4. Obesity effects on blastocyst cell lineage allocation. Can the authors revise this paragraph as the referral to figures is incorrect.

This has been amended to correct referral to the figures (lines 315-318).

5. Metabolic analysis. I advise the authors to look over this section too as according to figure 4 total cell number was higher in LP embryos compared to COP embryos (again a fundamental point of the manuscript?)

This section has been amended to reflect that LP embryos have higher cell numbers than COP embryos (line 322).

6. To revise the discussion in light of the above comments (should only involve first few sentences).

In amending the results (see Reviewer 1 Comment 5), the discussion is now consistent with the figures and results.

7. In Figure 4 legend, more explanation is required as to what is defined as an early blastocyst. If COP embryos are delayed by 7h to reach blastocyst stage then how does this impact on total cell number at Day 4? Should COP embryos be analysed at day 4.5? Please clarify the time of measurement in these two groups.

The definition of early blastocyst has been included in the methods (line 223-225) and the authors have now also stated the definition in the Figure legend. Both COP and LP embryos were analysed at day 4.5, as the method involves incubating compacted morulae for 24 hours and then selecting subsequent early blastocysts for analysis. The delay in development of COP embryos is likely to impact cell number, which is why the metabolic data have been expressed per cell, rather than embryo. The reviewer draws upon an important point that we highlight in the discussion. Namely, should the timing of metabolic analysis of obese parented embryos be the same as lean parented embryos, based on the differences in embryo kinetics and cell number.

Minor comments:
Line 177 change to Š²snap frozen for subsequent gene expression analysis². *Amended*

Line 198 remove ³Primer efficienciesŠ.² Sentence as you have already stated this in line 193 and 194. *Amended*
Line 208 remove extra spacing between ³were² and ³minus² *Amended*
Line 279 remove ³in². *Amended*
Line 345 remove ³an². *Amended*
Line 683 change ³tPF² to ³tPNF² in the figure legend. *Now replaced with tPNB in the figure legend and main text to be consistent and avoid confusion.*
Line 699 add open bar annotation for LP and shaded bars for COP in the figure legend. *Amended*

Reviewer: 2

Comments to the Author

The study examines the effect of combined parental obesity on preimplantation embryo development, differentiation and physiology. Notably, developmental speed, blastocyst lineage allocation and glucose handling are perturbed as a consequence of combined parental obesity. The authors also report a thinning of the zona pellucida as an additional early consequence of parental obesity.

Overall, the study is novel, timely, relevant and appears well-conducted and of interest to a wide audience of Human Reproduction. However, this referee has some major and a few minor concerns:

Major comments

1. In several places within the results the text does not tally with the significant differences marked on the figures as defined in the legends. Examples: line 273/274; lines 289/290; line 307. These issues need to be thoroughly checked and text or figure markings need to be adjusted before interpretation.

The authors have thoroughly checked the results text and figures and amended inconsistencies, as highlighted by both reviewers.

2. Interpretation of results and discussion: Please avoid describing non-significant results (eg line 333). The manuscript lacks reference to some important literature. Please check relevant publications involving eg Roger Sturmey, Jo Leroy, Pascale Chavatte-Palmer.

Descriptions of non-significant results have been removed from the text, where appropriate, to simplify the findings for the reader. The manuscript has been updated to include references to important literature (e.g. lines 76, 78, 373-374, 406-407, 441, 474).

3. Please include information on mating efficiency, ovulation efficiency and fertilisation rate in your 4 treatment groups.

Information on mating efficiency, ovulation rate and fertilisation rate has been included in the results section under 'Impact of parental obesity upon mating and fertilisation success' (Line 274-282).

4. Do you have evidence that 18s was a suitable reference gene that did not change with treatment? Please provide.

Prior to running analysis, a group of potential housekeeper genes were selected. These genes were run on all plates, allowing for the most consistent housekeeper gene to be selected. 18s rRNA produced the most consistent CT values, and it's consistency across treatments was further confirmed with the use of NormFinder software (Andersen et al., 2004). 18s produced a stability value of 0.022, with an intergroup variation level of +/- 0.011. The methods have been updated to include reference to this software (Line 215-216).

Minor comments:

line 164: define what you mean by 'on time'.

Updated to include definition, and reference to literature (Line 167).

line 177: 'stored for gene expression'.

Addressed in response to reviewer 1 comments

line 198: omit last sentence as repeat from above.

Amended

line 209: 'absence of DNA contamination'.

Amended

line 279: omit one 'in'

Amended

line 300-302: ... 'Figure 3b and d' .... 'Figure 3a and c'

Amended

line 307: I would guess the text should say the reverse (COP had less total cells than LOP)?

Correct, text has been amended (Line 322).

line 348-351: do you have any evidence for this statement?

The sentence has been rewritten and the statement amended to include a reference pertaining to the single parental obesity study in humans.

line 374: modify to ...'embryonic developmental timing' to make a clear link to text above

Amended

line 398-400: do you have any evidence for this statement in the literature?

This statement has been amended to reflect that this is a speculative statement.

line 436-438: I don't agree with this statement as the only evidence published here is serum glucose levels (no GTT, insulin levels, insulin stimulation...). Other similar models do report at least a pre-diabetic state (see eg literature by Schatten, Sun, Wei group etc). It is not just hyperglycaemia itself doing the damage. In addition, fatty acid metabolism will likely be perturbed, too. Please revise/rephrase.

This model of diet-induced obesity in C57BL6 mice has been published extensively as being non-diabetic (Bakos et al., 2011; Mitchell et al., 2011), including publications in Human Reproduction (Fullston et al., 2012). Furthermore, there is evidence that feeding this high fat diet fed to C57BL6 for a short period does not impact fasting blood glucose, glucose tolerance, insulin tolerance or plasma insulin levels (Fullston et al., 2013). The Schatten, Sun and Wei groups' model of diet-induced obesity involves feeding of a higher fat diet (60% kcal from fat) for a longer time period compared to the present model (21% kcal from fat) fed for 8 weeks, so inherently will show altered phenotypes. Indeed, this alternate model of diet-induced obesity highlights the varied impacts diet can have. We acknowledge that hyperglycaemia does not impact oocyte or embryonic development

alone, and that the levels of free fatty acid would also have impacts. The section has been updated to acknowledge this (Line 466-476).

Associate Editor's comments to Author:

Both reviewers have several remarks. The authors are encouraged to revise their manuscript accordingly. All of the are concerns are relevant and should be addressed in a revised version. The authors should in particular look careful into the concordance between the figures and the interpretation of data.

The authors have addressed all the comments raised by the reviewers, with specific attention given to consistency of findings stated in the figures and the text.

Minor comments:

P 6 l 124. In line with the relevant comment made by reviewer 1 concerning the distinction between high fat diet and/or obesity, the author should state the criteria for characterizing the mice as control or obese.

Please see the detailed response to reviewer one's comments. The authors have also highlighted the consistency of their findings with previous studies of diet-induced obesity (Line 126).

P 7, ll 164: what where the specific criteria for including embryos in the time-lapse analysis.

The criterion for inclusion in time-lapse analysis was reaching blastocyst stage in a time frame consistent with previously published literature. The text has been updated to clearly state this criterion (Line 167-169).

P 14, ll 360-362: As reviewer nr 2 comments, the authors should evaluate the use of references. The authors should include literature on human embryos if they intend to discuss clinical implications. At least one human study was not been able to confirm that maternal BMI influences timing (Bellver 2013). On the other hand a recent study in HR (Leary 2015) have reported the opposite findings, i.e faster development and reduced glucose consumption in embryos from obese women. The authors are encouraged to discuss these findings, in particular as they suggest the study to have clinical implication and in the abstract claims that human embryos are impacted in a similar fashion.

The text has been updated to include references and further discussion around the implications of this study for human embryos (e.g. Lines 372-374, 437-443). The recent study by Leary 2015 is of interest, as it suggests faster development and lower glucose uptake in embryos of obese mothers. As this is a human study, there are a number of complications related to the obese state. Potentially, the other complications of a high body mass index may impact embryonic development, resulting in faster development and lower glucose uptake compared to normal BMI parented embryos. Furthermore, the study involves only 7 women in the 'OW/OB' group having glucose consumption measured. It would be of interest to see this replicated in a larger group, considering the number of confounders present in human studies, before firm conclusions can be drawn.

In continuation, it P 15, ll 386. As an example of the above, this statement suggests that there is solid documentation that timing of development is associated with pregnancy, by referring to a review. The authors should consider modifying this statement, as the

documentation is debatable (as reviewed by Racowsky et at HRU, by Kirkegaard et al 2015 FS and a recent Cochrane review by Armstrong et al).

The section has been updated to reflect that there is no definitive documentation relating time-lapse analysis and increased pregnancy rates, highlighting literature that both supports and negates this proposition (Lines 404-408).

Figures: Apart from making sure that the figure and the text are coherent either author should consider applying legends explaining the bars in the figure itself.

More detail has been included in the figure legends to aid the reader's interpretation of the data.

Editor-in-Chief's comments:

Potentially important study. "Translation" to human clinical situation needs more attention since our readership consists, for a large part, of clinicians.

The authors appreciate the comment that this is a potentially important study. Further references to human studies, as well as the translation of the current findings to the human clinical situation have been added throughout the discussion.