REPRODUCTION
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A low-fat diet enriched in fish oil increased lipogenesis and fetal outcome of C57BL/6 mice

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Abstract

There is clear evidence that nutritional strategy employed during pregnancy has profound influence on the offspring health outcomes. However, the effect of the quality and the quantity of maternal fat intake on maternal metabolic profile during different stages of pregnancy and its impact on pregnancy sustainability is not known. Female C57BL/6 mice (7 weeks old) were fed diets varying in the quantity of fat (5% vs 11%) for two weeks prior to mating and throughout pregnancy. The 5% fat diet was enriched with longer chain omega (n)-3 polyunsaturated fatty acids (PUFA) from fish oil. Maternal plasma and tissues were collected before mating and during pregnancy at days 6.5, 12.5 and 18.5. Plasma lipids, glucose, insulin, progesterone and estradiol levels were measured. Cholesterol efflux capacity of maternal plasma as well as the mRNA expression of placental steroidogenic acute regulatory protein and hepatic lipogenic genes (acetyl-CoA carboxylase-1, fatty acid synthase, diacylglycerol acyltransferase-2 and stearoyl-CoA desaturase-1) was determined. Feto-placental weight and fetuses sustained throughout gestation were recorded. A low-fat maternal diet enriched with n-3 PUFA increased maternal plasma triacylglycerol and the mRNA expression of rate-limiting lipogenic enzymes, along with increasing cholesterol efflux capacity ($P < 0.05$), likely to meet fetal lipid demand during pregnancy. Furthermore, diet enriched with longer chain n-3 PUFA increased the maternal plasma concentration of progesterone and estradiol during pregnancy ($P < 0.05$), which coincides with an increase in the number of fetuses sustained till day 18.5. These novel findings may be important when designing dietary strategies to optimize reproductive capability and pregnancy outcomes.

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Introduction

The susceptibility of offspring to developing pathological conditions primarily originate from compromised intrauterine environment via nutritional insults (Barker *et al.* 1989, Perera & Herbstman 2011). We have previously shown that the quantity and the quality of fat consumed during pregnancy and lactation has a profound effect on the aortic function, as well as the expression of brain-derived neurotrophic factor in the offspring (Kanta *et al.* 2010, Balogun & Cheema 2014). Furthermore, we have established that the fatty acid composition of maternal diet has the potential to induce long-lasting changes in the tissue fatty acid composition of the offspring (Chechi *et al.* 2010); this is very important because fatty acids play a key role in maintaining metabolic functions (Martínez-Fernández *et al.* 2015). The quality as well as the quantity of maternal fat intake is capable of programming set points for several physiological and metabolic factors for the mother, and also in the developing embryo during pregnancy, thereby impacting the health of the mother, as well as that of the offspring (Martin-Gronert & Ozanne 2006).

As pregnancy progresses, complex metabolic adaptations occur, which allows the mother to support the growth and development of the fetus. For example, during pregnancy, there is an increase in insulin secretion at early gestation as insulin stimulates hepatic *de-novo* synthesis and storage of triacylglycerol (TG) (Benito *et al.* 1982, Wilcox 2005). Studies using knockout mouse models of acetyl-CoA carboxylase (*Acc*) and fatty acid synthase (*Fas*), the rate-limiting enzymes for endogenous lipid synthesis, showed increased embryonic death demonstrating the importance of lipogenesis during pregnancy (Chirala *et al.* 2003, Abu-Elheiga *et al.* 2005). Diacylglycerol acyltransferase-2 (*Dgat2*) and stearoyl-CoA desaturase-1 (*Scd1*) enzymes also play key role in hepatic lipid synthesis (Miyazaki *et al.* 2001, Zammit 2013). *Dgat2* enzyme catalyzes the final reaction for hepatic formation of TG (Yen *et al.* 2008, Zammit 2013). Interestingly, lipogenesis has been shown to be inhibited in *Scd1* knockout mice model despite increased expression of *Fas* (Miyazaki *et al.* 2001). An increase in TG synthesis during pregnancy contributes to fetal development by serving as a depot for fatty acids, which are released into fetal circulation via active feto-placental nutrient transport (Duttaroy 2009). Additionally, maternally derived cholesterol also crosses the placenta at early gestation to support fetal

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growth and development (Herrera 2002). Cholesterol is also the precursor for the synthesis of estradiol and progesterone (sex steroid hormones), which are essential for a successful pregnancy (Hu *et al*. 2010). Steroidogenic acute regulatory protein (*Star*) mediates cholesterol transfer within the mitochondrial, especially in the steroid-producing tissues such the ovary, testis, adrenal cortex and the placenta (Lin *et al*. 1995, Stocco & Clark 1996). Thus, maternal changes in lipid metabolism during pregnancy play an important role toward maintaining pregnancy and proper growth and development of the fetus.

The fetus also relies on the mother for the supply of essential fatty acids, especially docosahexaenoic acid (DHA), an n-3 PUFA, that is important for brain and eyes (retina) development (Neuringer *et al*. 1988, Innis 2007). The intake of maternal DHA has also been shown to reduce the risk of preterm delivery (Olsen & Secher 2002, Horvath *et al*. 2007), and low birth weight (Imhoff-Kunsch *et al*. 2012), especially in high-risk pregnancies. The essential fatty acid, alpha-linolenic acid (ALA), once obtained in the diet, is converted to DHA via elongation and desaturation. Since DHA is essential for brain and eyes development, the conversion of ALA to DHA is upregulated during pregnancy; however, this process is limited to about 9% in women (Burdge & Wootton 2002*a*, Childs *et al*. 2011). Thus, optimal growth and development of the fetus is dependent upon the nutritional, metabolic and hormonal environment provided by the mother.

Several studies, including research from our laboratory, have shown that both the quantity and the quality of maternal fat intake impact the health of the offspring (Chechi *et al*. 2010, Coletta *et al*. 2010, Balogun *et al*. 2013). However, the effect of the quality and the quantity of maternal fat intake on maternal metabolic profile during different stages of pregnancy and its impact on pregnancy sustainability is not known. We hypothesized that a low-fat maternal diet will increase lipogenesis during pregnancy to meet the requirements of the fetus. We further hypothesized that a maternal diet enriched with longer chain n-3 PUFA will increase pregnancy outcome in terms of sustaining the number of fetuses during gestation. There was no change in food intake, weight of fat pad and placental efficiency (data not shown). However, our findings have revealed for the first time that a low-fat maternal diet increased lipogenesis, and that a diet enriched in longer chain n-3 PUFA sustained a higher number of fetuses at late gestation.

Materials and methods

Animals and experimental design

All experimental procedures involving animals were carried out in accordance with the principles and guidelines of the Canadian Council on Animal Care and were approved by Memorial University's Animal Care Committee (approval no: 15-11-SC). Male and female C57BL/6 mice (seven weeks old) were purchased from Charles Rivers Laboratories, and were housed in separate cages under controlled temperature (21 ± 1°C) and humidity (35 ± 5%) conditions with a 12-h light/12-h darkness period cycle. Mice were kept on standard rodent chow pellets (Prolab RMH 3000) (PMI Nutrition, MO, USA) for one-week acclimatization period. After this period, female mice were randomly divided into two groups, and each group was fed with breeding chow diet varying in the quantity and quality of fat (Table 1): 5% (w/w) fat (Pico-Vac Lab Rodent Diet, 5061; LabDiet, MO, USA) and 11% (w/w) fat (Mouse Diet, 5015; LabDiet) for two weeks. Mating was carried out, and female mice were checked by 06:00 h the following morning for vaginal plug formation to confirm pregnancy. Pregnant mice were continued on the assigned diets throughout gestation. Fresh food and water was provided *ad libitum* every day. Body weight and food intake was recorded every day; no significant difference in food intake was observed (data not shown). Mice were sacrificed before pregnancy (non-pregnant), at early gestation (day 6.5), mid-gestation (day 12.5) and late gestation (day 18.5) using isoflurane. Figure 1 depicts each stage of pregnancy. Blood was collected by cardiac puncture in tubes containing EDTA (4.5 mM, pH 7.4), and was separated immediately into plasma and red blood cells (RBC). Tissues were removed and weighed at the time of sacrifice, snap frozen in liquid nitrogen and stored at −80°C until further analyses. Implantation sites and fetuses sustained throughout the gestation period were recorded. Uterine and fetal pictures were taken using Canon camera (SX500 IS). All effort was made to reduce the number of animals and to minimize animal suffering.

Table 1 Macronutrient and caloric composition of the experimental diets.

	5% Fat	11% Fat
Macronutrients (% w/w)		
Protein	20.0	18.9
Carbohydrate	52.9	51.8
Fat	5.0	11.1
Calories provided (%)		
Protein	24.6	19.8
Carbohydrate	62.1	54.2
Fat	13.2	26.1

Analyses of biochemical parameters and fatty acid composition

Lipids were extracted from the diets, RBC and liver samples according to the method of Folch and coworkers (1957) as per our previous publication (Chechi *et al*. 2010). Plasma biochemical parameters were quantified using commercially available kits according to the manufacturers' instructions: plasma and liver TG kit #236-17 (Genzyme Diagnostics, PEI, Canada); total cholesterol (TC) kit #234-60 (Genzyme Diagnostics, PEI, Canada); plasma glucose kit #10009582 (Cayman Chemical) and insulin (Mouse) ELISA Kit #KA3812 (Abnova Corporation, Taiwan). Plasma progesterone and

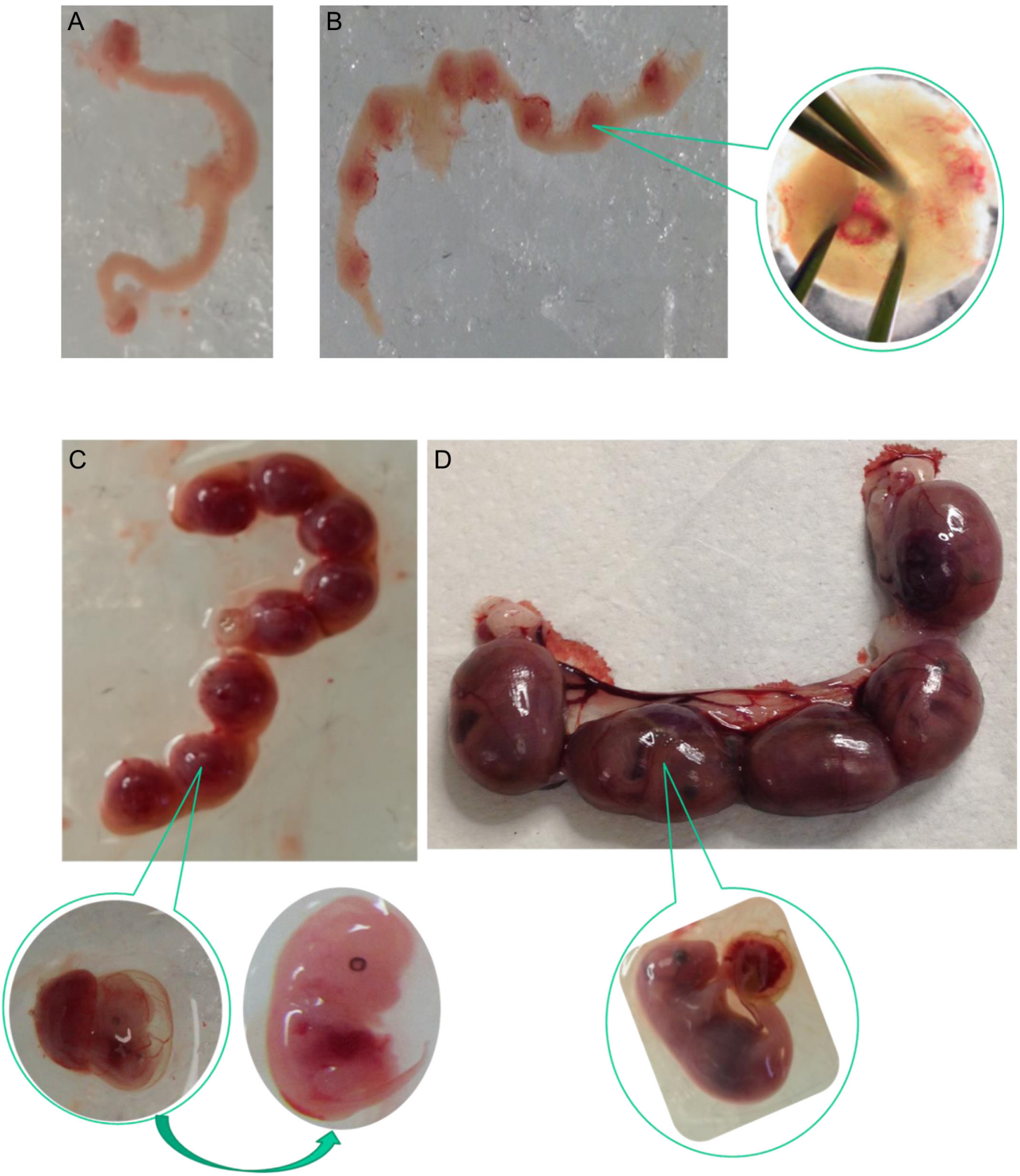

Figure 1 The uterus of a non-pregnant mouse (A), pregnant mouse at days 6.5 (B), 12.5 (C) and 18.5 (D).

estradiol concentrations were determined using Architect Systems (B7K770 and B7K720, respectively). The fatty acid composition of the extracted lipids was determined using gas chromatography–flame ionization detection according to our previously published method (Chechi *et al*. 2010). The fatty acid composition of the diets is given in Table 2.

Cholesterol efflux assay

Macrophage cholesterol efflux capacity assay of the plasma samples was determined using J774 cells according to our previously published method (Balogun *et al*. 2014). Briefly, the cells were seeded in 12-well plates at a density of

Table 2 Fatty acid composition of the diets.

Fatty acids (%)	**5% Fat**	**11% Fat**
C14:0	0.94	1.24
C16:0	14.77	20.98
C18:0	4.46	8.61
ΣSFA	20.17	30.82
C16:1	1.40	2.05
C18:1	21.40	31.73
C20:1	0.42	0.43
ΣMUFA	23.23	34.21
C18:2n6	48.85	31.87
C20:4n6	ND	0.18
Σn-6 PUFA	48.85	32.05
C18:3n3	5.78	2.72
C20:5n3	0.88	ND
C22:5n3	0.31	ND
C22:6n3	0.86	ND
Σn-3 PUFA	7.76	2.72
n-6:n-3	6:1	12:1

Data are expressed as weight percentage of the total extracted lipids. ΣMUFA, sum of monounsaturated fatty acids; Σn-3 PUFA, sum of omega-3 polyunsaturated fatty acids; Σn-6 PUFA, sum of omega-6 polyunsaturated fatty acids; n-6:n-3, Omega-6 to omega-3 ratio; ND, not detected; ΣSFA, sum of saturated fatty acids.

2×10^{-5} cells/well in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 1× antibiotic/anti-mycotic. The following day, the cells were labeled with RPMI supplemented with 1% FBS, 1 µCi/mL ^{3}(H)-cholesterol (Perkin Elmer), 2 µg/mL acyl-CoA:cholesterol acyltransferase inhibitor (Sandoz, QC, Canada) and 1× antibiotic/anti-mycotic for 24 h. Cells were equilibrated for 18 h in RPMI medium in the presence of liver X receptor agonist (1 µM) (Sigma), retinoic X receptor agonist (1 µM) (Sigma), retinoic acid (1 µM) (Sigma) and ATP-binding cassette A1 agonist (1 µM) (Sigma). Cholesterol efflux was initiated by treating cells with 2% plasma samples from both 5% ($n=8$) and 11% fat group ($n=8$) collected at different stages of pregnancy as the efflux acceptor or 0.2% bovine serum albumin (BSA) as the negative control for 5 h. At the end of the efflux interval, the medium was collected from each well and centrifuged at 402 ***g*** for 5 min. Supernatants were removed for liquid scintillation counting. Wells were washed twice with 1× PBS, and residual radioactivity in the cells was determined after scraping the cells in 1× PBS. Cholesterol efflux was calculated as ([^{3}H]-cholesterol in medium/[^{3}H]-cholesterol in medium + [^{3}H]-cholesterol in cells) × 100. All the efflux values were corrected by subtracting the percentage efflux at time zero (before active/passive efflux).

RNA extraction and real-time qPCR

Total RNA was extracted from liver and placenta samples using Trizol method (Chomczynski & Sacchi 1987). Genomic DNA contamination was removed by treating with DNAse enzyme (Promega). The concentration of the extracted RNA was determined using Nano Drop 2000 (Thermo Scientific). RNA integrity was assessed using 1.2% agarose gel. Synthesis of cDNA from the extracted RNA was carried out using reverse transcription as per our previous publication (Balogun & Cheema 2014). All primers used for qPCR were designed using

Table 3 Primers sequences.

Gene	**Primers sequence** (5′–3′)
Acc1 (S)	ggccagtgctatgctgagat
Acc1 (AS)	agggtcaagtgctgctcca
Fas (S)	ctgcggaaacttcaggaaatg
Fas (AS)	ggttcggaatgctatccagg
Dgat2 (S)	ctagctagttaggctaggtttcac
Dgat2 (AS)	caggaggatatagcgccagag
Scd1 (S)	agagtagctgagctttgggc
Scd1 (AS)	acaccccgacagcaatatccag
Star (S)	tgcccatcatttcattcatcctt
Star (AS)	aaaagcggtttctcactctcc
Actb (S)	cacgcagctcattgtagaagg
Actb (AS)	atggtgggaatgggtcagaag

Acc1, acetyl-CoA carboxylase 1; *Actb*, beta-actin; AS, anti-sense primer; *Dgat2*, diacylglycerol acyltransferase-2; *Fas*, fatty acid synthase; S, sense primer; *Scd1*, stearoyl-CoA desaturase-1; *Star*, steroidogenic acute regulatory protein.

NCBI primer blast (www.ncbi.nlm.nih.gov/tools/primer-blast/) (Accessed on 09/06/2016) and obtained from IDT technologies (IA, USA); primer sequences are given in Table 3. Amplification was performed using iQ SYBER Green Supermix (Biorad). The reactions were run at a reaction volume of 10 µL and 50 ng cDNA per reaction. Samples were run using the CFX96TM Real-Time System, while data output was managed using the CFX ManagerTM Software Version 3.0. The delta Ct values were recorded for each of the gene of interest and normalized with Beta-Actin (*Actb*) as the reference gene. The expression levels between the two groups were compared using the Livak method (Livak & Schmittgen 2001).

Statistical analysis

Data were analyzed using GraphPad Prism Software (version 5.0). Sample means were compared using two-way analysis of variance (ANOVA) to determine main effects of diet and time, and the interactions between them. Pairwise comparison using Bonferroni correction was used to determine differences among the groups when there was an observed interaction. Results are expressed as mean ± standard deviation (S.D.) for $n=8$ in each experimental group. Real-time qPCR data were $\log_{10}$ transformed and fatty acid composition data were arcsine transformed prior to statistical analyses. Differences were considered to be statistically significant if $P<0.05$. Pearson's correlation was used to compare the relationship between plasma cholesterol and sex steroid hormones (progesterone and estradiol).

Results

Effects of diets on maternal RBC fatty acid composition

The RBC fatty acid composition is given in Table 4. The 5% diet group showed lower levels of C18:0 and total saturated fatty acids (SFA) ($P<0.005$ and $P<0.0001$, respectively), compared to the 11% group. The 5% fat group also showed lower levels of C18:1 and total

Table 4 Fatty acid composition of maternal red blood cells.

	5% Diet			11% Diet			Main effect		
Fatty acids	Day 6.5	Day 12.5	Day 18.5	Day 6.5	Day 12.5	Day 18.5	Diet	Time	Diet*time
C14:0	1.70±0.15	1.44±0.43	1.54±0.12	1.23±0.15	1.46±0.07	1.25±0.23	$P<0.0001$	NS	NS
C16:0	29.28±0.99	29.17±1.43	30.30±1.97	29.42±0.99	29.62±0.41	30.58±0.24	NS	NS	NS
C18:0	13.84±0.57	14.31±0.51	14.60±1.00	17.38±0.57	15.81±0.52	15.91±1.02	$P<0.005$	NS	NS
∑SFA	44.82±0.84	44.92±1.91	46.44±2.72	48.04±0.84	46.88±1.02	47.73±1.10	$P<0.0001$	NS	NS
C16:1n7	0.91±0.20^{a}	0.67±0.15^{b}	0.66±0.25^{b}	0.82±0.20^{a}	0.71±0.24^{b}	0.52±0.22^{c}	NS	$P<0.05$	NS
C18:1	13.03±0.94	12.98±1.14	12.09±1.44	14.60±0.50	15.19±1.49	14.46±0.78	$P<0.0001$	$P<0.05$	NS
C20:1n9	0.41±0.31^{a}	0.24±0.08^{b}	0.27±0.20^{b}	0.35±0.05	0.25±0.07	0.34±0.05	NS	$P<0.05$	$P<0.05$
∑MUFA	14.35±0.50^{a}	13.89±1.01^{ab}	13.02±0.95^{b}	15.77±0.41	16.15±1.48	15.32±1.88	$P<0.0001$	$P<0.05$	NS
C18:2n6	14.77±0.39^{a}	12.04±1.10^{b}	10.54±3.68^{c}	12.61±0.39^{b}	10.69±0.73^{c}	10.72±0.24^{c}	NS	$P<0.05$	NS
C20:4n6	13.97±0.34^{b}	14.52±0.58^{ab}	15.75±0.90^{a}	14.55±0.51^{b}	16.54±1.48^{a}	15.18±1.06^{b}	NS	$P<0.01$	$P<0.05$
C22:4n6	1.24±0.09^{b}	1.17±0.31^{b}	1.48±0.69^{a}	1.95±0.09	2.20±0.29	2.04±0.03	$P<0.0001$	$P<0.05$	NS
∑n-6 PUFA	29.98±0.61^{a}	27.73±1.47^{b}	27.77±3.02^{b}	29.11±0.62^{a}	29.44±1.04^{a}	27.94±1.67^{b}	$P<0.0001$	NS	$P<0.05$
C18:3n3	0.37±0.34	0.29±0.07	0.25±0.01	0.20±0.04	ND	ND	$P<0.05$	NS	NS
C20:5n3	1.15±0.08	0.99±0.03	0.94±0.03	0.49±0.05^{c}	0.85±0.06^{b}	0.98±0.02^{a}	$P<0.05$	$P<0.05$	$P<0.001$
C22:5n3	1.21±0.15	1.37±0.28	1.19±0.08	0.61±0.08	0.88±0.37	0.56±0.03	$P<0.0001$	NS	NS
C22:6n3	7.59±0.39^{c}	8.74±1.38^{b}	9.68±0.51^{a}	4.79±0.39^{e}	5.03±0.21^{e}	6.45±1.80^{d}	$P<0.0001$	0.001	NS
∑n-3 PUFA	10.32±0.30^{b}	11.96±1.48^{a}	12.53±0.57^{a}	6.09±0.51^{d}	6.76±0.21^{d}	7.99±1.71^{c}	$P<0.0001$	$P<0.001$	$P<0.0001$
N6/N3	2.9:1	2.4:1	2.3:1	4.8:1	4.4:1	3.5:1			

Data are expressed as weight percentage of the total extracted lipids. Values are expressed as mean±S.D., $n=8$. Main effects and interactions were determined by 2-way ANOVA after arcsine transformation.
Mean values within a row with unlike superscript letters (a, b and c) were significantly different for each group ($P<0.05$).
∑MUFA, sum of monounsaturated fatty acids; ∑n-3 PUFA, sum of omega-3 polyunsaturated fatty acids; ∑n-6 PUFA, sum of omega-6 polyunsaturated fatty acids; ND, not detected; NS, not significant; ∑SFA, sum of saturated fatty acids.

monounsaturated fatty acids (MUFA), compared to the 11% group ($P<0.0001$), while C16:1n7 decreased with time of gestation in both groups ($P<0.05$). Moreover, the 5% diet group revealed a significant interaction between diet and time of gestation for C20:1; there was a significant decrease from days 6.5 to 18.5 ($P<0.05$) in the 5% group. There was an independent effect of time of gestation on linoleic acid (C18:2n6; LA) and arachidonic acid (C20:4n6; AA); LA decreased as gestation progressed from days 6.5 to 18.5 for both dietary groups ($P<0.05$), while AA increased with time of gestation in the 5% group only ($P<0.01$). The 5% group revealed a higher amount of alpha-linolenic acid (C18:3n3; ALA), eicosapentaenoic acid (C20:5n3; EPA), docosapentaenoic acid (C22:5n3; DPA) and DHA, compared to the 11% group ($P<0.0001$). Both diet and time of gestation showed a significant interaction for EPA and total n-3 PUFA; EPA increased as the gestation progressed from days 6.5 to 18.5 in 11% group ($P<0.0001$). Interestingly, ALA was not detected at days 12.5 and 18.5 in the 11% diet group. Diet and time of gestation also had an independent effect on DHA; there was a significant increase from days 6.5 to 18.5 in both dietary groups ($P<0.001$); 5% group had higher DHA, compared to the 11% group ($P<0.0001$).

Effects of diets on maternal plasma lipids profile

The plasma TG was significantly higher in the 5% group, compared to the 11% group ($P<0.0001$) (Fig. 2A). There was also an interaction between diet and time of gestation on the plasma TG ($P<0.005$) to reveal an increase from days 6.5 to 18.5 in the 5% group. However, there was no significant difference in plasma TG in the 11% group as gestation progressed from days 6.5 to 18.5. There was a significant time-dependent decrease in plasma TC (Fig. 2B) from days 6.5 to 18.5 ($P<0.0001$) in both dietary groups; this effect was more pronounced in the 5% group, compared to the 11% group (42.7% vs 29.4% decrease). Furthermore, the cholesterol efflux capacity was significantly higher in the 5% group, compared to the 11% group ($P=0.0002$; Fig. 2C). Time of gestation also had an effect on cholesterol efflux capacity ($P<0.0001$), where day 12.5 showed a significantly lower cholesterol efflux capacity in both dietary groups, compared to days 6.5 and 18.5.

Effects of diets on maternal glucose and insulin levels

Diet had an independent effect on plasma insulin and glucose where 5% group showed higher levels, compared to the 11% group ($P<0.0001$; Fig. 3A and B, respectively). There was also an interaction between time of gestation and diet ($P<0.05$) for plasma insulin and glucose in the 5% group to show higher levels at day 12.5; however, there was no change in the 11% group.

Effects of diets on maternal hepatic lipid concentrations

Diet had an independent effect on liver TG concentrations ($P<0.05$; Fig. 4A), revealing higher TG in the 5% group, compared to the 11% group. The liver TG concentration peaked at day 12.5 for both dietary groups, and

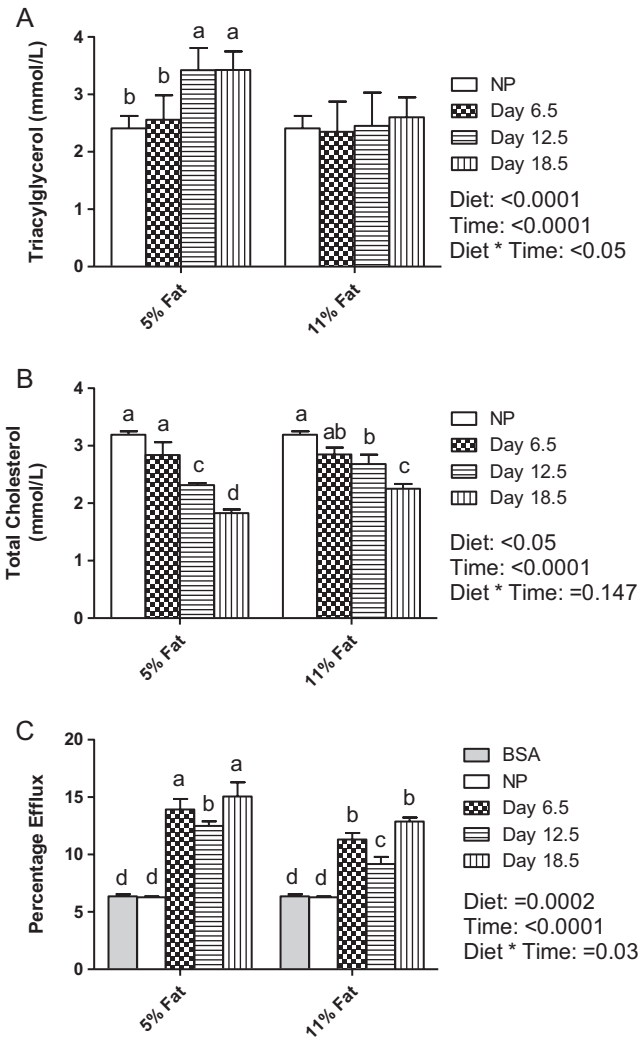

Figure 2 Effects of diets varying in the quantity and the quality of fat on maternal plasma lipid profile at different stages of pregnancy: plasma triacylglycerol (A), total cholesterol (B) and cholesterol efflux capacity (C) were measured for non-pregnant (NP) mice and during gestation at days 6.5, 12.5 and 18.5 as explained in the 'Materials and methods' section. Values are presented as means ± S.D., $n = 8$ at each stage of pregnancy. Data were assessed using two-way ANOVA to determine the main effects and the interactions of diet and time; pairwise comparison using Bonferroni correction was used to determine differences when there was an observed interaction. Letters (a, b and c) represent significant difference between stages of pregnancy in each diet groups. $P < 0.05$ was considered significant; BSA, bovine serum albumin.

decreased thereafter in both dietary groups. Contrary to liver TG, there was no change in liver cholesterol concentration in the 5% group as gestation progressed (Fig. 4B); however, there was a significant effect of time of gestation on liver TC in the 11% group revealing a significant decrease at day 12.5 ($P < 0.05$).

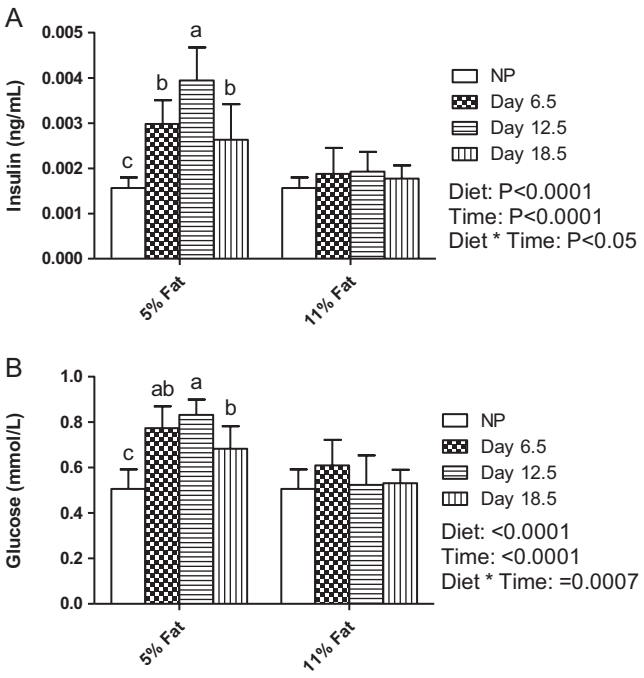

Figure 3 Effects of diets varying in the quantity and the quality of fat on maternal plasma insulin (A) and glucose levels (B) were measured for non-pregnant (NP) mice and during gestation at days 6.5, 12.5 and 18.5 as explained in the 'Materials and methods' section. Values are presented as means ± S.D., $n = 8$ at each stage of pregnancy. Data were assessed using two-way ANOVA to determine the main effects and the interactions of diet and time; pairwise comparison using Bonferroni correction was used to determine differences when there was an observed interaction. Letters (a, b and c) represent significant difference between stages of pregnancy in each diet groups. $P < 0.05$ was considered significant.

Effects of diets on maternal mRNA expression of hepatic lipogenic genes

There was an independent effect of diet on the mRNA expression of *Acc1*, revealing a higher expression in the 5% group, compared to the 11% group ($P < 0.05$; Fig. 5A). There was also an independent effect of time on the mRNA expression of *Acc1* ($P < 0.05$); *Acc1* mRNA expression increased significantly as gestation progressed from days 6.5 to 18.5 in the 5% group. Interestingly, there was no change in the mRNA expression of hepatic *Acc1* in the 11% group. Similarly, diet had an independent effect on the mRNA expression of *Fas*; the expression was significantly higher in the 5% group, compared to the 11% group ($P < 0.05$; Fig. 5B). Time of gestation also had an independent effect on the mRNA expression of *Fas* ($P < 0.05$) in the 5% group, revealing a significant increase as gestation progressed to day 18.5. Similar to *Acc1*, there was no change in the hepatic mRNA expression of *Fas* in the 11% group. Diet had an independent effect on both *Dgat2* and

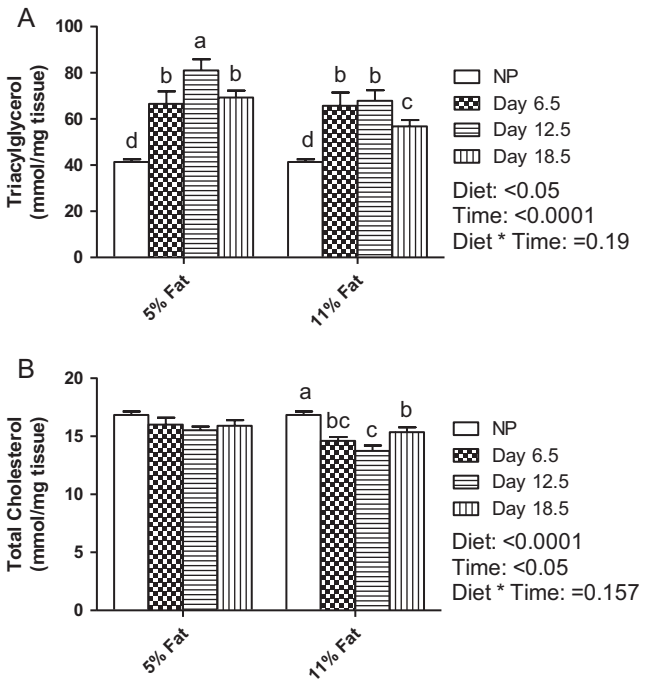

Figure 4 Effects of diets varying in the quantity and the quality of fat on maternal hepatic lipids concentration at different stages of pregnancy: liver triacylglycerol (A) and total cholesterol (B) were measured for non-pregnant (NP) mice and during gestation at days 6.5, 12.5 and 18.5 as explained in the 'Materials and methods' section. Values are presented as means ± S.D., $n = 8$ at each stage of pregnancy. Data were assessed using two-way ANOVA to determine the main effects and the interactions of diet and time; pairwise comparison using Bonferroni correction was used to determine differences when there was an observed interaction. Letters (a, b and c) represent significant difference between stages of pregnancy in each diet groups. $P < 0.05$ was considered significant.

Scd1 (Fig. 5C and D, respectively), revealing a higher expression in the 5% group ($P < 0.05$), compared to the 11% group.

Effects of diets on maternal plasma sex-hormones level and placental Star *mRNA expression at different stages of pregnancy*

There was an independent effect of diet on maternal plasma progesterone ($P < 0.05$; Fig. 6A), revealing a higher level in the 5% group, compared to the 11% group, especially at days 12.5 and 18.5. There was also an independent effect of time of gestation ($P < 0.0001$), where maternal plasma progesterone levels increased significantly in both groups as gestation progressed. There was a significant inverse correlation between maternal plasma progesterone concentration and plasma cholesterol levels in both the 5% (Fig. 6B) and the 11% group (Fig. 6C). The maternal plasma estradiol concentration was also significantly higher in the 5% group ($P < 0.001$) at days 12.5 and 18.5, respectively, compared to the 11% group (Fig. 6D). There was a significant interaction between diet and time of gestation; estradiol concentrations increased significantly in both dietary groups as gestation progressed ($P < 0.0001$). There was also an inverse correlation between maternal plasma estradiol concentration and plasma cholesterol levels in both the 5% (Fig. 6E) and the 11% group (Fig. 6F).

Diet had a significant effect on the mRNA expression of *Star* in the placenta, revealing higher expression in the 5% diet group, compared to the 11% (Fig. 7; $P < 0.05$). There was also an independent effect of time; the mRNA expression of *Star* increased in the placenta as gestation progressed from mid- to late gestation. However, the mRNA expression of *Star* was higher in the 5% group at both mid- and late gestation ($P < 0.05$), compared to the 11% group.

Effects of diets on pregnancy outcomes

Fetal and placental weight increased significantly from days 12.5 to 18.5 in both dietary groups ($P < 0.0001$ and $P < 0.05$, respectively); however, there was no effect of diet on both fetal and placental weight (Table 5). Both diet and time had a significant interaction on the whole uterine weight ($P < 0.05$) to reveal an increase as gestation progressed ($P < 0.0001$) in both groups; however, the 5% group showed higher uterine weight, compared to the 11% group ($P < 0.05$). Interestingly, diet had an independent effect on fetal number; number of fetuses sustained from days 6.5 to 18.5 was significantly higher in the 5% group, compared to the 11% group ($P < 0.05$; Fig. 8).

Discussion

The quality as well as the quantity of fat consumed during pregnancy has the potential to program set points for several physiological and metabolic events in the mother, with a concomitant impact on the health of the offspring (Martin-Gronert & Ozanne 2006, Hartil *et al*. 2009, Jones *et al*. 2009, Williams *et al*. 2014). We have previously shown that a maternal diet high in n-3 PUFA has protective effects on the cardiovascular health of the offspring (Balogun *et al*. 2013, 2014), and that the quality of maternal diet alters the tissue fatty acid composition of the offspring (Chechi *et al*. 2010). We have also shown previously that supplementing maternal diet with n-3 PUFA during pregnancy enriches offspring RBC with DHA (Balogun *et al*. 2013). Similar to our previous findings, we found that females fed the 5% diet that contained higher levels of n-3 PUFA showed higher amounts of EPA, DPA and DHA, compared to the 11% group. It was interesting that ALA was not detected at days 12.5 and 18.5 in the 11% fat group, which coincided with an increase in EPA and DHA. The 11% diet only contained ALA as a source of n-3 PUFA, thus it

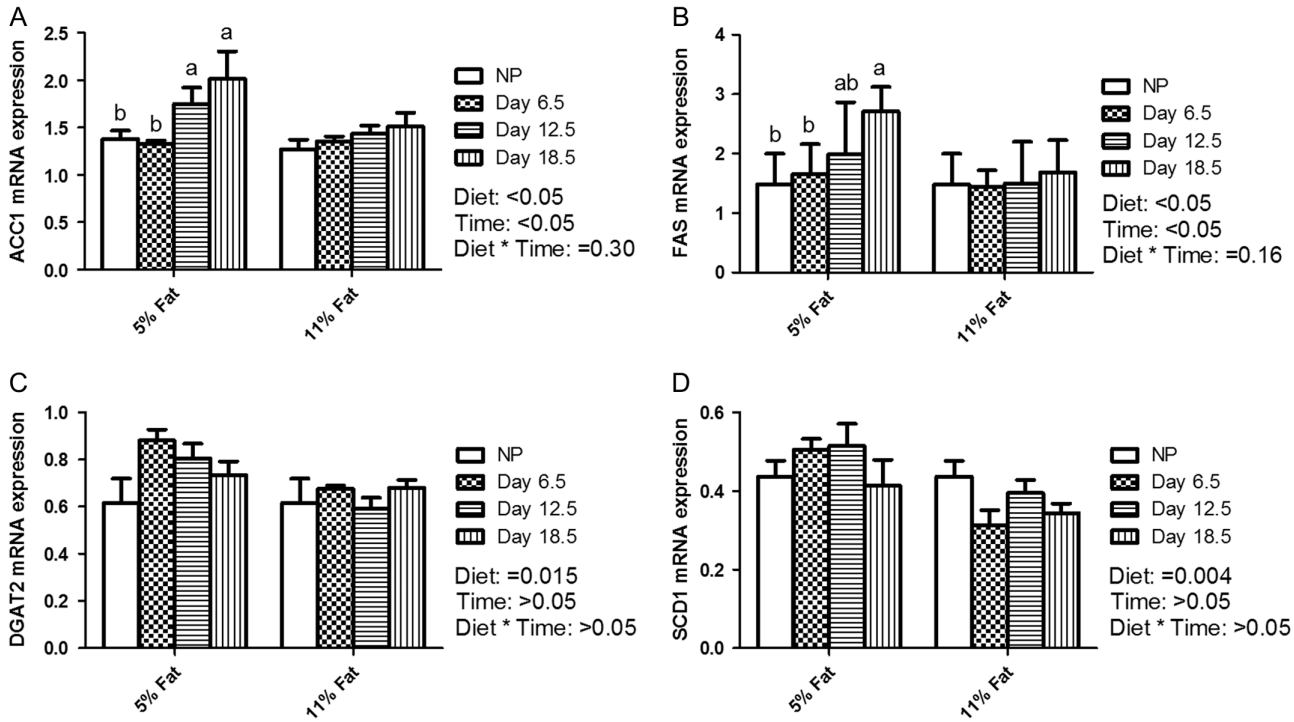

Figure 5 Effects of diets varying in the quantity and the quality of fat on maternal mRNA expression of hepatic lipogenic genes at different stages of pregnancy: The gene expression of acetyl-CoA carboxylase, *Acc1* (A); fatty acid synthase, *Fas* (B); diacylglycerol acyltransferase-2 (*Dgat2*) and stearoyl-CoA desaturase-1 (*Scd1*) was determined in non-pregnant (NP) and during gestation at days 6.5, 12.5 and 18.5 as explained in the 'Materials and methods' section. Values are presented as means ± S.D., $n = 8$ at each stage of pregnancy. The mRNA expression of *Acc1*, *Fas*, *Dgat2* and *Scd1* was normalized with *Actb* as the reference gene. Data were assessed using two-way ANOVA to determine the main effects and the interactions of diet and time; pairwise comparison using Bonferroni correction was used to determine differences when there was an observed interaction. Letters (a and b) represent significant difference between stages of pregnancy in each diet groups. $P < 0.05$ was considered significant.

is obvious that ALA is being converted to longer chain n-3 PUFA as gestation progressed to provide these fatty acids for fetal growth and development. On the other hand, the 5% group showed no change in ALA from days 6.5 to 18.5. Studies have shown that dietary EPA and DHA downregulate the conversion of ALA to longer chain n-3 PUFA by up to 70% (Burdge *et al*. 2003, Pawlosky *et al*. 2003, Arterburn *et al*. 2006); this would explain the detection of ALA throughout gestation in the 5% group as this diet contained longer chain n-3 PUFA. Although the conversion of ALA to DHA is generally limited in women and often not detectable in men (Burdge & Wootton 2002*b*, Burdge & Calder 2005, Hussein *et al*. 2005), the conversion becomes highly efficient during pregnancy (Burdge & Wootton 2002*b*, Childs *et al*. 2011). The rate-limiting step in the conversion of ALA to DHA is the conversion of intermediate DPA to DHA (Arterburn *et al*. 2006). Our data revealed lower DPA in the 11% group; this may be due to a higher conversion of DPA to DHA to increase the availability of DHA for fetal brain at late gestation. DHA is critical for brain and eyes (retina) development (Neuringer *et al*. 1988, Innis 2007). DHA accumulation in the brain has been shown to be most rapid during the last trimester and at the first year of birth; fetus accrues approximately 70 mg DHA per day during third trimester (Clandinin *et al*. 1980). As anticipated, the highest amount of DHA was observed at day 18.5 in both dietary groups. Children from mothers with high intake of DHA during pregnancy has been shown to have higher cognitive capability and better problem-solving skills compared to those with low intake (Judge *et al*. 2007, Dunstan *et al*. 2008). Our findings have established that feeding a diet containing longer chain n-3 PUFA shows higher levels of EPA and DHA in RBC, compared to the diet without longer chain n-3 PUFA, suggesting that the most effective way to supply longer chain n-3 PUFA is by providing these specific fatty acids in the diet.

As pregnancy progresses, metabolic changes occur in the mother to increase the levels of circulating lipids to supply to the fetus (Qureshi *et al*. 1999, Ghio *et al*. 2011, Emet *et al*. 2013). An increase in maternal TG during pregnancy has been shown to contribute to embryo development as it serves as a carrier for fatty acids, which are later released and transferred into fetal circulation (Duttaroy 2009). Our data revealed that both plasma and hepatic TG were significantly higher in the 5% group, compared to the 11% group, likely to meet

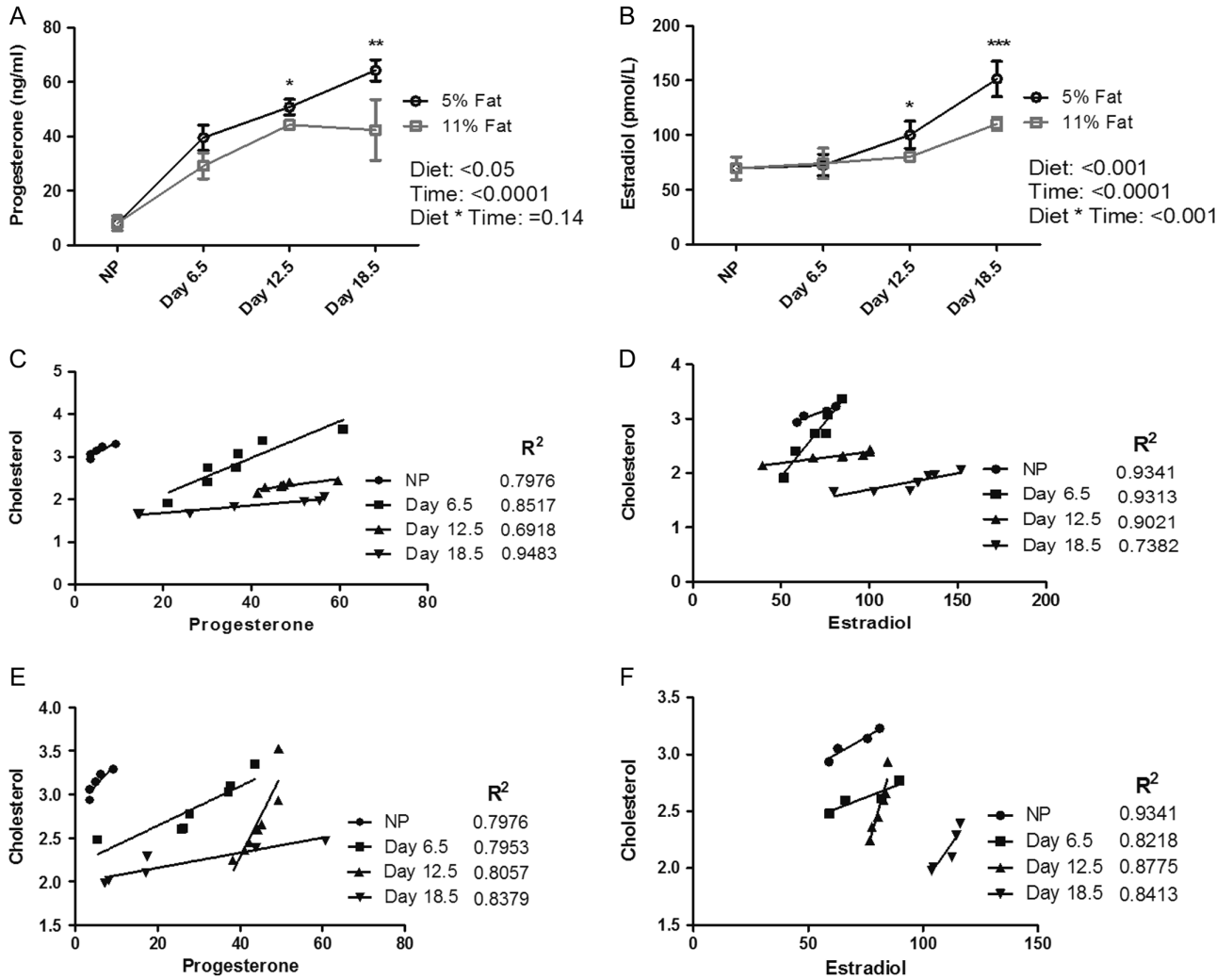

Figure 6 Effects of diets varying in the quantity and the quality of fat on maternal plasma sex steroid hormones at different stages of pregnancy: Plasma progesterone (A) and estradiol (D) were measured in non-pregnant (NP) and during gestation at days 6.5, 12.5 and 18.5 as explained in the 'Materials and methods' section. Values are presented as means ± S.D., $n = 8$ at each stage of pregnancy. Data were assessed using two-way ANOVA to determine the main effects and the interactions of diet and time. Pairwise comparison using Bonferroni correction was used to determine differences when there was an observed interaction. $^{*}P < 0.05$ and $^{**}P < 0.001$ represent significant difference between stages of pregnancy in each dietary group. Pearson's correlation analyses between plasma cholesterol and progesterone in the 5% (B) and the 11% group (C). Pearson's correlation analyses between plasma cholesterol and estradiol concentration in the 5% (E) and the 11% group (F).

fetal fat requirement. A study by Nakashima (2009) revealed an increase in maternal plasma TG in response to a maternal low-fat diet during pregnancy. We also observed a time-dependent increase in plasma TG levels during pregnancy; maternal plasma TG peaked at day 18.5 in the 5% group. This is consistent with the findings of Qureshi and coworkers (1999), who reported that maternal plasma TG increased significantly during the second trimester and reached maximum in the third trimester. An established mechanism for increased plasma TG concentration is via lipogenesis in the liver (Kersten 2001). We observed a higher mRNA expression of lipogenic genes (*Acc1*, *Fas*, *Dgat2* and *Scd1*) in the liver obtained from the 5% group, supporting our observation that the increase in plasma and hepatic TG is due to increased lipogenesis. Interestingly, there was no significant change in plasma TG concentration across gestation time period in the 11% group.

We also found a significantly higher level of insulin in the 5% group, which may have played a role in increasing lipogenesis (Kersten 2001). During pregnancy, maternal lipids are used as the primary energy source to spare amino acids and glucose for fetal use (Ghio *et al*. 2011). Although the fetus has been shown to have a considerably high capacity to adapt to changes in glucose supply during pregnancy, however, lower maternal plasma glucose level has been associated with reduced fetal development (Scholl *et al*. 2001). We observed a higher plasma glucose level in the 5% group, compared to the 11% group, which could have a profound effect on the

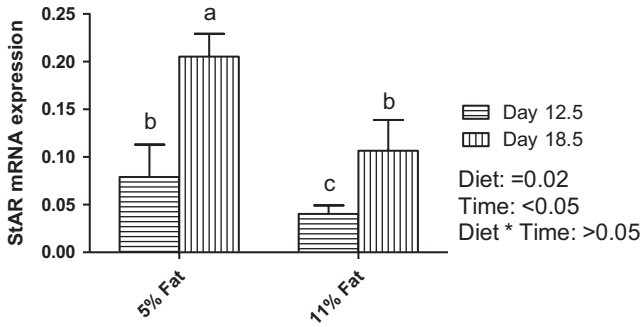

Figure 7 Effects of diets varying in the quantity and the quality of fat on maternal mRNA expression of placental steroidogenic acute regulatory protein (*Star*) at different stages of pregnancy: The mRNA expression of *Star* were measured at days 12.5 and 18.5 as explained in the 'Materials and methods' section. Values are presented as means ± S.D., $n = 8$ at each stage of pregnancy. The mRNA expression of *Star* was normalized with *Actb* as the reference gene. Data were assessed using two-way ANOVA to determine the main effects and the interactions of diet and time; pairwise comparison using Bonferroni correction was used to determine differences when there was an observed interaction. Letters (a, b and c) represent significant difference between stages of pregnancy in each diet groups. $P < 0.05$ was considered significant.

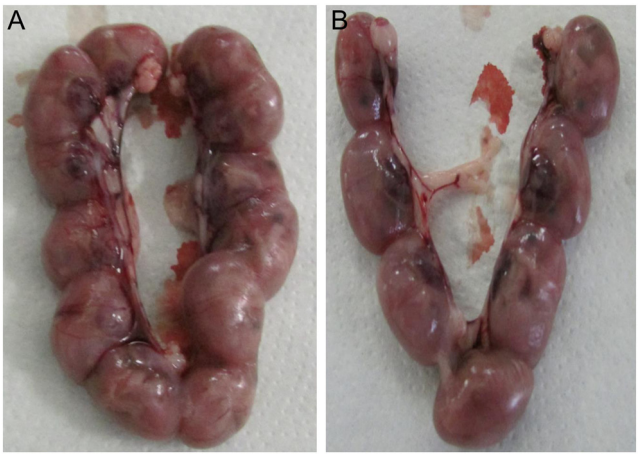

Figure 8 Number of fetuses sustained till day 18.5 in the 5% group (A) and 11% group (B).

development of the fetus. Maternal glucose levels have been shown to decrease slightly during third trimester (Riskin-Mashiah *et al*. 2011); we found a similar trend; however, the reduction in maternal plasma glucose was only significant in the 5% group.

There was a time-of-gestation dependent decrease in plasma cholesterol in both groups from days 6.5 to 18.5; however, the percentage decrease was higher in the 5% group, compared to the 11% group (42.7% vs 29.4%). Maternal cholesterol is an important source of fetal cholesterol at early gestation, especially for cell membranes formation (Krause & Regen 2014). However, the significance of maternal cholesterol to fetal development decreases as gestation progresses, owing to the ability of fetal tissues to synthesize cholesterol (Herrera 2002). We found a consistent decrease in maternal cholesterol levels as gestation progressed to day 18.5 suggesting that cholesterol is being delivered to fetal circulation, as it plays a key role in regulating cascade of activities required for optimal fetal development (Miller 1998). We also found an increase in cholesterol efflux capacity of plasma obtained from mothers fed the 5% diet that contained higher levels of longer chain n-3 PUFA. This was consistent with our previous studies to show that a diet high in n-3 PUFA increases cholesterol efflux capacity (Balogun *et al*. 2014).

Our findings revealed an inverse correlation between maternal plasma cholesterol and the concentration of progesterone and estradiol. It is well known that cholesterol is a precursor for the synthesis of steroid hormones. An increase in the level of cholesterol during pregnancy makes cholesterol available for the synthesis of progesterone and estradiol, which are indispensable in creating a suitable uterine environment for implantation and pregnancy maintenance (Miller 1998). *Star* is a rate-limiting regulator of steroid hormones synthesis by mediating cholesterol transfer within the mitochondrial in the steroid-producing tissues (Stocco & Clark 1996). However, the placenta becomes the primary site for estradiol and progesterone synthesis during pregnancy

Table 5 Pregnancy outcomes.

Pregnancy	5% Fat			11% Fat			Main effect		
Outcomes	Day 6.5	Day 12.5	Day 18.5	Day 6.5	Day 12.5	Day 18.5	Diet	Time	Diet*time
Fetal weight (g)	N/A	0.09 ± 0.02^{b}	1.11 ± 0.12^{a}	N/A	0.09 ± 0.01^{b}	1.08 ± 0.12^{a}	NS	$P < 0.0001$	NS
Placental weight (g)	N/A	0.59 ± 0.14^{b}	0.86 ± 0.15^{a}	N/A	0.54 ± 0.14^{b}	0.70 ± 0.20^{a}	NS	$P < 0.05$	NS
Whole uterine weight (g)	0.29 ± 0.07^{d}	2.85 ± 0.60^{c}	13.48 ± 3.14^{a}	0.32 ± 0.05^{c}	$2.89 \pm .36^{c}$	9.45 ± 4.53^{b}	$P < 0.05$	$P < 0.0001$	$P < 0.05$
Implantation/ fetal number	8.25 ± 1.63	7.71 ± 1.70	8.00 ± 1.83	8.43 ± 0.10^{a}	6.43 ± 1.23^{b}	5.14 ± 0.24^{b}	$P < 0.05$	NS	NS

Values are presented as mean ± S.D., $n = 8$ dams at each stage of pregnancy. Data were analyzed using two-way ANOVA to determine the main effects and the interactions of diet and time. Pairwise comparison using Bonferroni correction was used to determine differences when there was an observed interaction.
Letters (a, b and c) represent significant difference between stages of pregnancy in each dietary group. $P < 0.05$ was considered significant.
N/A, not available; NS, not significant.

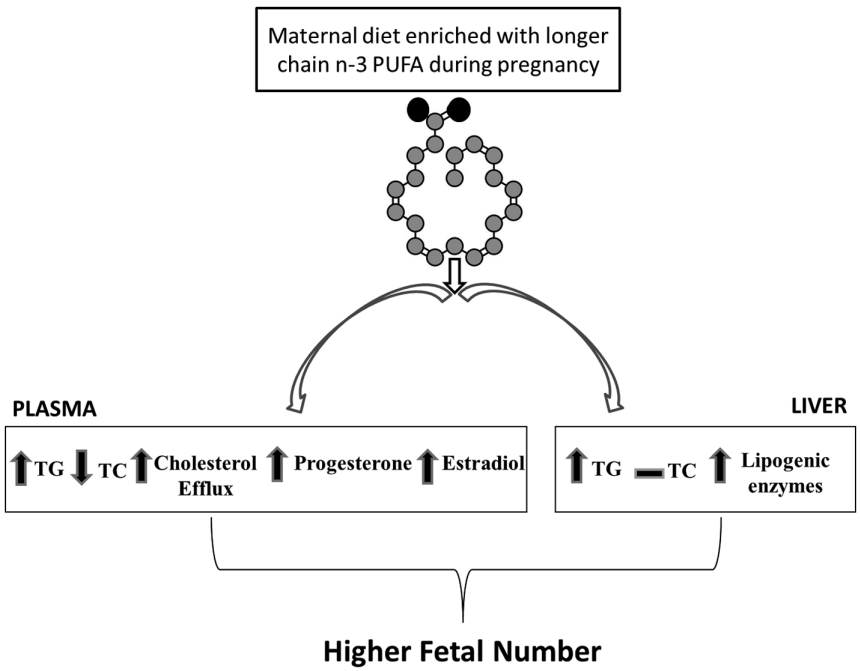

Figure 9 Schematic representation of the effects of a low-fat maternal diet supplemented with longer chain n-3 PUFA on maternal metabolic profile and fetal outcome. n-3 PUFA: Omega-3 polyunsaturated fatty acids; TC, total cholesterol; and TG, triacylglycerol.

(Yivgi-Ohana *et al*. 2009). Approximately 83% of spontaneous abortions has been directly associated with low levels of progesterone during pregnancy (Hahlin *et al*. 1990). Although progesterone treatment is controversial, a handful of studies have attempted the use of progesterone to prevent threatened abortions and to treat recurrent miscarriages (Palagiano *et al*. 2004, El-Zibdeh 2005, Yassaee *et al*. 2014). Impaired progesterone synthesis and action has also been associated with preterm birth in both human and mice, partly due to its ability to suppress the expression of inflammatory cytokines at the materno–fetal interface (Mendelson 2009, Blanks & Brosens 2012). Progesterone also regulates uterine quiescence by preventing contractions that could disturb the growing embryo (Blanks & Brosens 2012). In addition, oral administration of both progesterone and estradiol has been considered to reduce miscarriage rates (Tonguc *et al*. 2011). Increased production of estradiol is very critical at mid-pregnancy as it has been shown that progesterone alone could not maintain pregnancy at this stage (Barkley *et al*. 1979). The demand for progesterone and estradiol increases as gestation progresses (Barkley *et al*. 1979, Milligan & Finn 1997); interestingly, this was also observed in our results. However, our results revealed that the level of progesterone and estradiol in maternal plasma was consistently higher in the 5% group, compared to the 11% group, and this coincides with an increase in the mRNA expression of *Star* in the placenta at mid- and late gestation. Our finding suggests that besides the possible contribution from plasma cholesterol for progesterone and estradiol synthesis, the presence of n-3 PUFA in the 5% group may also be regulating the synthesis of these hormones. Recently, a study by Richardson and coworkers (2013) showed that feeding cows with dietary n-3 fatty acids increased serum progesterone levels. Although the mechanism through which n-3 PUFA might be regulating the synthesis of progesterone and estradiol has not been comprehensively examined, our results show for the first time that higher level of these hormones may elicit higher number of fetuses as the number of fetuses sustained till day 18.5 was significantly higher in the 5% group, compared to the 11% group.

In conclusion, our findings demonstrate for the first time that a low-fat maternal diet enriched with longer chain n-3 PUFA increased the mRNA expression of rate-limiting enzymes for lipogenesis and increased cholesterol efflux, likely to meet fetal lipid demand during pregnancy. In addition, our findings indicate that supplementing maternal diet with longer chain n-3 PUFA increased the maternal plasma concentration of progesterone and estradiol during pregnancy, which may be responsible for an increase in the number of fetuses sustained till day 18.5 as proposed in Fig. 9. These novel findings may be important when designing dietary strategies to optimize reproductive capability and maternal and fetal health.

Declaration of interest

There are no known conflicts of interest associated with this publication.

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