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The effect of maternal dietary fat content and omega-6 to omega-3 ratio on offspring growth and hepatic gene expression in the rat

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Abstract

Omega-6 fatty acids have been shown to exert pro-adipogenic effects whereas omega-3 fatty acids ~~appear to~~ work in opposition. Increasing intakes of LA (linoleic acid; omega-6) vs ALA (alpha-linolenic acid; omega-3) in Western diets has led to the hypothesis that consumption of this diet during pregnancy may be contributing to adverse offspring health. This study investigated the effects of feeding a maternal dietary LA:ALA ratio similar to that of the Western diet (9:1) compared to a proposed 'ideal' ratio (~1:1.5), at two total fat levels (18% vs 36% fat w/w), on growth and ~~fat deposition~~lipogenic gene expression in the offspring. Female Wistar rats were assigned to one of the four experimental groups throughout gestation and lactation. Offspring were culled at 1 and 2 weeks of age for ~~blood and tissue~~ sample collection. Offspring of dams consuming a ~~high~~-36% fat diet were ~20% lighter than those exposed to a ~~low~~-18% fat diet ($P<0.001$). Male, but not female, liver weight at 1 week was ~13% heavier, and had increased glycogen ($P<0.05$), in offspring exposed to high LA ($P<0.01$). Hepatic expression of lipogenic genes suggested an increase in lipogenesis in male offspring exposed to a ~~high~~-36% fat maternal diet and in female offspring exposed to a low LA diet, via increases in the expression of *Fasn* and *Srebf1*. Sexually dimorphic responses to altered maternal diet appeared to persist until two weeks-of-age. In conclusion, whilst maternal total fat content predominantly affected offspring growth, fatty acid ratio and total fat content had sexually dimorphic effects on offspring liver ~~morphology~~ weight and composition.

Introduction

Accumulating evidence suggests that the nutritional environment experienced by an individual during fetal and early infant development has long-lasting impacts on their metabolic health [1]. In the context of the global epidemic of obesity and nutritional excess, there has been considerable interest in determining the effects of maternal overnutrition on the metabolic health of the offspring. The majority of these studies have utilised animal models and have consistently reported that maternal high-fat feeding during pregnancy has detrimental effects on the metabolic health of both the mother and her offspring [2,3]. As a result, excess maternal fat consumption has been implicated as a key contributor to metabolic programming of long-term health and disease risk.

There is increasing evidence, however, that the impact of a high-fat diet on the metabolic health of the offspring depends not only on the amount of fat in the diet, but also on the fatty acid composition [4,5]. There has been particular interest in the role of two classes of polyunsaturated fatty acids (PUFA), due to the substantive increase in the amounts of omega-6 PUFA, predominately linoleic acid (LA), being consumed in the diets of many Western countries over the past few decades [6,7]. This increase in the intake of LA ~~intakes~~ has not been accompanied by substantial changes in the consumption of omega-3 PUFA such as alpha-linolenic acid (ALA) and has therefore resulted in increases in the ratio of omega-6:omega-3 PUFA consumed in the diets of many Western countries [6,8].

The increasing dominance of omega-6 over omega-3 PUFA in modern Western diets has considerable biological significance, since the omega-6 and omega-3 fatty acid families utilise the same enzymes for production of longer chain bioactive derivatives such as arachidonic acid (AA; omega-6), eicosapentaenoic acid (EPA; omega-3), docosapentaenoic acid (DPA; omega-3) and docosahexaenoic acid (DHA; omega-3), and also compete for incorporation into cell membranes. As a result, excess consumption of LA leads to a decrease in the production and incorporation of omega-3 fatty acids through simple substrate competition, and this effect is exacerbated when total dietary PUFA is high [9,10]. The omega-3 and omega-6 long-chain polyunsaturated fatty acids (LCPUFA) derivatives also have opposing physiological actions, with those from the omega-3 family predominately exhibiting anti-inflammatory properties (for example via the suppression of the pro-inflammatory transcription factor nuclear factor kappa B and activation of the anti-inflammatory transcription factor peroxisome proliferator activated receptor γ [11]) and those

from the omega-6 family exhibiting more pro-inflammatory and pro-adipogenic properties [12]. This has led to the hypothesis that the increasing ratio of omega-6 to omega-3 fatty acids in modern Western diets may have negative effects on conditions characterised by low-grade inflammation, including obesity and the metabolic syndrome, and may potentially be contributing to an intergenerational cycle of obesity [8].

Data from observational studies in humans and animal models provide supporting evidence that suggests high intakes of omega-6 PUFA during pregnancy could have negative effects on metabolic health of the progeny [13,14,15]. However, the results of these studies have not been consistent. The results of pre-clinical studies are also limited by the use of diets with much higher omega-6:omega-3 PUFA ratios and/or absolute PUFA contents than those encountered in typical human diets. Furthermore, offspring often continue to have access to the same diet as their mother so that any effects observed cannot be clearly attributed to dietary fatty acid exposure during the gestation and lactation periods [16,17,18]. The aim of this study, therefore, was to investigate the effects of feeding a maternal dietary LA:ALA ratio similar to that of the Western diet (9:1) [6], compared to a proposed 'ideal' ratio of 1:1.5 [19,20] on growth and ~~fat deposition~~lipogenic gene expression ~~of~~ in the offspring. Since total dietary PUFA intake also influences PUFA metabolism [9,10], we also investigated the effect of feeding each dietary fat ratio at either 18% fat w/w (in line with dietary recommendations [21]) or at a higher fat level of 36% fat w/w. A rat model was utilised to achieve the study objectives by allowing for tight control of dietary manipulation as well as invasive end points.

Materials and Methods

Animals

All animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 under Home Office licence and were approved by the Animal Ethics Committee of the University of Nottingham, UK. Virgin female Wistar rats (n=30; 75-100g; Charles River, UK) were housed on wood shavings in individually ventilated cages under a 12 hour light/12 hour dark cycle at a temperature of 20-22°C and had *ad libitum* access to food and water throughout the experiment. Female rats were allowed to acclimatise to the unit for 1-2 weeks, during which time they were fed standard laboratory chow (2018 Teklad Global 18% Protein Rodent Diet, Harlan Laboratories, UK). After acclimatisation, a tail vein blood sample was taken from each animal for the determination of fatty acid status. The rats were then randomly

assigned to one of 4 dietary groups (details provided below). Animals were maintained on their allocated diet for a four week 'pre-feeding' period, after which they were mated. Conception was confirmed by the presence of a semen plug and this was recorded as day 0 of pregnancy. Animals were housed in individual cages and remained on their respective diets throughout pregnancy and lactation.

Litters were standardised to 8 pups within 24 hours of birth (4 males and 4 females, where possible). At 1 and 2 weeks of age, one randomly selected male and one randomly selected female from each litter were culled via cervical dislocation and exsanguination for blood and tissue collection. At 3 weeks of age, the remaining offspring were weaned and dams were then euthanised by CO_2 asphyxiation and cervical dislocation for collection of maternal blood and tissues. All dams were weighed and had feed intake measured daily throughout the experiment and offspring bodyweight was measured weekly.

Diets

Diets were designed to provide either a high (9:1, high LA) or low (1:1.5, low LA) ratio of LA (cis/cis isomer) to ALA, achieved by altering the amounts of flaxseed and sunflower oil included in the fat component of the feed. The levels of saturated and monounsaturated fatty acids were comparable in all diets, achieved by adjusting the amounts of coconut (saturated fat source) and macadamia (monounsaturated fat source) oils in the diets. For each level of LA, diets were developed to contain~~ing~~ either 18% fat (w/w), in line with government recommendations [21], or 36% fat (w/w) to highlight any additive effects ~~were developed~~ (38.6 vs 63.5% of dietary energy respectively). This resulted in four experimental diets (n=6-9 per dietary group); high LA (18% fat), high LA (36% fat), low LA (18% fat) and low LA (36% fat). The list of ingredients and final fatty acid composition of the four experimental diets have been reported previously [5].

Blood sample and tissue collection

Blood samples were collected from dams prior to the start of the experiment and after the 4 week 'feed-in' period (tail vein sample) and at the end of lactation (via cardiac puncture following CO_2 asphyxiation and cervical dislocation). Truncal blood samples were also collected from one randomly selected male and one randomly selected female at 1 and 2 weeks of age. In all cases, samples of whole blood (~30µl) from non-fasted animals were spotted onto PUFAcoat™ dried blood spot (DBS) collection paper [22], allowed to dry at room temperature

and stored at -20°C for subsequent fatty acid analysis. Maternal tissues were weighed and samples of whole liver, retroperitoneal and gonadal adipose tissues collected. Offspring body and organ weights were measured and whole liver samples were collected from one randomly selected male and female pup at both time points. At 2 weeks of age, samples of gonadal and retroperitoneal fat were also collected from one male and one female pup per litter. All tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until determination of gene expression by quantitative reverse transcriptase PCR (qRT-PCR).

Fatty acid methylation and analysis

Fatty acid composition in maternal and fetal blood was determined as previously described [22]. Briefly, whole DBS samples were directly transesterified with 2ml of 1% H_2SO_4 in methanol and the fatty acid methyl esters (FAME) were extracted with heptane. Samples were separated and analysed by a Hewlett-Packard 6890 gas chromatograph (GC) equipped with a capillary column (30m x 0.25mm) coated with 70% cyanopropyl polysilphenylene-siloxane (BPX-70; 0.25µm film thickness) which was fitted with a flame ionization detector (FID). FAMEs were identified in unknown samples based on the comparison of retention times with an external lipid standard (Standard 463, Nu-check prep Inc., MN, USA) using Agilent Chemstation software (Agilent Technologies Australia Pty Ltd). Individual fatty acid content was calculated based on peak area and response factors normalised to total fatty acid content and expressed as a percentage of total fatty acids.

Isolation of RNA and cDNA synthesis and quantitative reverse transcription real-time PCR (qRT-PCR)

RNA was isolated from crushed snap-frozen samples of ~25mg of liver using the Roche High Pure Tissue kit (Roche Diagnostics Ltd., UK). Adipose RNA was extracted, after homogenisation of ~100mg of tissue with MagNA lyser green beads and instrument (Roche Diagnostics Ltd.), using the RNeasy Mini Kit (QIAGEN Ltd., UK). RNA concentration was determined using a Nanodrop 2000 (Thermo Scientific) and RNA quality was evaluated by agarose gel electrophoresis. cDNA was synthesised using a RevertAid™ reverse transcriptase kit (Thermo Fisher Scientific, UK) with random hexamer primers.

Lipogenic pathway and adipokine target genes were chosen based on previous data from our laboratory that indicated that these genes were sensitive to changes in the maternal diet[23] and included; peroxisome proliferator-activated receptor gamma (*Pparg*), sterol regulatory

element-binding protein (variant 1c; *Srebf1*), fatty acid synthase (*Fasn*), lipoprotein lipase (*Lpl*) and leptin (*Lep*), with β-actin (*Actb*) as the housekeeper. Primer efficiency ranged from 85%-108% and sequences have previously been published elsewhere [5]. Adipocyte and hepatic gene expression was quantified using SYBR Green (Roche Diagnostics) in a Light-Cycler 480 (Roche Diagnostics). Samples were analysed against a standard curve of a serially diluted cDNA pool to produce quantitative data and expression was normalised to the housekeeping gene using LightCycler® 480 software (version 1.5.1) as previously described [24]. The expression of the housekeeper gene was not different between treatment groups.

Determination of liver DNA, protein and glycogen content

For determination of DNA and protein content of liver samples, approximately 100mg of frozen crushed sample was added to 1ml of 0.05M trisodium citrate buffer. Samples were homogenised and centrifuged at 2500rpm for 10 minutes at 4°C. Supernatant was used for further analyses. DNA concentration (ug/ml) was measured using a Hoechst fluorimetric method and protein content (ug/well), modified for a 96 well plate format, was measured as described by Lowry *et al.* [25]. Measurements were normalised to the exact amount of tissue used for measurements. Liver glycogen was measured using the Colorimetric Glycogen Assay Kit II (Abcam Ltd.) according to manufacturer's instructions.

Statistical analysis

Data are presented as mean ± SEM. Data were analysed using the Statistical Package for Social Sciences (Version 24, SPSS Inc.). The effect of maternal dietary fatty acid ratio and maternal dietary fat content on maternal dependent variables was assessed using a two-way ANOVA, with dietary LA:ALA ratio and dietary fat content as factors and dams were used as the unit of analysis. Where longitudinal data were analysed, as with maternal feed, protein and energy intakes, the impact of maternal dietary LA:ALA ratio and maternal dietary fat content was analysed using a two-way repeated-measures ANOVA. Offspring data were analysed using a two-way ANOVA, with maternal dietary LA:ALA ratio and fat content as factors; where there was no overall effect of sex, male and female offspring data were combined. Where data were not normally distributed, analyses were performed on log10 transformed data. A value of $P<0.05$ was considered to be statistically significant.

Results

Maternal dietary intakes

There were no differences in feed intake of dams between treatment groups before or during pregnancy. During lactation, dams receiving the 36% fat diets had a lower average daily feed intake than those receiving the 18% fat diets, irrespective of dietary LA:ALA ratio (P<0.001; Fig 1a). Energy intake was similar between groups throughout the experiment (Fig. 1b). Protein intake prior to and during pregnancy was affected by both dietary LA:ALA ratio and fat content (P<0.05; Fig. 1c), however, these effects were small and inconsistent. During lactation, protein intake was affected by dietary fat content only (P<0.001; Fig. 1c), such that mothers receiving ~~high~~ 36% fat diets ~~(36% fat)~~ consumed 24% less protein on average compared to those consuming ~~lower (~~18%~~)~~ fat diets, irrespective of dietary LA:ALA ratio. As expected, all dams consumed more food, energy and protein during lactation than before and during pregnancy regardless of dietary group (P<0.001).

Maternal fatty acid profile

There were no differences in the proportions of either saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), omega-6 (Fig. 2a) or omega-3 PUFA (Fig. 2b) in whole blood samples collected from the dams prior to the commencement of dietary intervention. After 4 weeks on their respective diets, the blood fatty acid profiles were significantly different between treatment groups and largely reflected the composition of the experimental diets. Thus, dams fed on high LA diets had higher proportions of LA (1.2 fold) and AA (1.4 fold) compared to those consuming a low LA diet (P<0.001; Fig. 2c). Conversely, dams fed the low LA diets had a 5.5 fold higher proportion of ALA and an 8.5 fold higher proportion of EPA compared to those consuming a high LA diet (P<0.001; Fig. 2D). These changes were independent of the total fat content of the diet. DPA and DHA levels after the 4 week pre-feeding period were influenced by both dietary LA:ALA ratio and total fat content. Thus, the relative proportions of DPA were higher in dams fed the low LA compared to high LA diets (P<0.001), and marginally higher in dams consuming the 18% vs 36% fat diets (P<0.05). DHA proportions were also higher in the low LA group (P<0.001) but, unlike DPA, were modestly but significantly higher in dams consuming the 36% fat vs 18% fat diets (P<0.05; Fig. 2D). Total blood MUFA proportions were higher (1.3-fold) in dams consuming the low LA diet, irrespective of dietary fat content (P<0.001; Fig. 2C).

The blood fatty acid profile of the dams at the end of lactation, after a further 6 weeks on their respective experimental diets, were similar to those observed after the first 4 weeks of dietary intervention. A notable difference, however, was that at this time point, relative proportions of DHA, as a percentage of total lipids, were not different between dietary groups (Fig. 2F). LA (1.5-fold), AA (1.8-fold) and total omega-6 (1.5-fold) were all higher in dams consuming a high LA diet irrespective of dietary fat content (P<0.001; Fig. 2E). Conversely, total omega-3 levels were 3-fold higher in dams consuming a low LA diet, irrespective of dietary fat content (P<0.001). The proportions of ALA were also higher in the groups consuming the low LA diets and in rats consuming the 36% vs 18% fat diets in the low LA group only (P<0.05; Fig 2F). DPA proportions were higher in the groups consuming the low LA diets, however, unlike ALA, DPA proportions were lower, rather than higher, in dams consuming the 36% fat diets in the low LA group only (P<0.001; Fig. 2F). EPA proportions were higher in groups consuming a low LA diet compared to those consuming a high LA diet (P<0.001; Fig. 2F). EPA proportions were also affected by total dietary fat content, and were lower in dams consuming a ~~high~~ 36% fat ~~(36% fat)~~ diet compared to an ~~lower (~~18% fat~~)~~ diet (P<0.001; Fig. 2F). Maternal blood total MUFA levels at the end of lactation were 1.4-fold higher in the dams consuming a low LA diet irrespective of dietary fat content (P<0.001; Fig. 2E).

Maternal weight, body composition and gene expression

There were no significant differences in dam bodyweight between dietary groups prior to the commencement of the dietary intervention or at any time during the experiment (data not shown). Dams consuming the 36% fat diets had heavier lungs relative to bodyweight at the end of lactation compared to those consuming the 18% fat diets, independent of the LA:ALA ratio (P<0.05). There were no differences in the relative weight of the heart, liver, brain, kidney, gonadal or retroperitoneal fat pads between experimental groups (Table 1).

Analysis of mRNA expression of lipogenic genes indicated that hepatic (3-fold) and gonadal fat (7-fold) expression of *Fasn* was higher in dams consuming an 18% fat diet, compared to those on a 36% fat diet, irrespective of dietary fatty acid ratio (P<0.01). The mRNA expression of *Lpl*, *Pparg* and *Srebf1* was not, however, affected by either dietary fat content or ratio in either hepatic or gonadal fat tissues (Table 1). Expression of leptin mRNA in gonadal adipose tissue was not significantly different between treatment groups.

Birth outcomes and offspring bodyweights

There were no differences between dietary groups in terms of litter size or sex ratio of pups (Table 2). Birth weight was lower in offspring of dams fed a 36% fat vs 18% fat diets, independent of the dietary LA:ALA ratio (Table 2). The lower body weight in offspring of dams fed the 36% fat diet persisted during the sucking period such that offspring of dams fed the 36% fat diets remained lighter than offspring of dams fed on 18% fat diets at both 1 and 2 weeks of age; again this was independent of dietary LA:ALA ratio (P<0.001; Table 3).

Offspring fatty acid profile

At 1 week of age, proportions of AA (2.1 fold) were lower in the offspring of the low LA compared to high LA dams (P<0.001), and in offspring of dams consuming the 36% fat vs 18% fat diets (1.4 fold; P<0.001; Fig. 3A). Blood ALA proportions were 5.9 fold higher in offspring of dams in the low LA groups compared to high LA groups (P<0.001; Fig. 3B). Offspring EPA and DPA proportions were also higher in the low LA group compared to the high LA group. Blood EPA was also influenced by total dietary fat content, but only in offspring of dams fed the low LA diet, in which EPA levels were lower in offspring of dams fed the 36% fat diets compared to the 18% fat diets (EPA, P<0.001; DPA, P<0.01; Fig. 3B). DHA proportions were not different between groups at 1 week of age (Fig. 3B). MUFA proportions were higher (1.2-fold) in offspring of dams in the low LA groups (P<0.001), consistent with the pattern in maternal blood. However, unlike maternal MUFA, offspring MUFA levels were also affected by maternal dietary fat content and were 1.2-fold higher in offspring of dams fed the 36% fat vs 18% fat diets (P<0.001; Fig. 3A). At 1 week of age offspring of dams in the 36% fat diet groups also had lower blood proportions of SFA, irrespective of LA:ALA ratio of the maternal diet (P<0.01; Fig. 3A).

The fatty acid profiles of the offspring at 2 weeks of age were similar to those observed at 1 week. Thus, blood AA (1.9 fold) and total omega-6 (1.6 fold) proportions were lower (Fig. 3C) and ALA (6.3 fold), EPA (4.7 fold), DPA (2.4 fold) and total omega-3 PUFA (3-fold) proportions (Fig. 3D) were higher in offspring of dams in the low LA group compared to high LA groups, irrespective of maternal dietary fat content (P<0.001). Proportions of LA were higher in offspring of dams fed the 36% fat diets compared to those fed 18% fat diets in the high LA group only (P<0.05; Fig 3C), while EPA and DPA proportions were lower in the 36% compared to the 18% fat diet groups, independent of the dietary LA:ALA ratio (P<0.001; Fig. 3D). Unlike findings at 1 week of age, the DHA levels in 2 week old offspring of dams consuming a 36% fat diet were lower (P<0.05) when compared to 18% fat groups, irrespective

of maternal dietary fatty acid ratio. As at 1 week, SFA proportions were lower (1.2-fold) in offspring of dams fed a ~~high~~ 36% fat ~~(36%)~~ diet, independent of the LA:ALA ratio (P<0.001). MUFA proportions were 1.2 fold higher in offspring of dams fed the low LA diets, and 1.2 fold higher in offspring of dams who consumed a 36% fat vs 18% diet (P<0.001; Fig. 3C).

Offspring organ weight and liver composition

At 1 week of age, heart weight relative to bodyweight was higher in female offspring of dams receiving a ~~high (~~36%~~)~~ fat diet compared to the 18% fat diet, independent of the dietary LA:ALA ratio (P<0.05). There were no differences in the relative weight of lung or kidney at 1 week of age and no differences in the relative weight of the heart, lung, liver, gonadal or retroperitoneal fat pads in the offspring at 2 weeks of age between treatment groups in either male or female offspring (Table 3).

Liver weight at 1 week appeared to be influenced by the LA:ALA ratio of the diet to a greater extent than total fat level, at least in males. Thus, male offspring of dams consuming the high LA diets had increased liver weights compared to offspring of dams receiving a low LA diet (P<0.01), irrespective of total dietary fat content. The glycogen content of the livers was also higher in male offspring of dams consuming the high LA diets at 1 week of age (P<0.05). No effect of maternal diet on offspring liver protein or DNA concentration was observed (Table 4). These differences were not present in females at 1 week of age and no differences in glycogen content were observed at two weeks of age in male offspring. DNA concentration in females at two weeks of age was marginally increased (1.1-fold) in offspring exposed to a ~~high~~ 36% fat diet, irrespective of maternal dietary fatty acid ratio (P<0.05).

Hepatic gene expression

At 1 week of age, hepatic *Fasn* expression was influenced by maternal dietary intervention in a sex specific manner. Thus, in males, *Fasn* expression was higher in offspring of dams consuming a ~~high~~ 36% fat ~~(36%)~~ diet irrespective of maternal LA:ALA ratio (P<0.05). In female offspring, however, *Fasn* expression was higher in offspring of dams consuming a low LA diet, independent of dietary fat content (P<0.05). Hepatic *Lpl* mRNA expression in male offspring at 1 week of age was also influenced by maternal dietary fat content, with higher expression in offspring of dams consuming a 36% fat diet vs ~~a low~~ 18% fat diet (P<0.05). In female offspring, hepatic *Srebf* expression, similar to that of *Fasn*, was higher in offspring of dams consuming a low LA diet at 1 and 2 weeks of age (P<0.01). Female hepatic expression

of *Pparg* was lower in offspring of dams consuming a low LA diet at 2 weeks of age ($P<0.05$). There were no differences in the expression of *Fasn* or *Lpl* in female offspring, or expression of any hepatic genes in male offspring at this time point (Table 3).

Discussion

This study has demonstrated that altering the fat content and/or LA:ALA ratio of the maternal diet during pregnancy and lactation resulted in significant alteration in the circulating fatty acid profile of dams in the absence of any significant effects on maternal bodyweight or body composition. Exposure to a ~~high~~ 36% fat diet during gestation and lactation was, however, associated with lower offspring bodyweight from birth, which persisted to 2 weeks of age. This suggests that increased dietary fat intake during pregnancy and lactation can compromise growth of the progeny, irrespective of the type of fat consumed. In addition, alterations in the fat content and/or composition of the maternal diet had transient effects on offspring body composition and hepatic gene expression, effects which were also sex-specific.

Maternal fatty acid profiles after 4 weeks on the experimental diets largely reflected dietary composition, confirming that the dietary intervention had the desired effect on maternal circulating fatty acid composition. These changes persisted after a further 6 weeks of exposure to the diets and, as expected, the dietary LA:ALA ratio had a greater impact on the maternal blood omega-6 and omega-3 status than total dietary fat content. Consistent with previous studies [5,9,26,27], decreasing the dietary LA:ALA ratio resulted in substantial increases in relative maternal ALA and EPA levels but only a very modest increase in DHA proportions after a 4-week exposure, and no difference compared to the higher LA:ALA ratio after 10 weeks. Interestingly, and independent of dietary LA:ALA ratio, dams appeared to be more efficient at converting DPA to DHA when total dietary fat load was higher. One possibility ~~is~~ could be that this is simply a result of the higher amount of substrate (i.e. ALA) available for conversion to the longer chain derivatives such as DPA and DHA in diets containing higher total fat levels. This effect did not, however, persist after a further 6 weeks of dietary exposure, at which point EPA and DPA were lower in dams consuming a low LA 36% fat diet compared to a low LA 18% fat diet. This may be a result of saturation of the PUFA metabolic pathway when total fat, and therefore PUFA, levels were higher [10,28]. This apparent decrease in capacity to convert ALA through to EPA and DHA during consumption of a ~~high~~ 36% fat diet coincides with the decreased protein intake observed in these groups. It is possible that the lower consumption of protein in rats fed on the 36% fat diets may have contributed to reduced

conversion of ALA, since previous studies have shown reduced desaturase, particularly Δ6-desaturase, expression in the mammary gland [29] and liver [30] of rats exposed to a low protein diet. Maternal whole blood MUFA proportions appeared to be influenced by dietary LA:ALA ratio, however, this is most likely a result of the slightly higher MUFA content of the low LA diets.

Offspring fatty acid profiles at 1 and 2 weeks of age largely reflected maternal profiles with maternal dietary LA:ALA ratio exhibiting the strongest effect on offspring circulating fatty acid proportions. However, the total fat content of the maternal diet appeared to have a greater influence on the blood fatty acid composition of the offspring ~~than~~as opposed to that observed in the dams. Of particular interest was the finding that the proportion of both EPA and DPA in offspring at 1 week of age were higher in the low LA (18% fat) vs the low LA (36% fat) group, and that this effect persisted at 2 weeks of age despite ALA levels being increased in the low LA (36% fat) group at this time point. DHA was not different between groups at 1 week of age but was lower in offspring exposed to a ~~high~~ 36% fat diet at 2 weeks of age. As with the maternal fatty acid profiles, this again may be a result of saturation of the PUFA metabolic pathway at higher total PUFA intakes, and is in line with findings from numerous studies, both human and animal, that indicate that simply increasing the quantity of substrate, i.e. ALA, is not an effective strategy for increasing concentrations of its long-chain derivatives, in particular DHA [26,27,31,32].

The total dietary fat content of the maternal diet also had an influence on the proportion of SFA in the offspring, such that offspring of dams consuming ~~high~~ 36% fat diets exhibited lower SFA proportions than offspring of dams consuming the ~~lower~~18% fat diets. Unlike the fetus, where fatty acid composition is largely related to maternal dietary intake, during suckling, offspring fatty acid composition is largely determined by the composition of the milk, which may not fully reflect maternal fatty acid intakes. In a study by Mohammad *et al.* [33], for example, women consuming diets with a higher total fat content (55%en vs. 25%en) exhibited reduced SFA concentrations (C6:0-C14:0) in breast milk but not in maternal plasma. While milk composition was not assessed in the current study, this raises the possibility that SFA content of the milk may have been lower in those dams consuming the 36% fat diets, which could in turn explain the lower SFA status of the offspring. Alternatively, it may be that increasing the fat content of the diets resulted in an increased conversion of SFA to MUFA, since high-fat feeding has been associated with increased expression of the enzyme responsible

for conversion of SFA to MUFA, stearoyl-CoA desaturase 1 (SCD-1) [34] and could therefore be the reason for the observed effect of fat content on offspring MUFA levels in this study. It is important to note, however, that circulating fatty acid profiles are a product of both dietary fatty acid intake as well as tissue fatty acid production and release. Whilst the collection of blood samples from animals in the fed state suggests that the dietary fraction of fatty acids would provide a greater contribution to the fatty acid profile of both dams and offspring, the influence of hepatic synthesis of fatty acids should not be overlooked as a contributor to the observed differences.

Despite significant shifts in maternal fatty acid profiles and increased fat content of the 36% fat diets, we saw no differences in maternal bodyweight or fat deposition. This is consistent with our previous study [5] and is likely a result of the reduced feed intakes of the dams to compensate for the increased energy density of the higher fat diets, a phenomenon consistently seen with dietary intervention trials using rodents [35]. Despite the lack of an effect on maternal weight gain and fat deposition, bodyweight was reduced in offspring of dams receiving a ~~high-~~36% fat ~~(36% fat)~~ diet, irrespective of maternal dietary LA:ALA ratio. This phenotype was consistent across sexes and persisted from birth to 2 weeks of age. Variable results have been reported in this regard with some studies reporting no effects [36,37,38] or increased weight [39]. This finding was, however, consistent with many other studies that reported decreased fetal [40,41], birth [42] and weaning weight [43] in offspring of dams exposed to a ~~high-~~36% fat diet during gestation and lactation periods. The differential effects of different ~~high-~~36% fat diets on offspring growth is likely due to differences in composition of the diet as well as periods of exposure between studies [3]. In those studies that have reported lower offspring weights in offspring fed a high-fat diet, lower protein intakes in dams consuming a high-fat diet have been cited as a likely contributing factor. Further to this, protein restricted diets have been associated with impaired mammary gland development [29,44] leading to impaired milk synthesis [44], and this may also have contributed to reduced offspring growth observed during the suckling period. It is important to note however, that the reduction in protein intake in ~~high-fat~~ dams consuming a 36% fat diet in the current study were more modest (10-25%) than those typically used in low-protein diet studies (~50% reduction) [45,46,47,48].

The lower *Fasn* expression in the liver and adipose tissue of dams exposed to a ~~high-~~36% fat diet is consistent with the established role of this enzyme in suppressing lipogenesis in times of energy excess [49]. Surprisingly, this change did not appear to be mediated through changes

in maternal *Srebf1* mRNA expression, a known regulator of *Fasn* expression [50]. It is important to note that since only mRNA expression was measured, we cannot comment on any differences in protein expression or activity of this transcription factor although mRNA and protein levels have been shown to be closely correlated [23]. Following this up at the protein level is a major priority for future study. In the offspring, however, hepatic *Fasn* expression was not downregulated by exposure to a maternal ~~high~~ 36% fat diet but was actually higher in male offspring of dams consuming the 36% fat compared to the 18% fat diets at 1 week of age and was accompanied by an increase in *Lpl* expression. In female offspring, however, hepatic *Fasn* and *Sbrepf1* expression at 1 week were influenced by maternal dietary fatty acid ratio, rather than total fat content, with both genes upregulated in offspring of dams fed the low LA diets. In both cases, the upregulation of *Sbref1*, *Fasn* and *Lpl* genes would be expected to be associated with an upregulation of both lipogenesis and fatty acid uptake. It is worth mentioning that differences in hepatic expression of lipogenic genes in male offspring were consistently associated with maternal dietary fat content whereas differences in female hepatic expression were consistently associated with maternal dietary fatty acid ratio. This suggests that female offspring are more sensitive to changes in the types of maternal dietary fat whereas male offspring are more sensitive to gross maternal fat consumption. Sex specific effects associated with the programming of disease hypothesis have been frequently reported [51]. The mechanism by which sex influences these effects, however, remains to be elucidated within a larger perspective, as well as within the context of this study.

We found no evidence that these alterations in hepatic gene expression translated to increases in liver weight, however whether there was any effect on hepatic fat content remains to be determined. In both male and female offspring, relative liver weight was increased in offspring of dams fed the high LA diet. In an aim to further elucidate the source of this increased weight, we measured liver DNA, protein and glycogen composition. Similar to liver weight, glycogen levels were increased in offspring of dams fed the high LA diets. This increase in glycogen, however, was not sufficient enough to completely account for the differences observed in liver weight but may be a contributing factor. Consideration of DNA and protein content of the tissue did not indicate significant changes to cell size or number. More detailed analysis is required to further elucidate the mechanism by which high maternal dietary omega-6 may impact upon offspring liver ~~morphology and~~ physiology.

The majority of the hepatic mRNA expression differences, as well as gross differences in liver weight and composition, appeared to be transient and were no longer present at 2 weeks of age. A notable exception was the lower expression of *Srebf1* mRNA and higher expression of *Pparg* in females of dams exposed to a high LA diet compared to the low LA diet, with a similar trend observed in males. Although found in relatively low concentrations in the liver, activation of *Pparg* has been shown to increase hepatic lipid storage and is elevated in models of hepatic steatosis [52]. As such, decreased *Pparg* expression can alleviate some of the symptoms of hepatic steatosis leading to a reduced liver weight in conjunction with a reduction in hepatic triglyceride content [53]. Thus, our finding that female offspring of dams exposed to a high LA diet tended towards to have an increased liver weight at one week of age followed by increased hepatic *Pparg* expression at two weeks of age may suggest that the increase in *Pparg* expression is a potential response to the increased liver growth observed a week earlier. Alternatively, parallels may be drawn to the effect of low protein diets where fluctuations between an increased and decreased lipogenic capacity, chiefly mediated by altered *Srebf1* expression, occur in early life only to settle into a pattern of upregulated lipogenesis at a later life stage [23]. Further studies would be needed to directly evaluate this hypothesis.

In conclusion, we have demonstrated that exposure to a ~~high~~ 36% fat diet during gestation and lactation is associated with persistent growth restriction in both male and female offspring irrespective of maternal dietary fatty acid composition. Growth restriction has been associated with a plethora of metabolic disturbances later in life [54,55,56] and transient alterations in gene expression have been suggested as a mechanism for programming changes in metabolic processes within tissues as well as the morphology of the tissues themselves [1] . In this study, offspring are still exposed to the experimental diets via the dams milk, and further studies in offspring at older ages are required to assess whether the changes in growth, hepatic gene expression and liver weights in the current study are associated with phenotypic changes that persist once offspring are no longer exposed directly to the altered diet composition. In addition, analysis of lipogenic pathway and adipokines targets at the protein level, as well as whole transcriptome analysis, may yield useful information about their regulation and the extent to which these experimental diets programme other metabolic and regulatory pathways in the liver. ~~Further to this~~Finally, the longevity of these perturbations into later life, especially when presented with secondary metabolic challenges such as aging, prolonged high-fat feeding or in the case of female offspring, pregnancy, remains to be elucidated.

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Conflicts of Interest

None

Author Contributions

SCL-E, BSM and MJE participated in study design. SAVD carried out the study (assisted by GG), data analysis and preparation of the manuscript which was revised and approved by SCL-E, BSM, MJE and GG.

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675

Table 1. Maternal organ weights and gene expression

	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)
Bodyweight (g)	308.75 ± 9.82	288.73 ± 14.95	303.29 ± 11.11	302.23 ± 7.85
Heart (% BW)	0.35 ± 0.01	0.36 ± 0.01	0.35 ± 0.01	0.35 ± 0.01
Lungs (% BW)[a]	0.45 ± 0.02	0.51 ± 0.04	0.42 ± 0.02	0.48 ± 0.02
Kidney (% BW)	0.78 ± 0.02	0.83 ± 0.03	0.82 ± 0.02	0.79 ± 0.02
Liver (% BW)	5.01 ± 0.10	4.80 ± 0.28	5.28 ± 0.10	4.88 ± 0.09
Brain (% BW)	0.59 ± 0.02	0.59 ± 0.02	0.58 ± 0.02	0.59 ± 0.02
Gonadal Fat (% BW)	1.88 ± 0.35	2.02 ± 0.39	1.65 ± 0.19	1.61 ± 0.11
Retroperitoneal Fat (% BW)	0.76 ± 0.13	0.76 ± 0.13	0.76 ± 0.08	0.85 ± 0.15
Liver mRNA Expression				
Fasn[a*]	20.98 ± 6.17	7.03 ± 1.26	25.08 ± 8.12	9.45 ± 1.63
Lpl	0.20 ± 0.04	0.19 ± 0.04	0.19 ± 0.04	0.13 ± 0.01
Pparg	0.63 ± 0.22	0.78 ± 0.18	0.41 ± 0.09	0.70 ± 0.16
Srebf1	3.52 ± 0.91	2.56 ± 0.64	7.85 ± 2.57	3.39 ± 0.61
Gonadal Fat mRNA Expression				
Fasn[a*]	1.29 ± 0.64	0.18 ± 0.05	2.50 ± 1.16	0.37 ± 0.14
Lpl	0.90 ± 0.23	0.87 ± 0.06	1.56 ± 0.41	1.48 ± 0.46
Ppparg	0.91 ± 0.23	1.22 ± 0.20	1.12 ± 0.13	1.16 ± 0.18
Srebf1	1.80 ± 0.48	1.56 ± 0.31	3.43 ± 1.16	2.21 ± 0.62
Lep	0.49 ± 0.08	1.00 ± 0.29	1.10 ± 0.31	1.38 ± 0.25

All values are mean ± SEM and n=6-9 per dietary group. The effect of dietary fatty acid ratio and dietary fat content were assessed using a two-way ANOVA. [a] indicates a significant effect of dietary fat content (P<0.05, *P<0.01). Although not statistically significant there was some evidence that maternal Srebpf1 expression was influenced by the LA (P=0.08) and fat content (P=0.06) of the diet.

682 **Table 2. Birth outcomes**

	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)
n	6	8	7	9
Litter Size	12.83 ± 1.19	13.00 ± 1.21	13.14 ± 0.40	13.33 ± 1.08
Sex Ratio (male/female)	1.01 ± 0.23	0.97 ± 0.24	1.27 ± 0.28	1.13 ± 0.26
Male Birthweight (g)[a]	6.19 ± 0.53	5.19 ± 0.18	5.66 ± 0.14	5.36 ± 0.11
Female Birthweight (g)[a]	5.60 ± 0.37	4.85 ± 0.21	5.26 ± 0.14	5.07 ± 0.12

683 All values are mean ± SEM. The effect of dietary fatty acid ratio and dietary fat content was
684 assessed using a two-way ANOVA. [a] indicates a significant effect of maternal dietary fat
685 content ($P<0.05$).

686 **Table 3. Offspring organ weights and hepatic gene expression**

Experimental Group	Male				Female			
	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)
1 Week Offspring								
Bodyweight (g)	17.52 ± 1.22[a]	12.85 ± 1.16[b]	16.61 ± 0.41[a]	14.20 ± 0.63[b]	15.79 ± 1.11[a]	12.44 ± 1.17[b]	15.66 ± 0.66[a]	13.40 ± 0.56[b]
Heart (% BW)	0.59 ± 0.07	0.67 ± 0.06	0.58 ± 0.04	0.64 ± 0.02	0.56 ± 0.02[a]	0.70 ± 0.06[b]	0.57 ± 0.06[a]	0.69 ± 0.04[b]
Lungs (% BW)	1.87 ± 0.05	1.73 ± 0.04	1.89 ± 0.05	1.90 ± 0.06	1.96 ± 0.11	1.92 ± 0.05	1.88 ± 0.12	1.93 ± 0.05
Kidney (%BW)	1.27 ± 0.08	1.34 ± 0.05	1.19 ± 0.09	1.22 ± 0.02	1.25 ± 0.04	1.38 ± 0.06	1.21 ± 0.10	1.26 ± 0.03
Liver (% BW)	3.17 ± 0.16[a]	3.39 ± 0.13[a]	2.81 ± 0.12[b]	2.89 ± 0.09[b]	3.18 ± 0.10	3.20 ± 0.27	2.96 ± 0.13	2.99 ± 0.05
Liver *Fasn*	0.21 ± 0.08[a]	0.24 ± 0.05[b]	0.18 ± 0.02[a]	0.38 ± 0.04[b]	0.15 ± 0.02[a]	0.22 ± 0.03[a]	0.32 ± 0.06[b]	0.35 ± 0.08[b]
Liver *Lpl*	1.09 ± 0.38[a]	1.26 ± 0.25[b]	0.76 ± 0.15[a]	2.01 ± 0.38[b]	1.26 ± 0.24	1.37 ± 0.46	1.59 ± 0.28	1.81 ± 0.35
Liver *Pparg*	0.40 ± 0.16	0.30 ± 0.07	0.46 ± 0.14	0.38 ± 0.08	0.51 ± 0.11	0.52 ± 0.13	0.62 ± 0.16	0.41 ±0 .06
Liver *Srebpf1*	0.63 ± 0.16	0.56 ± 0.09	0.51 ± 0.10	0.74 ± 0.10	0.44 ± 0.06[a]	0.44 ± 0.05[a]	0.64 ± 0.11[b]	0.80 ± 0.12[b]
2 Week Offspring								
Bodyweight (g)	39.76 ± 1.67[a]	31.78 ± 2.17[b]	39.89 ± 0.59[a]	31.56 ± 1.49[b]	37.77 ± 1.55[a]	31.70 ± 2.05[b]	38.49 ± 0.93[a]	30.75 ± 1.29[b]
Heart (% BW)	0.60 ± 0.01	0.60 ± 0.02	0.61 ± 0.03	0.63 ± 0.01	0.67 ± 0.06	0.67 ± 0.01	0.65 ± 0.03	0.61 ± 0.02
Lungs (% BW)	1.33 ± 0.20	1.26 ± 0.05	1.25 ± 0.07	1.42 ± 0.07	1.28 ± 0.07	1.32 ± 0.05	1.26 ± 0.08	1.32 ± 0.06
Kidney (%BW)	1.05 ± 0.02	1.02 ± 0.03	1.06 ± 0.02	1.00 ± 0.03	1.17 ± 0.04	1.15 ± 0.04	1.14 ± 0.01	1.05 ± 0.02
Gonadal Fat (%BW)	0.22 ± 0.06	0.18 ± 0.02	0.19 ± 0.02	0.18 ± 0.01	0.24 ± 0.02	0.21 ± 0.02	0.23 ± 0.02	0.24 ± 0.03
Retroperitoneal Fat (%BW)	0.36 ± 0.01	0.41 ± 0.04	0.41 ± 0.02	0.39 ± 0.02	0.33 ± 0.03	0.27 ± 0.02	0.29 ± 0.03	0.27 ± 0.01
Liver (% BW)	3.01 ± 0.06	3.08 ± 0.14	3.11 ± 0.02	3.03 ± 0.02	3.18 ± 0.09	3.15 ± 0.09	3.23 ± 0.05	3.01 ± 0.10
Liver ~~FAS~~*Fasn*	0.17 ± 0.01	0.18 ± 0.02	0.19 ± 0.02	0.20 ± 0.02	0.19 ± 0.02	0.20 ± 0.03	0.22 ± 0.03	0.24 ± 0.03
Liver ~~LPL~~*Lpl*	1.70 ± 0.25[a]	1.81 ± 0.29[b]	1.60 ± 0.13[a]	2.44 ± 0.23[b]	1.25 ± 0.16	1.89 ± 0.16	2.01 ± 0.29	1.81 ± 0.08
Liver ~~PPARγ~~*Pparg*	0.56 ± 0.17	0.66 ± 0.10	0.48 ± 0.10	0.42 ± 0.07	0.79 ± 0.25[a]	0.58 ± 0.07[a]	0.31 ± 0.06[b]	0.43 ± 0.07[b]
Liver ~~SREBP1c~~*Srebf1*	0.74 ± 0.02	0.71 ± 0.08	0.83 ± 0.06	0.80 ± 0.05	0.68 ± 0.07[a]	0.68 ± 0.05[a]	0.83 ± 0.06[b]	0.95 ± 0.10[b]

687 All values are mean ± SEM. A two-way ANOVA was used to analyse results with maternal dietary fatty acid ratio and maternal dietary fat
688 content as factors. Different superscripts denote values which are significantly different ($P<0.05$). n=4-9 per dietary group. All comparisons are
689 made within sex group.

690 **Table 4. Offspring liver composition**

Experimental Group	Male				Female			
	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)
1 Week Offspring								
Liver DNA (µg/mg tissue)	0.48 ± 0.06	0.54 ± 0.04	0.56 ± 0.06	0.52 ± 0.03	0.51 ± 0.03	0.51 ± 0.04	0.50 ± 0.04	0.52 ± 0.02
Liver Protein (mg/g tissue)	119.2 ± 12.8	137.7 ± 8.9	135.6 ± 5.2	129.8 ± 4.9	123.8 ± 4.5	138.8 ± 8.3	128.6 ± 3.3	129.5 ± 5.2
Liver Glycogen (µg/mg tissue)	12.71 ± 0.70[a]	11.26 ± 1.86[a]	9.72 ± 1.32[b]	8.64 ± 0.76[b]	9.70 ± 0.89	7.73 ± 0.88	9.00 ± 1.43	11.27 ± 1.80
2 Week Offspring								
Liver DNA (µg/mg tissue)	0.59 ± 0.03	0.53 ± 0.05	0.56 ± 0.04	0.51 ± 0.03	0.52 ± 0.02[a]	0.61 ± 0.05[b]	0.52 ± 0.03[a]	0.57 ± 0.01[b]
Liver Protein (mg/g tissue)	115.1 ± 3.6	129.9 ± 13.5	130.2 ± 10.0	117.9 ± 9.4	117.2 ± 9.7	132.3 ± 9.1	120.7 ± 9.4	120.6 ± 6.5
Liver Glycogen (µg/mg tissue)	9.45 ± 0.61	7.48 ± 0.54	8.35 ± 0.98	9.30 ± 1.75	-	-	-	-

691 All values are mean ± SEM. A two-way ANOVA was used to analyse results with maternal dietary fatty acid ratio and maternal dietary fat
692 content as factors. Different superscripts denote values which are significantly different ($P<0.05$). n=4-9 per dietary group. All comparisons are
693 made within sex group.

694 **Figures**

695

696 **Figure 1.** Maternal average daily (A) feed intake, (B) energy intake and (C) protein intake
697 during pre-feeding, pregnancy and lactation fed on either a high LA (18% fat) diet (closed
698 circles), high LA (36% fat) diet (open circles), low LA (18% fat) diet (closed squares) and a
699 low LA (36% fat) diet (open squares). Values are means ± SEM and n=6-9 per group. The
700 effects of dietary fatty acid ratio and dietary fat content were determined using a two-way
701 repeated measures ANOVA. * indicates a significant effect of dietary fat content (** P<0.01,
702 *** P<0.001). † indicates a significant interaction between dietary fat content and fatty acid
703 ratio.

704

705 **Figure 2.** Maternal whole blood fatty acids profile at (A/B) baseline (C/D) after 4 weeks on
706 experimental diet and (D/E) at the end of lactation (3 weeks post-partum). Values are means
707 ± SEM and n=6-9 per group. The effects of dietary fatty acid ratio and dietary fat content
708 were determined using a two-way ANOVA (*P<0.05, **P<0.01, ***P<0.001). † indicates a
709 significant interaction effect (P<0.05).

710

711 **Figure 3.** Offspring whole blood fatty acids profile at (A/B) one week of age and (C/D) at
712 two weeks of age. Values are means ± SEM and n=11-17 per group. The effects of maternal
713 dietary fatty acid ratio, maternal dietary fat content and sex were determined using a three-
714 way ANOVA. No effect of sex was found for any of the fatty acids measured and so male
715 and female samples were combined for further analysis. * Indicates significant difference
716 (*P<0.05, **P<0.01, ***P<0.001). † indicates a significant interaction effect (P<0.05).

Figure 1.

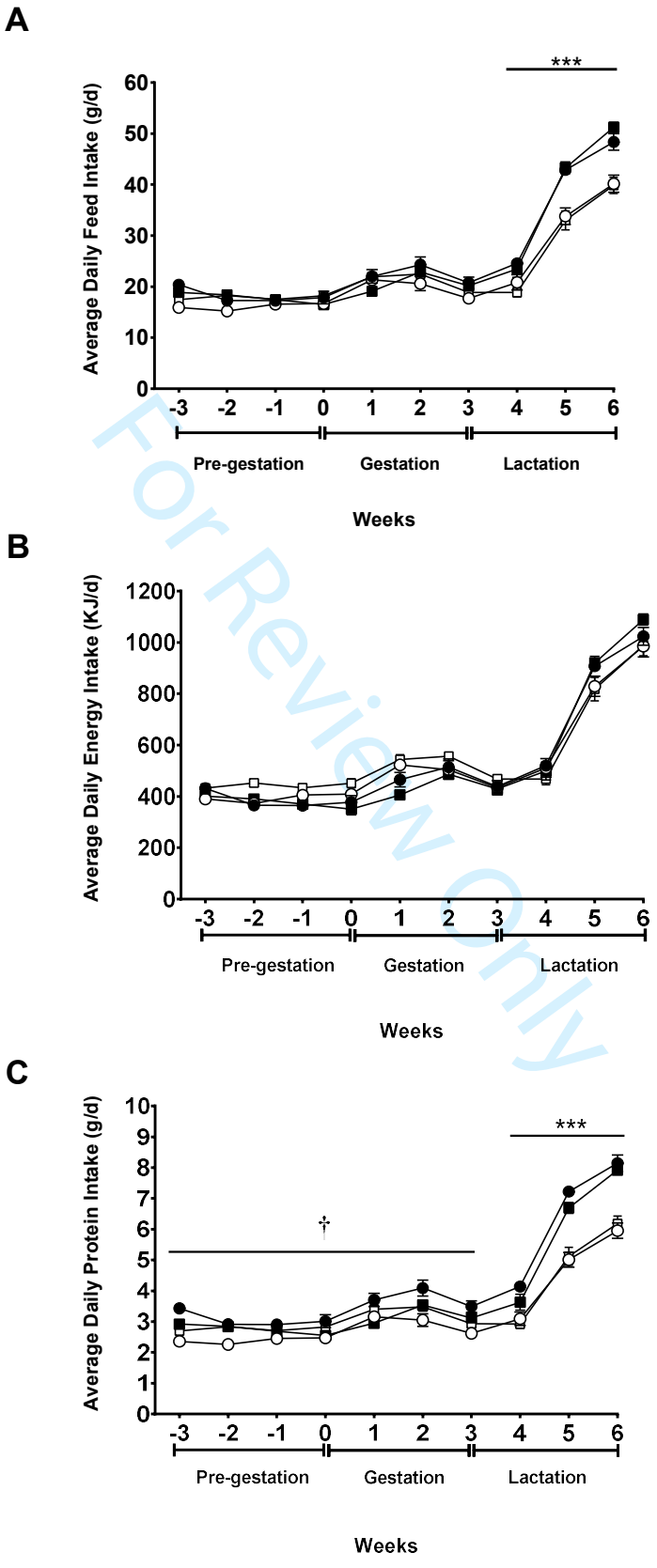


Figure 2.

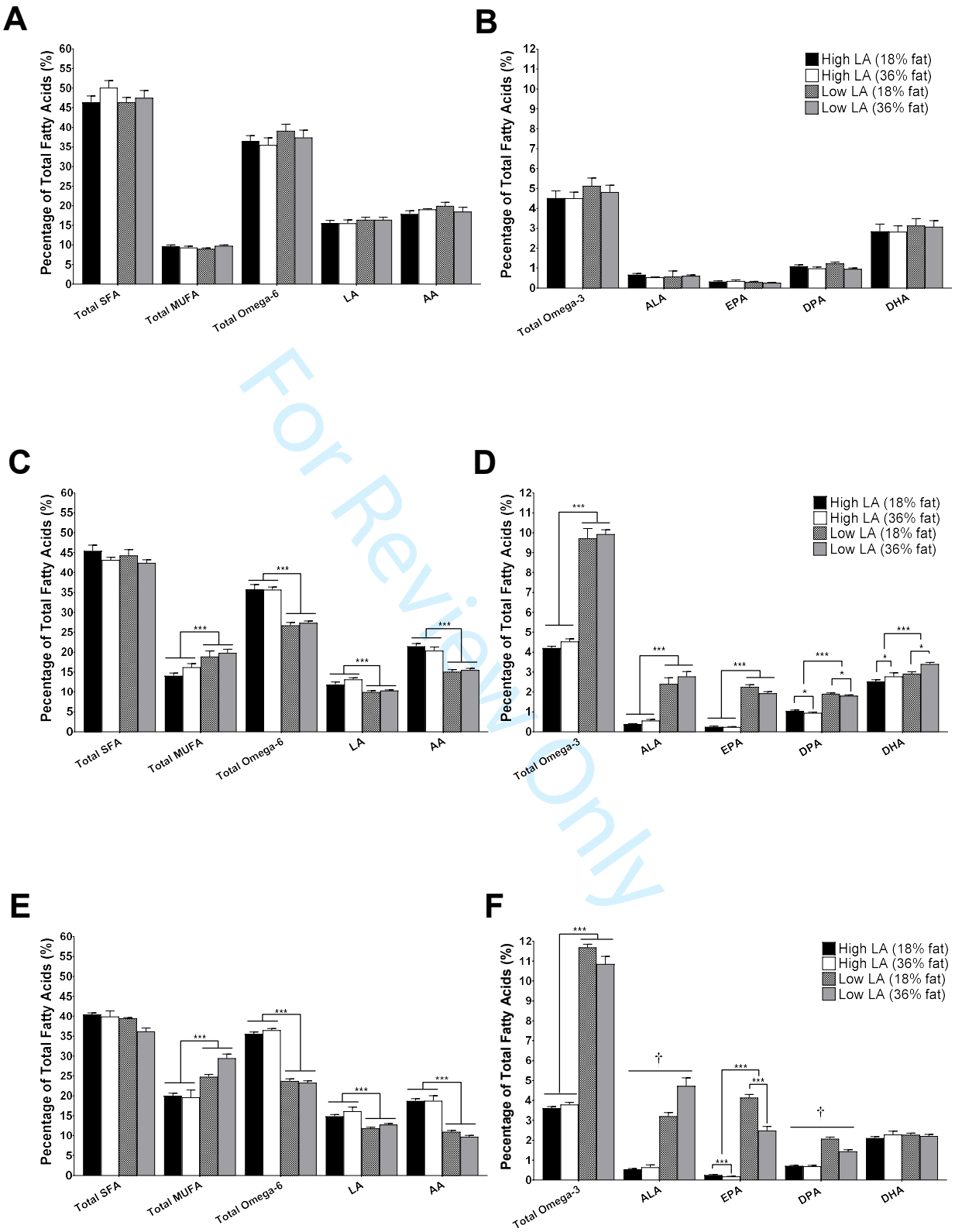
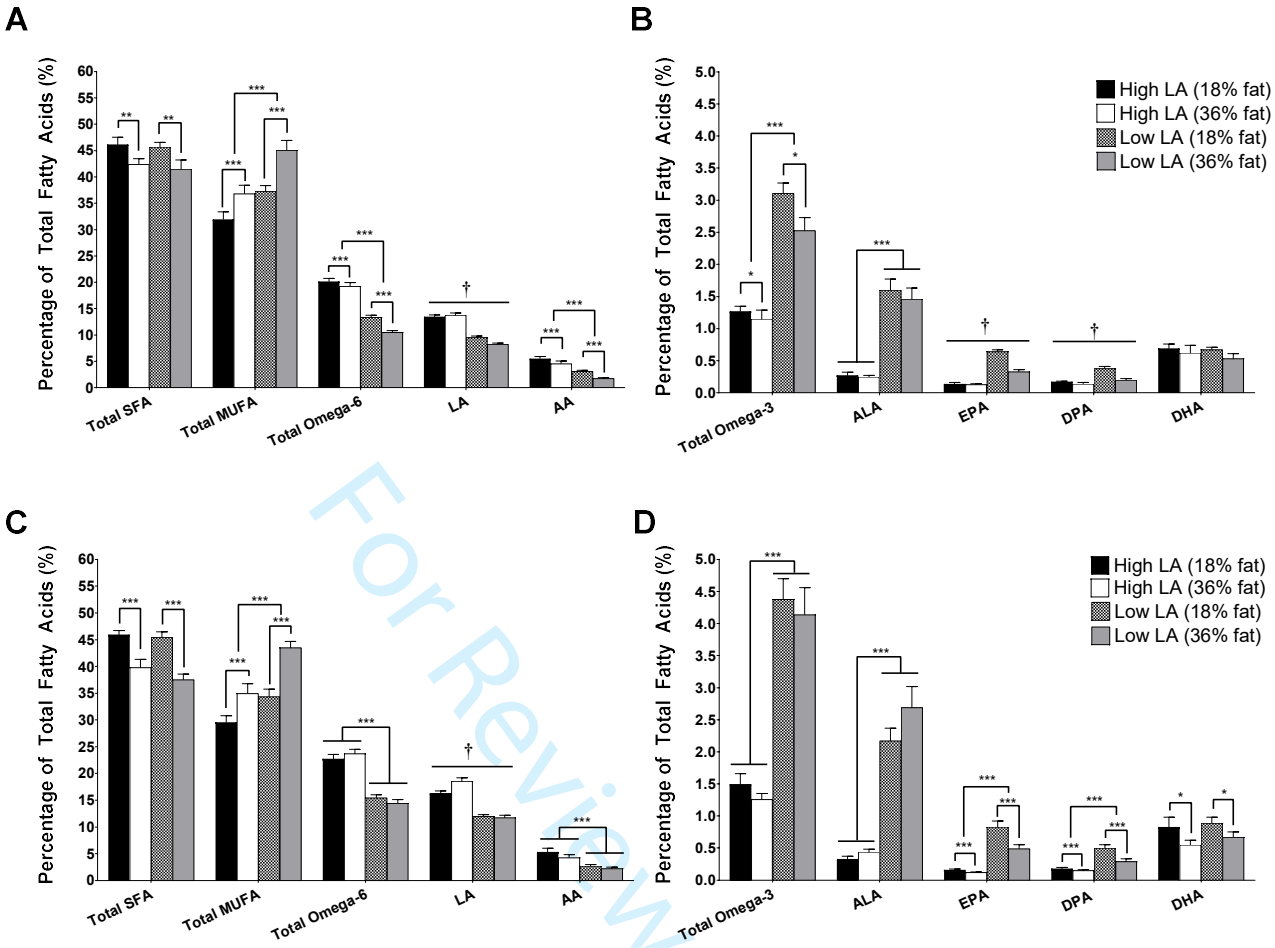


Figure 3.



ARRIVE

The ARRIVE Guidelines Checklist

Animal Research: Reporting In Vivo Experiments

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	ITEM	RECOMMENDATION	Section/ Paragraph
Title	1	Provide as accurate and concise a description of the content of the article as possible.	Title
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	Abstract
INTRODUCTION			
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.	a. Introduction (para. 1-5) b. Introduction
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	Introduction
METHODS			
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	Materials and methods
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	a. Materials and methods (section: diets) b. Materials and methids c. Materials and methods
Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	a/b/c/d. Materials and methods (all sections)
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	a/b. Materials and methods (section: animals)

The ARRIVE guidelines. Originally published in *PLoS Biology*, June 2010[1]

Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	a/b/c. Materials and methods (section: animals/diets)
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	a/b/c. Materials and methods (sections: Animals/diets/ statistical
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	a/b. Materials and methods (section: animals)
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	Introduction (para. 5)
Statistical methods	13	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	a/b. Materials and methods (section: Statistical analyses)
RESULTS			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	Results
Numbers analysed	15	a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%[2]). b. If any animals or data were not included in the analysis, explain why.	a/b. Results (Figures and tables)
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	Results (figures and
Adverse events	17	a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.	a/b. N/A
DISCUSSION			
Interpretation/ scientific implications	18	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results[2]. c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.	a/b. Discussion c. N/A
Generalisability/ translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	Discussion
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	Funding

References:
1. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
2. Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332.