Effect of maternal obesity on estrous cyclicity, embryo development and blastocyst gene expression in a mouse model

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STUDY QUESTION: Does maternal obesity affect estrous cyclicity, embryo development and blastocyst gene expression in mice?

SUMMARY ANSWER: Maternal obesity alters estrous cyclicity and causes the down-regulation of two key metabolite receptors (*Slc2a1* and *Ldlr*) in blastocysts recovered from diet-induced obese females, but embryo development is not affected.

WHAT IS KNOWN ALREADY: Maternal obesity reduces fertility because of effects in the periconception period, but its negative influence is on estrous cyclicity, oocyte quality or embryo development.

STUDY DESIGN, SIZE AND DURATION: This was a randomized study based on a mouse model for obesity. Twenty-one outbred NIH Swiss mice were used and obesity was induced by a diet high in fat administered for 12 weeks prior to breeding to control males.

MATERIAL, SETTING AND METHODS: Females were fed either a control diet (C, $n = 9$) or a diet high in fat [diet-induced obesity (DiO), $n = 12$] for 12 weeks, and were then co-housed with fertile males. Mice that failed to breed during 20 consecutive days were considered infertile. Control and diet-induced obese females that demonstrated vaginal plugs were euthanized 3.5 days after mating, blood was sampled for glucose and hormone measurements, corpora lutea counted and embryos recovered; the relative mRNA abundance of 11 candidate genes was determined in blastocysts by qPCR.

MAIN RESULTS AND THE ROLE OF CHANCE: Five DiO females failed to breed and displayed anovulatory ovaries (DiOI), whereas the other seven DiO females (DiOF) could breed, albeit over an extended period compared with controls. DiOF weighed significantly less than DiOI. Both groups had elevated serum insulin compared with C, although blood glucose level was only significantly higher than that in controls in the infertile DiOI group. Adiponectin was lower in the DiOI and leptin higher in both the DiOI and DiOF mice than in C. DiOF ovulated the same number of oocytes as C, and embryo development to blastocyst was normal. The expression of genes encoding metabolic hormone receptors (*Insr*, *Igf1r*, *Igf2r*, *Adipor1*, *Adipor2* and *Lepr*) and key metabolic enzymes (*Gapdh*, *Cpt1a* and *Sod2*) did not differ between DiOF and C blastocysts, but that of metabolite receptors (*Slc2a1* and *Ldlr*) was down-regulated in DiOF. To limit the role of chance, the experiments were conducted in a defined laboratory setting with the proper controls, and the animals were randomly assigned to each experimental group. Moreover, a P -value of < 0.05 was chosen to determine whether the differences observed between the groups were statistically significant.

LIMITATIONS AND REASONS FOR CAUTION: The results obtained may not fully extrapolate to humans. Also, as follicular activity was not monitored while breeding, so the extended breeding period for DiOF group might be explained by behavioral abnormalities occurring in normal cycling animals.

WIDER IMPLICATIONS OF THE FINDINGS: DiO alters the estrous cycle in the mouse model and demonstrates a role of obesity in infertility. The data also suggest that in an outbred, genetically diverse population, such as the human, individual susceptibility to obesity and associated infertility induced by diet exists. The apparently normal development to blastocyst observed in fertile, obese females suggests that preimplantation embryos can resist potentially adverse outcomes caused by an oversupply of fatty acids and glucose under *in vivo* conditions.

This metabolic plasticity may, in part, be due to an ability to down-regulate metabolite transporters, thereby preventing excessive nutrient uptake.

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Introduction

Obesity is considered by the World Health Organization as a global epidemic (2003) and can lead to a wide range of secondary pathologies, including female infertility (Metwally et al., 2008a; Veleva et al., 2008). Female reproductive function can be affected by obesity at different levels, including the control of ovulation, oocyte development, embryo development, endometrial development, embryo implantation and fetal development (Brewer and Balen, 2010).

Grossly obese women display a menstrual disturbance rate 3-fold greater than in women of normal weight (Hartz et al., 1979), but obesity-linked alterations in reproductive function differ greatly between individuals (Brewer and Balen, 2010). The circulating levels of a number of metabolites, such as glucose, triglycerides and free fatty acids, and hormones such as insulin (Brothers et al., 2010), leptin (Schneider et al., 2000) and adiponectin (Groth, 2010) are known to affect the hypothalamic–pituitary–ovarian axis, and therefore may vary between obese fertile and infertile women. However, the complex interactions between different parameters, including interventions, such as ovarian stimulation protocols, make it difficult to draw conclusions solely on the basis of retrospective human studies. Animal models can, therefore, provide further insights into the pathophysiology of the phenomenon.

On the other hand, obesity may alter the hormone or nutrient levels in the milieu where oocytes and embryos develop, resulting in direct detrimental changes on metabolism (Robker et al., 2009). Although there is *in vitro* evidence for negative effects of an excess of certain metabolites, such as glucose (Moley et al., 1998; Bermejo-Alvarez et al., 2012), and a number of different lipids (Nonogaki et al., 1994; Leroy et al., 2010; Wonnacott et al., 2010; Van Hoeck et al., 2011) and various hormones (Arias-Alvarez et al., 2011) on oocyte and embryo development in animal models, the *in vivo* effect of obesity remains controversial. Obese women have been reported to have significantly poorer quality embryos than lean women (Carrell et al., 2001), and another group observed a similar effect, but only in women younger than 35 years (Metwally et al., 2007). However, others have been unable to demonstrate a significant correlation between embryo quality and BMI (Fedorcsak et al., 2001; Spandorfer et al., 2004; Dechaud et al., 2006; Esinler et al., 2008; Shalom-Paz et al., 2011) and a retrospective analysis of 6500 IVF cycles observed no difference in embryo quality across the BMI strata (Bellver et al., 2010). The study of preimplantation embryo pathophysiology is particularly challenging because of the limited amount of biological material available for analysis, but recent refinements in molecular techniques allow accurate gene expression analysis in such small samples by quantitative RT–PCR (qRT–PCR) (Bermejo-Alvarez et al., 2011a).

In the present study, our goal was to determine the effect of high-fat-diet-induced obesity (DiO) on the ovarian function and preimplantation embryo development in an outbred mouse strain. Ovarian function was evaluated on the basis of estrus cyclicity and ovulation rates. We also attempted to correlate abnormal ovarian function with altered serum levels of glucose, insulin, adiponectin and leptin. Finally, we also evaluated whether embryos from fertile obese mice developed normally to the blastocyst stage and whether these embryos exhibited altered expression of a number of candidate genes whose function is linked to the metabolic and hormonal changes associated with obesity.

Materials and Methods

Induction of obesity and breeding

All animal experiments were approved by the University of Missouri ACUC committee (ACUC Protocol Number 6154) and performed in accordance with the NIH Animal Care and Use guidelines. Outbred mice (NIH Swiss, Harlan, Madison, WI) were maintained in a 12 h:12 h light:dark cycle under standard conditions ($25 \pm 2^\circ\text{C}$ and $50 \pm 10\%$ humidity), with *ad libitum* access to water and food. This strain was chosen because it is outbred and in this sense, mirrors the genetic diversity evident in human populations. Additionally, NIH Swiss mice are susceptible to DiO (Rosenfeld et al., 2003; Alexenko et al., 2007). The female mice (21 NIH Swiss mice at 8 weeks of age) were housed at three animals/polypropylene cage ($32 \times 18 \times 24$ cm) and fed a control diet (Purina 5001, St Louis, MO, 'C', $n = 9$) or a diet very high in saturated fat (Research diets D12492, 'DiO', $n = 12$) for 12 weeks. Body weights were recorded on an every other week basis. After 12 weeks on the diets, the females were naturally bred to NIH Swiss males of proven fertility. During the breeding period, the pairs were maintained on the same diet, i.e. Purina 5001 for 'C' or Research diets D12492 for 'DiO'. The females were observed every morning for the presence of a vaginal plug indicating successful mating. Accordingly, the parameter 'days to breed' was used as an indirect means of determining whether the animals exhibited normal ovarian cyclicity. In this regard, normal cycling females should breed in a period not exceeding the duration of a normal estrous cycle (5 days). Those females that were not bred during 20 consecutive days were considered infertile. The lack of ovarian activity in this group of infertile females was further confirmed by the absence of signs of follicular activity [i.e. corpora hemorrhagica or corpora lutea (CL)] post-mortem.

Blood and embryo collection

Three days after plug detection (3.5 days post coitum) or 20 days after unsuccessful breeding, the females were weighed and anesthetized with an intraperitoneal combination of ketamine (VEDCO, MO, USA) (0.1 g/kg) and xylazine (Lloyd, CA, USA) (0.01 g/kg), supplemented with a subcutaneous injection of buprenorphine (PharmaForce, OH, USA) (0.1 mg/kg).

Blood samples (around 0.7 ml/mouse) were drawn by cardiac puncture at around mid-phase of the light cycle from anesthetized females, which were then immediately euthanized in accordance with the recommendations of the Panel of Euthanasia of the American Veterinary Medical Association (http://www.avma.org/issues/animal_welfare/euthanasia.pdf). The blood was allowed to clot for 30 min at room temperature, and then the samples were centrifuged (10 min; 4°C; 1500g). The serum was stored at -20°C until analysis. The abdominal cavity was exposed and the reproductive tract and mesenteric fat removed. The fat was weighed and ovaries were examined to determine the number of CL. Any embryos were flushed from the uterus in pre-warmed (37°C) CZBH medium (Sigma, MO, USA) and counted. Embryo development was evaluated, and blastocysts snap frozen in liquid N₂ in groups representing each individual female. Blastocysts samples were stored at -80°C until analysis.

Analysis of serum parameters

Glucose levels were determined in blood immediately after extraction by Contour blood glucose test strips (Bayer, Tarrytown, NY, USA; detection range 70–333 mg/dl, inter-assay variation 2.3%). Levels of individual hormones in serum were measured in duplicates on a single day with commercially available mouse ELISA kits by following the manufacturers' instructions (Insulin, Mercodia 10-1247-01, Uppsala, Sweden; Adiponectin Assaypro EMA2500-I, St Charles, MO, USA; Leptin, Assaypro EML2001-I, St Charles, MO, USA). The detection ranges were 0.2–6.5 µg/ml, 0.7–25 µg/ml and 0.4–30 ng/ml and the intra-assay variations 2.7, 4.5 and 6.9% for insulin, adiponectin and leptin, respectively.

Gene expression analysis

Poly(A) RNA was extracted from each group of blastocysts by using the Dynabeads mRNA Direct Extraction KIT (DynaL Biotech, Oslo, Norway) with minor modifications (Bermejo-Alvarez *et al.*, 2011b). Immediately after extraction, the reverse transcription reaction was carried out with qScript cDNA Supermix (Quantabiosciences, Gaithersburg, MD, USA) in a total volume of 20 µl. Retrotranscription was performed following the manufacturer's instructions: the samples were incubated for 5 min at 25°C, followed by 1 h at 42°C and 5 min at 85°C. After cDNA synthesis, the samples were diluted to 55 µl in 10 mM Tris-HCl (pH 7.5). The quantification of all mRNA transcripts was carried out in the 15 samples by qPCR with two repetitions for all genes of interest (Table I). Experiments were conducted to contrast the relative levels of each transcript and the housekeeping gene, histone *H2afz*, in each sample. PCR was performed by adding a 2 µl aliquot of each cDNA sample to the PCR mix containing the specific primers to amplify transcripts for *H2afz*, insulin receptor (*Insr*), insulin-like growth factor 1 receptor (*Igf1r*), insulin-like growth factor 2 receptor (*Igf2r*), adiponectin receptor 1 (*Adipor1*), adiponectin receptor 2 (*Adipor2*), leptin receptor (*Lepr*), solute carrier family 2 (facilitated glucose transporter), member 1 (*Slc2a1*, previously known as *Glut1*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), low-density lipoprotein receptor (*Ldlr*), carnitine palmitoyltransferase 1a, liver (*Cpt1a*) and superoxide dismutase 2, mitochondrial (*Sod2*, previously known as *Mnsod*). Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table I. For quantification, qRT-PCR was performed as described previously (Bermejo-Alvarez *et al.*, 2011b). PCR conditions were tested to achieve efficiencies close to 1. The comparative cycle threshold (CT) method was used to quantify expression levels. Values were normalized to the endogenous control, *H2afz*. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to a doubling of the amplified PCR product. According to the comparative CT

method, the Δ CT value was determined by subtracting the *H2afz* CT value for each sample from each gene CT value of the sample. The calculation of $\Delta\Delta$ CT involved using the highest sample Δ CT value, i.e. the sample with the lowest target expression, as an arbitrary constant to subtract from all other Δ CT sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$.

Statistical analysis

Data were analyzed by using the SigmaStat (Jandel Scientific, San Rafael, CA) software package. Differences between groups with respect to body weight at individual timepoints, days to successful breeding, serological parameters, number of oocytes and blastocysts recovered and relative mRNA abundance for candidate genes were analyzed by one-way analysis of variance (ANOVA, $P < 0.05$) followed by the Holm-Sidak *post hoc* test. The weight gain of the mice over time was also analyzed by a repeated measure analysis ($P < 0.05$). The correlation between body weight and mesenteric fat weight with metabolic parameters (glucose, insulin, adiponectin and leptin) was analyzed by Pearson Correlation.

Results

Induction of obesity and reproductive parameters

The mice on the high-fat diet weighed significantly more than the mice on the control diet after just 2 weeks. As determined by repeated measure analysis, the weight difference between the two groups continued to widen through 12 weeks of age ($P < 0.05$, Fig. 1) and was considered to represent DiO. At this stage, attempts were made to breed all females to fertile stud males. Five mice out of 12 in the DiO group failed to breed during the subsequent 20 days. These acyclic animals (DiO infertile, DiOI) were significantly heavier than the remaining seven fertile females from the DiO group (DiO fertile, DiOF) (53.9 ± 3.8 versus 41.6 ± 0.9 g, $P < 0.05$, Table II). Four of these DiOI females weighed >50 g at the time of euthanasia (Fig. 2). Furthermore, the ovaries of the DiOI females showed no signs of either follicular activity or recent ovulations, clearly indicating that the absence of an estrous cycle was accompanied by a lack of follicular development. The seven cyclic animals in the DiOF group were heavier (41.6 ± 0.9 versus 31 ± 0.8 g, $P < 0.05$, Table II) and required a greater number of days to mate (7.3 ± 1.6 versus 2.9 ± 0.5 , $P < 0.05$, Table II, Fig. 2) than control females. However, the number of oocytes ovulated (as assessed by the number of CL) did not differ between Control and DiOF groups (Control 12 ± 0.9 versus DiOF 13.4 ± 0.9 , Table II). In both fertile groups, all the embryos had reached the blastocyst stage when flushed from the oviduct, and the number of CL reflected the final blastocyst recovery in all females (Table II), indicating that embryo development to the blastocyst stage was not affected by DiO.

Serum glucose and hormone levels

In order to determine possible markers for obesity-linked anovulatory infertility and possible physiological causes of the disruption of cyclicity, blood glucose levels and levels of the metabolism-related hormones insulin, adiponectin and leptin were determined in the three groups of females described earlier. Blood glucose level was significantly higher in DiOI than in the other two groups (Table III).

Table I Details of primers used for qPCR.

Gene	Primer sequence (5'-3')	Fragment size (bp)	GenBank accession no.
<i>H2afz</i>	AGGACAACCAGCCACGGACGTGTG CCACCACCAGCAATGGTAGCTTTG	209	NM_016750.2
<i>Insr</i>	GTCAGTGGCCGACCATGCC TTCCTGGGGAGAGCCCTCGC	241	NM_010568.2
<i>Igf1r</i>	CACAGCACGCCAGTGGAGGG GAGGAGCAGCTGAGGGGGCT	218	NM_010513.2
<i>Igf2r</i>	CCCGGAGTAGGGGCTGCTT CGTCGCTTGCTCACAGGCCA	270	NM_010515.2
<i>Adipor1</i>	CCACTCCCAAGCACCGGCAG CTGCTGCCACCACCAGGACG	267	NM_028320.3
<i>Adipor2</i>	TGCCCTCTATGCGGCCCGTA TGCAGCCCCCGCCAATCATG	167	NM_197985.3
<i>Lepr</i>	ACCCAAGGCAACGCAGGACG CCCTCTTTCCGCCTCCGGGA	157	NM_146146.2
<i>Slc2a1</i>	GCAGCAGCCTGTGTACGCCA CCGTTCCAGCAAGGCCAGGG	172	NM_011400.3
<i>Gapdh</i>	TGGTGCTGCCAAGGCTGTGG GGCAGGTTTCTCCAGGCGGC	121	NM_008084.2
<i>Ldlr</i>	CGGCTTCCGGTTGGTGACC GGACCTCGTGGCGTTGGTG	203	NM_010700.2
<i>Cpt1a</i>	AGTGAGGGTGGGGTGGCCAG GCGGTCAGGCGGCTTTGACT	194	NM_013495.2
<i>Sod2</i>	GGGCGCCTCTCATGCATGCA CCGGGGGCTGAAAAGATGGC	235	NM_013671.3

Figure 1 Body weight divergence of mice on the control and high-fat (DiO) diets over 12 weeks (mean $\pm$ SEM). Asterisks denote significant differences between control and DiO females based on ANOVA ($P < 0.05$).

However, insulin levels were significantly higher in both obese groups, DiOI and DiOF, compared with the control, but no significant differences were observed between DiOF and DiOI (Table III). Adiponectin followed an opposite tendency, being significantly decreased in DiOI compared with the control group, whereas the DiOF group did not differ significantly from either of the other two groups (Table III).

Leptin levels differed significantly between the three groups (Table III). Finally, to analyze the differences between the two obese subgroups, i.e. fertile and infertile, we examined the correlation of body weight and mesenteric fat weight of individual animals with their serum levels of glucose, insulin, adiponectin and leptin. Only leptin levels were significantly correlated with body weight and mesenteric fat.

Relative mRNA abundance of selected candidate genes

In order to gain some insight into whether obesity and the accompanying changes in metabolites and hormones had direct effects on the preimplantation embryos from DiOF females, we determined the relative expression level of four genes (*Insr*, *Adipor1*, *Adipor2* and *Lepr*) encoding receptors for the metabolic hormones previously analyzed (insulin, adiponectin and leptin, respectively), both insulin-like growth factors (*Igf1r* and *Igf2r*), four genes implicated in glucose and lipid transport and metabolism (*Slc2a1*, *Gapdh*, *Ldlr* and *Cpt1a*) and one gene involved in oxygen radical detoxification (*Sod2*) by real-time qPCR. The relative mRNA abundance of the genes encoding for hormone receptors did not vary among groups (Fig. 3A). Two genes with roles in the transport of glucose (*Slc2a1*) and lipids (*Ldlr*) were down-regulated in the DiOF group compared with the control (*Slc2a1*: 1 ± 0.1 versus 1.38 ± 0.1 ; *Ldlr*: 1 ± 0.1 versus 1.24 ± 0.1).

Table II Effect of DiO on body weight, mesenteric fat weight, days to breed, number of corpora lutea and blastocysts recovered 3.5 days post coitum.

Group	Body weight (g)	Mesenteric fat (g)	Days to breed	Corpora lutea observed/blastocysts recovered
Control	31.0 ± 0.8 ^a	0.51 ± 0.09 ^a	2.9 ± 0.5 ^a	12 ± 0.9 ^a
DiOF	41.6 ± 1 ^b	3.93 ± 0.40 ^b	7.3 ± 1.6 ^b	13.4 ± 0.9 ^a
DiOI	53.9 ± 3.8 ^c	7.61 ± 1.05 ^c	N/A ^c	0 ^b

N/A: not applicable, as these animals failed to breed. DiOF: DiO fertile. DiOI, DiO infertile.
Different letters indicate significant differences between treatments based on one-way ANOVA ($P < 0.05$).

Figure 2 Relationship between days required to observe a copulatory plug and body weight for mice maintained on the control and DiO diets for 12 weeks. Individual data are for Control (orange diamonds, $n = 9$), fertile obese (DiOF, dark blue squares, $n = 7$) and infertile obese females that failed to breed over a 20-day period (DiOI, light blue triangles, $n = 5$).

However, there were no differences in expression of downstream genes in glucose and lipid metabolism such as *Gapdh* (which encodes an essential glycolytic enzyme), *Cpt1a* (whose product catalyzes the rate limiting step of beta-oxidation) and *Sod2* (encodes the mitochondrial isoform of superoxide dismutase).

Discussion

The present study has analyzed the effects of obesity associated with consumption of a high-fat diet on ovulation and preimplantation embryo development in outbred NIH Swiss mice. DiO in the mouse largely mimics the metabolic syndrome observed in human obesity (Buettner *et al.*, 2007) and has been widely used to study the effects of obesity on reproductive function (Rosenfeld *et al.*, 2003; Alexenko *et al.*, 2007; Robker, 2008; Brothers *et al.*, 2010; Igosheva *et al.*, 2010; Jungheim *et al.*, 2010). As with obese women, who experience irregular menstrual cycles (Hartz *et al.*, 1979; Balen *et al.*, 1995; Hirschberg, 2009), these mice exhibited marked disruption of estrous cyclicity. Perhaps most informative is the fact that the severity of the condition was directly related to the individuals susceptibility to gain weight and, specifically, accumulation of mesenteric fat, and not to the diet *per se*. Thus, those females that had become grossly obese, exceeding 50 g in weight by the end of the 12 weeks on the diet, and had doubled their accumulation of mesenteric fat

relative to their obese but fertile counterparts, were not receptive to males, presumably due to failures in the hypothalamic–pituitary–ovarian axis, as the ovaries showed no signs of recent follicular activity. Unlike women, obese female mice seldom, if ever, exhibit polycystic ovaries (Walters *et al.*, 2012). Although we did not analyze gene expression in mesenteric fat, it seems likely that mesenteric adipose tissue contributed to the observed defects in estrous cyclicity, as the blood levels of two hormones secreted primarily by adipose tissue, adiponectin and leptin were significantly altered. For example, some of the metabolic dysfunction in mice on a high-fat diet may be due to inflammation in mesenteric fat (Lam *et al.*, 2012). Also a comparison of various adipose tissues in mice fed a diet has shown mesenteric fat to be the most metabolically active (Hageman *et al.*, 2010), and the mesenteric fat transcriptome is highly responsive to the fat content of the diet (Bolduc *et al.*, 2010). Finally, women with polycystic ovary syndrome (PCOS) who resume ovulation during a lifestyle program lose more body weight and, particularly, more abdominal fat than those who do not resume ovulation (Kuchenbecker *et al.*, 2011).

On the other hand, despite being fed the same high-fat diet, a second subgroup of females was less susceptible to the diet, as they weighed around 40 g by the end of the diet exposure period, and were fertile. Estrous cyclicity was not abolished in these moderately obese females, but the cycle, determined by the number of days taken to observe a vaginal plug, was significantly longer than in the control group, as has been observed by others for obese mice (Brothers *et al.*, 2010) and rats (Akamine *et al.*, 2010). Although it seems likely that the extended breeding period for DiOF females is due to alterations in ovarian cyclicity, ovarian activity *per se* was not monitored. Accordingly, we cannot rule out that the extension in time to breed obese females was due to their reduced libido or failure to respond appropriately to the breeder males. Even if this were the case, such phenotypes are generally considered to be under the control of gonadal steroid hormones (Flanagan-Cato, 2011) and consistent with an ovarian defect. However, the ovarian defect may not be the primary cause for the altered estrous cyclicity, but the consequence of metabolic disruption at the level of the hypothalamus or pituitary (see below). It is important to note that two previous studies from this laboratory, in which NIH Swiss mice were maintained on the same high-fat diet did not lead to the same extreme increases in weight and disruption or loss of estrous cyclicity observed in the present study. The most likely explanation is that these mice were repeatedly bred from an early age, suggesting that the multiple pregnancies and lactations counteracted the tendency of the mice to gain

Table III Blood and serum parameters in control, fertile obese (DiOF) and infertile (DiOI) obese mice.

Group	Glucose (mg/dl)	Insulin (μg/ml)	Adiponectin (μg/ml)	Leptin (ng/ml)
Control	176 ± 7 ^a	0.45 ± 0.02 ^a	4.66 ± 0.36 ^a	2.05 ± 0.34 ^a
DiOF	200 ± 18 ^a	0.87 ± 0.15 ^b	3.99 ± 0.23	11.37 ± 0.59 ^b
DiOI	272 ± 32 ^b	1.22 ± 0.38 ^b	3.13 ± 0.21 ^b	22.69 ± 2.19 ^c

Different letters indicate significant differences between treatments based on one-way ANOVA ($P < 0.05$).

Figure 3 Relative mRNA abundance for candidate genes expressed in blastocysts recovered from Control and DiOF dams. Data (mean ± SEM) are for control (black, $n = 9$) and DiO (white, $n = 7$). **(A)** Values for genes encoding for metabolic hormone; **(B)** genes implicated in metabolite transport (*Scd2a1* and *Ldlr*), key metabolic pathways (*Gapdh* and *Ldlr*) and ROS detoxification (*Sod2*). Different letters indicate significant differences between groups based on ANOVA ($P < 0.05$).

excessive weight (Rosenfeld et al., 2003; Alexenko et al., 2007). It seems, therefore, that the severity of infertility is closely linked to the degree of obesity and that fertility is not as severely affected when the weight gain is moderated (Rosenfeld et al., 2003; Alexenko et al., 2007). In this sense, another study that employed a similar experimental design to our study, i.e. weight gain without the modulating effect of pregnancy and lactation, also reported anovulatory infertility associated with obesity (Brothers et al., 2010).

The differences in systemic levels of glucose and metabolism-related hormones provide some insights into the physiological mechanisms that might underpin the altered estrus cyclicity observed in the obese females. Glucose was significantly elevated only in the infertile obese females (DiOI), whereas insulin was significantly higher in both fertile (DiOF) and infertile (DiOI) obese females compared

with the controls. Presumably, the DiOF mice were better able to control their glucose levels than DiOI through increasing insulin secretion, which suggests that DiOI mice may have developed insulin resistance. Ovarian insulin resistance disrupts estrous cycles in obese rats through a reduction in the insulin receptor substrate/phosphatidylinositol 3-kinase/AKT intracellular pathway (Akamine et al., 2010). Similarly, deletion of the insulin receptor substrate-2 gene (*Irs2*) in mice leads to infertility and small anovulatory follicles (Burks et al., 2000) and fertility is recovered in obese female mice by impairing insulin signaling in the pituitary (Brothers et al., 2010). Consistent with such studies, obese women who lose weight and resume ovulation experience a reduction in insulin resistance (Clark et al., 1995), and insulin resistance in obese women has been correlated with gonadotrophin resistance (Homburg et al., 1996). Thus, insulin resistance most

likely contributes to the infertility of the DiOI mice, either directly, as a result of the tissues in the hypothalamic–pituitary–ovarian axis being unable to respond to insulin, or indirectly, because the tissue systems are maladapted to the high concentrations of glucose that accompany insulin resistance.

Changes in adiponectin levels may also influence ovarian cyclicity. Adiponectin tends to be lower in women with anovulatory PCOS compared with weight-matched controls or women with ovulatory PCOS (Carmina *et al.*, 2009; Toulis *et al.*, 2009). Adiponectin has been implicated in the regulation of steroidogenesis in bovine (Maillard *et al.*, 2010), porcine (Ledoux *et al.*, 2006) and rat (Chabrolle *et al.*, 2007) follicular cells, operating in close association with insulin (Ledoux *et al.*, 2006) and insulin-like growth factor I (Chabrolle *et al.*, 2007; Maillard *et al.*, 2010). The lowered adiponectin levels in DiOI and the trend towards such decreases in DiOF mice are consistent with effects on ovarian cyclicity and with the infertility in the DiOI group. However, adiponectin seems unlikely to be the dominant player in obesity-related infertility in view of the fact that mice lacking the adiponectin gene are fully fertile (Ma *et al.*, 2002). Nevertheless, lowered adiponectin levels may play a role in infertility by enhancing insulin resistance (Nawrocki *et al.*, 2006).

Leptin has long been known to have multiple roles in female reproductive physiology, and the ablation of the leptin gene in mouse results in infertility (Zhang *et al.*, 1994). Leptin has been implicated in the control of gonadotrophin release (Pasquali and Gambineri, 2006). While leptin is essential to maintain fecundity, excessive levels may also impair reproduction. For instance, elevated intrafollicular leptin in rats inhibits ovarian estradiol (E_2) synthesis (Zachow and Magoffin, 1997) and ovulation (Duggal *et al.*, 2000). Furthermore, leptin inhibits the IGFI augmentation of FSH-stimulated E_2 production during *in vitro* culture of human granulosa cells (Agarwal *et al.*, 1999). In this context, the significantly higher levels of circulating leptin observed in DiOI mice relative to the two fertile groups in our experiments could, therefore, be taken as evidence that excessive leptin can have a negative impact on fertility and ovarian function despite the fact that it is generally considered to be a pro-fecundity hormone.

Despite the alterations on estrous cyclicity, obese fertile mice (DiOF) ovulated a normal number of oocytes per cycle and their fertilized zygotes developed normally to blastocysts. Obesity has been suggested to affect early embryo development, but available data are far from a consensus. Thus, despite some published data showing lower fertilization rates (Salha *et al.*, 2001; van Swieten *et al.*, 2005; Sneed *et al.*, 2008) and clinical pregnancy rates (Salha *et al.*, 2001; Maheshwari *et al.*, 2007; Metwally *et al.*, 2008b; Jungheim *et al.*, 2009) for oocytes retrieved from obese women, other reports found no effect of obesity on either fertilization success (Lashen *et al.*, 1999; Wittemer *et al.*, 2000; Fedorcsak *et al.*, 2004; Dokras *et al.*, 2006; Esinler *et al.*, 2008; Bellver *et al.*, 2010; Shalom-Paz *et al.*, 2011; Vilarino *et al.*, 2011) or pregnancy outcome (Lashen *et al.*, 1999; Wittemer *et al.*, 2000; Dokras *et al.*, 2006; Matalliotakis *et al.*, 2008; Shalom-Paz *et al.*, 2011; Koning *et al.*, 2012). Accordingly, it has been suggested that the lower pregnancy rates observed in obese women do not result from a reduction in embryo quality, but from alterations in the uterine environment (Bellver *et al.*, 2010). *In vitro* studies in animal models have shown detrimental effects of high concentrations of certain metabolites and hormones whose systemic levels are increased in obesity. Such factors have included glucose

(Moley *et al.*, 1998; Bermejo-Alvarez *et al.*, 2012), several fatty acids (Nonogaki *et al.*, 1994; Leroy *et al.*, 2010; Wonnacott *et al.*, 2010; Van Hoeck *et al.*, 2011) and leptin (Arias-Alvarez *et al.*, 2011). However, it is unclear whether the concentrations used *in vitro* mimic those encountered in the reproductive fluids, as the recovery of physiological oviductal fluid is challenging (Velazquez *et al.*, 2010) and there may exist complex interactions between different metabolites that may not occur *in vitro*. Some *in vivo* studies in animal models have also suggested a decrease in oocyte quality associated with obesity that has been attributed to alterations in oocyte maturation and mitochondrial function (Robker, 2008; Igosheva *et al.*, 2010; Jungheim *et al.*, 2010). In turn, these abnormalities were suggested to lead to impaired embryo development. Such outcomes appear to contrast with our results presented in Table II. However, in two of these earlier studies (Robker, 2008; Jungheim *et al.*, 2010) *in vivo* preimplantation embryo development to blastocyst was not assessed, while in the third (Igosheva *et al.*, 2010) there were no differences on blastocyst recovery and blastocyst cell numbers between obese and lean mice. A fourth study did report lowered developmental rates of embryos derived from oocytes recovered from obese mice (Minge *et al.*, 2008), but this apparent detrimental effect could have been influenced by the suboptimal *in vitro* culture conditions employed, since only ~70% of cleaved control zygotes developed to blastocyst. Moreover, a later study from the same group failed to observe any effect on either development to blastocyst or blastocyst morphology as an outcome of maternal obesity (Wu *et al.*, 2010). Clearly, the inference that oocyte quality and embryo development are affected by maternal obesity remains questionable, particularly as some reports failed to note differences in litter size or numbers of abnormal pups born when obese and lean females were compared (Rosenfeld *et al.*, 2003; Alexenko *et al.*, 2007; Brothers *et al.*, 2010).

Finally, we determined whether there was an embryonic response to maternal obesity and accompanying hormone and metabolite levels in terms of gene expression. We observed that the transcript levels of relevant hormone receptors (*Insr*, *Igf1r*, *Igf2r*, *Adipor1*, *Adipor2* and *Lepr*) did not differ between blastocysts recovered from obese and control females. We cannot exclude the possibility that DiOF embryos may have altered concentrations of the enzymes themselves or post-translational modifications, but the small amount of biological material present in the groups of blastocysts screened precluded reliable protein quantification by either western blotting or other proteomic technologies.

The lack of significant change in *Lepr* gene expression contrasts with an *in vitro* study with bovine blastocysts in which similar fold changes in leptin concentrations as those observed here between C and DiOF females were accompanied by up-regulation of *LEPR* (Arias-Alvarez *et al.*, 2011). In contrast to *Lepr*, two genes encoding two metabolite receptors (*Ldlr* and *Slc2a1*) were down-regulated in blastocysts obtained from the obese mice. This regulatory modulation may be linked to an increase in oviductal fluid levels of their target metabolites. *Slc2a1* is a glucose receptor whose down-regulation in the embryo may be linked to the high glucose serum levels in DiOF females. In addition, the increased insulin levels in DiOF would also likely influence glucose concentrations in the reproductive tract. *Ldlr* is a lipid receptor that internalizes low-density lipoproteins, whose levels are known to increase in mice fed a high-fat diet (Neyrinck *et al.*, 2012). Taken together, these data suggest that a protective mechanism operating

against excessive nutrient uptake by the blastocyst had come into play. Consistent with this inference, the expression of two genes encoding enzymes catalyzing key steps in glucose and lipid metabolism (*Gapdh* and *Cpt1a*) did not differ among groups. *Gapdh* encodes an essential glycolytic enzyme and its expression level correlates with the activity of anaerobic glycolysis in bovine preimplantation embryos (Bermejo-Alvarez et al., 2010). Transcript levels of *Cpt1a*, the gene encoding carnitine palmitoyltransferase I, correlate with the rate of fatty acid oxidation (Linher-Melville et al., 2011). Consistent with a lack of evidence for alterations in glucose or lipid metabolism, the expression of the mitochondrial isoform (*Sod2*) of the major antioxidant defense system (superoxide dismutases) (Fukai and Ushio-Fukai, 2011) also remained unaltered.

In summary, these results suggest that DiO in the mouse model reduces fertility through alterations in estrous cyclicity, manifested as either blocked or prolonged estrous cycles, depending on body weight gain. DiO did not affect the number of oocytes ovulated and embryo development to blastocyst in the obese, fertile females. Evidence supports the view that insulin resistance underlies the infertility of obese females. The expression levels of genes for key metabolic markers for anaerobic glycolysis, fatty acid metabolism and oxidative stress in blastocysts were not affected by maternal obesity, possibly because of an adaptive, protective mechanism as evidenced by the down-regulation of various nutrient receptors in embryos derived from diet-induced obese females.

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Authors' roles

P.B.A. conceived and performed the experiments and data analysis. All the authors contributed to discussing the data and writing the manuscript. R.M.R. and C.S.R. funded the study.

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Conflict of interest

None declared.

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