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RESEARCH PAPER

Linoleic and α-linolenic fatty acid consumption over three generations exert cumulative regulation of hepatic expression of genes related to lipid metabolism

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Abstract The essential fatty acids, omega-3 and omega-6, consumed during pregnancy can benefit maternal and offspring health. For instance, they could activate a network of genes related to the nuclear receptor peroxisome proliferator-activated receptor α (Ppara) and sterol regulatory element binding transcription factor 1 (Srebf1), which play a role in fatty acid oxidation and lipogenesis. The present study aimed to investigate the effects of diets with different omega-3/omega-6 ratio consumed over three generations on blood biochemical parameters and hepatic expression of Ppara- and Srebf1-related genes. During three consecutive generations adult Wistar rats were evaluated in the postpartum period (21 days after parturition). Regardless of prenatal dietary omega-3/omega-6 ratio, an upregulation in liver tissue was observed for Rxra, Lxra and Srebf1 and a downregulation for Fasn in all the evaluated generations. The diet with higher omega-3/omega-6 ratio decreased triacylglycerol serum levels and resulted in a constant non-esterified fatty acid level. Our results indicated that the PUFAs effect on the modulation of genes related to fatty acid oxidation and lipogenesis is cumulative through generations.

Keywords PUFAs · Ppara · Rxra · Lxra · Srebf1 · Fasn

Introduction

The programming of health begins before birth and is influenced by maternal health and nutrition. Alterations in the structure and function of maternal tissues during pregnancy can cause changes in fetal metabolic status (Newnham et al. 2002) leading to greater susceptibility of the offspring to some metabolic syndromes such as obesity, hypertension, diabetes and cardiovascular diseases (Langley-Evans 2006). The mechanisms by which a metabolic disorder or unbalanced nutrition during pregnancy

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influence gene expression and the phenotype of the offspring are mediated by epigenetics (Simmons 2011).

Recently, it has been proposed that nutrients such as long-chain fatty acids could influence offspring development and heath (Cetin et al. 2009). They are major sources of energy, acting to control the fluidity, permeability and conformation of cellular membranes. The polyunsaturated fatty acids (PUFAs) are especially important because they serve as precursors of important bioactive compounds such as prostacyclins, prostaglandins, thromboxanes and leukotrienes besides their role in structural functions (Haggarty 2010). During pregnancy in mammals, PUFAs are required for embryo (Haggarty et al. 2006) and oocyte development (Matorras et al. 1998), cell growth (Sellmayer et al. 1996) and differentiation (Ding et al. 2002). In this regard, studies have suggested the need for feeding different ratios of omega-6 (ω-6) and omega-3 (ω-3) during pregnancy (Simopoulos 2002; Simopoulos et al. 1999; Kris-Etherton et al. 2000). For instance, offspring of dams fed with a high-lard (i.e., saturated fat) diet during pregnancy and lactation were more susceptible to metabolic syndrome, including dyslipidemia, increased blood pressure and adiposity (Armitage et al. 2005). Recently, it was demonstrated that a high omega-3 diet reversed the hypertension, hyperlipidemia and adiposity that was induced by consumption of a high fat diet from weaning in 6-month-old male Wistar rats (Zulkafli et al. 2013).

A number of studies have demonstrated the potential of the ω-3 family of PUFAs to regulate key transcription factors controlling hepatic lipid metabolism (Jump 2008; Di Minno et al. 2012). The main target nuclear receptors for this fatty acids are the peroxisome proliferator-activated receptors (Ppar α, β, γ1 and γ2), sterol regulatory element binding transcription factor 1 (Srebf1), retinoid X receptor alpha (Rxrα) and nuclear receptor, subfamily 1, group H, member 3 (Nr1h3), also known as liver X receptor alpha (Lxrα) (Jump et al. 2005; Jump 2008).

Ppara is the most abundant PPAR isotype in the liver (Pegorier et al. 2004), and when activated by PUFAs it enhances the expression of genes related to fatty acid oxidation (Jump 2008) such as carnitine palmitoyltransferase 1a (Cpt1a), which is the hepatic Cpt isoform that catalyzes the transport of long-chain acyl-CoA from cytoplasm to the mitochondrial matrix by the conversion of long-chain acyl-CoA to acylcarnitines in order to go through β-beta oxidation (Sadana et al. 2007). Acyl-CoA oxidase 1 (Acox1) is another enzyme that takes part in the catabolism of straight-chain acyl-CoAs through peroxisomal β-oxidation (Kassam et al. 2001), and acyl-CoA dehydrogenase very long chain (Acadvl) is a target gene also involved in the mitochondrial β-oxidation pathway (Spiekerkoetter and Wood 2010). The modulation of Ppara target gene transcription is due to the binding of the activated heterodimer Ppar/Rxr to a specific DNA sequence, the peroxisome proliferator responsive element (PPRE) (Olefsky 2001).

While PUFAs promote Ppar activation, enhancing fatty acid oxidation, they suppress Srebf1, inhibiting lipogenesis (Jump 2008). Studies demonstrated that a decrease in Srebf1 transcription leads to hepatic inhibition of critical enzyme for lipogenesis (Teran-Garcia et al. 2007), as fatty acid synthase (Fasn) and acetyl-CoA carboxylase alpha (Acaca) (Ronnebaum et al. 2008).

Some investigations have evaluated the effect of prenatal nutrition with PUFAs on metabolic pathways and gene regulation of lipid metabolism, and whether alterations could be transmitted between generations (Armitage et al. 2005; Burdge et al. 2007; Moon et al. 2013). Apart from previously established evidence that dietary PUFAs have an effect on the regulation of hepatic gene expression related to lipid metabolism, the multigenerational effect of the ω-3/ω-6 ratio during pregnancy on the offspring metabolism has not yet been established. Therefore, our objective was to evaluate the effect of diets with different ω-3/ω-6 ratio in successive generations on blood parameters and hepatic expression level of some nuclear receptors and target genes involved in lipid metabolism.

Table 1 Ingredient composition of the experimental diets (g/kg diet)

Ingredient	AIN-93G (g/kg diet)	
	H	C
Cornstarch	407.15	397.48
Casein	200.00	200.00
Dextrinized cornstarch	132.00	132.00
Sucrose	100.00	100.00
Soybean oil	0.00	70.00
Flaxseed oil	60.33	0.00
Fiber	50.00	50.00
Mineral mix	35.00	35.00
Vitamin mix	10.00	10.00
L-Cystine	3.00	3.00
Choline bitartrate	2.50	2.50
Tert-butylhydroquinone	0.014	0.014

Materials and methods

Animals, diets and experimental procedures

Male and female Wistar rats, 8 weeks old, were obtained from Central Vivarium/UFPel. Animals were housed individually in a temperature- (21–23 °C) and humidity-controlled (60–70 %) room with 12:12 h light–dark

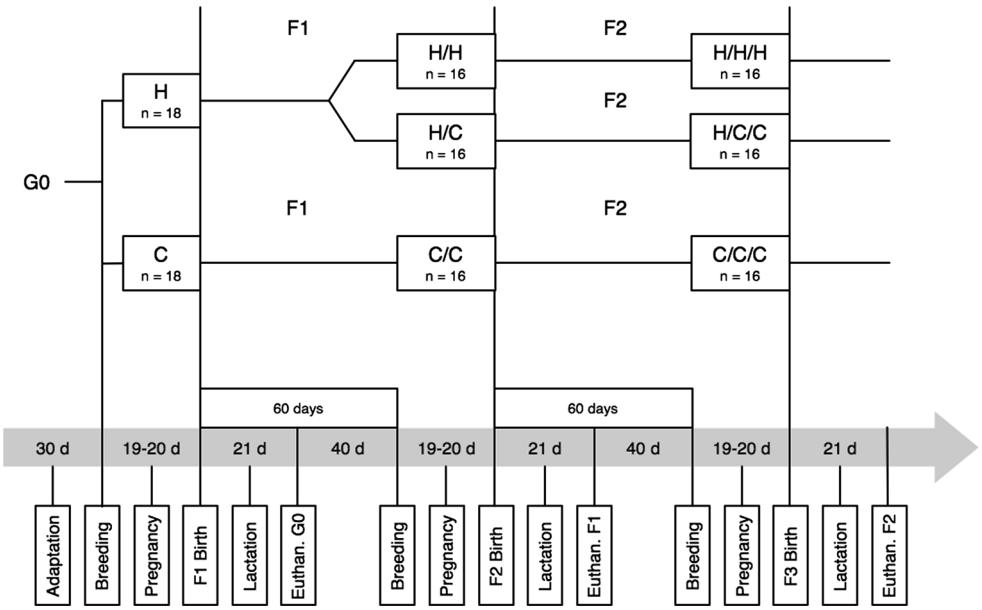

Fig. 1 Schematic design describing the division of the groups and all the experimental procedures in a chronological line

cycling (lights from 6 a.m. to 6 p.m.) and free access to a pelleted diet and water. The diets were elaborated in accordance with AIN-93G recommendations (AIN-93G for growth, pregnancy and lactation) (Reeves et al. 1993; Table 1). Food intake was recorded daily.

The founder generation (G0) was composed of thirty-six females that were randomly assigned to one of two groups: (1) rats fed with a diet elaborated with flaxseed oil, with a high ω-3/ω-6 ratio (2.44/1) (H Group, $n = 18$), and (2) rats fed with a control diet elaborated with soybean oil, with lower ω-3/ω-6 ratio (0.07/1) (C Group, $n = 18$). During the experiment, the males received only the control diet. The animals were acclimatized to housing and diets for 30 days. Shortly thereafter, the females were mated in a male:female ratio of 1:3 for 3 days. Dam weight was recorded weekly. The number and weight of offspring were recorded at birth, and offspring development was assessed by weighing the group of pups weekly. From the G0 offspring, female progeny were sorted at weaning (21 days) to compose the F1 generation and then divided into three groups: (1) females from the H group that continued receiving the diet with high ω-3/ω-6 ratio (H/H, $n = 16$); (2) females from the H group that began to receive the control diet (H/C, $n = 16$); and (3) females from the C group that continued to receive the diet with lower ω-3/ω-6 ratio (C/C, $n = 16$). Sixteen males were selected from the C group. The animals were fed the diets for 60 days and then were mated as described above. From the F1 offspring, female progeny were selected at weaning (21 days) to compose the F2 generation, and the diet groups were maintained: (1) H/H/H, $n = 16$; (2) H/C/C, $n = 16$; and (3) C/C/C, $n = 16$. To generate the F3 generation, we followed the same approach as above for the F2 generation. A schematic design of the experimental groups can be found in Fig. 1. All females were evaluated for pregnancy rate, number of pups per litter and average weight at birth.

Diet analysis

Every week a portion of the diet was randomly sampled for chemical analyses and fatty acid profile. The fat was isolated by the Bligh and Dyer method (Bligh and Dyer 1959) and the fatty acid profile analyzed by gas chromatography.

Blood sample and biochemical analysis

At the weaning day, females (with approximately 110 days old) were fasted for 12 h overnight, and then they were anaesthetized and euthanized according to the protocol approved by the University Animal Care and Use Committee. Blood was collected by intracardiac puncture in clean and EDTA-FK containing tubes. The samples were collected for measurement of plasma concentration of glucose, triacylglycerol (TAG) and non-esterified fatty acids (NEFA). Upon collection, samples were centrifuged at $3{,}000 \times g$ for 15 min. Plasma was harvested and stored at −80 °C until analyzed. The metabolites were analyzed using commercial biochemical assay kits, Glucose PAP Liquiform and Triacylglycerol (Labtest Diagnostica, Brazil) in a visible light spectrophotometer (Biospectro SP 220) and NEFA (Wako, USA) in a microplate reader (Thermo Plate Reader). All samples were analyzed in a single batch and intra-assay coefficient of variation was below 10 %.

Table 2 Primers sequence used for qRT-PCR, source and accession number at NCBI site

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Source	Accession no.
Ppara	TGGAGTCCACGCATGTGAAG	CGCCAGCTTTAGCCGAATAG	Somm et al. (2009)	NM_013196.1
Rxra	GGACACCAAACATTTCCTGCC	GATGTGCTTGGTGAAGGA	Gillio-Meina et al. (2009)	NM_012805.2
Lxra	AGGAGTGTCGACTTCGCAAA	CTCTTCTTGCCGCTTCAGTTT	Cha and Repa (2007)	NM_031627.2
Srebf1	GGAGCCATGGATTGCACATT	GGCCCGGGAAGTCACTGT	Cha and Repa (2007)	XM_213329.6
Acox1	GGCATGTAACCCGTAGCACT	GCCCAACTGTGACTTCCATT	Baarine et al. (2012)	NM_017340.2
Cpt1a	CTCCGCCTGAGCCATGAAG	CACCAGTGATGATGCCATTCT	Yang et al. (2011)	NM_031559.2
Acadvl	ATGACCCTGCCAAGAATGAC	TCCCAGGGTAACGCTAACAC	Buettner et al. (2006)	NM_012891.2
Fasn	CAGGTGTGTGATGGGAAGG	CCGTACACTCACTCGAGGC	Hansmannel et al. (2006)	NM_017332.1
Acaca	GCCATCCGGTTTGTTGTCA	GGATACCTGCAGTTTGAGCCA	Ronnebaum et al. (2008)	NM_022193.1
Ncor1	AGTCGCTACAGCCCAGAGTC	CTCCTCTCTGGGGATTTTCC	Malik et al. (2010)	XM_577103.4
Actb	TGTCACCAACTGGGACGATA	GGGGTGTTGAAGGTCTCAAA	Bonefeld et al. (2008)	NM_031144.3
Gapdh	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	Nelissen et al. (2010)	NM_017008.4
Rn18 s	ACGGACCAGAGCGAAAGCAT	TGTCAATCCTGTCCGTGTCC	Bonefeld et al. (2008)	NR_046237.1

Tissue collection, RNA isolation and qRT-PCR analyses

Six female per group, with 21 days postpartum, were euthanized in each of the three generations (G0, F1 and F2). Immediately after euthanasia the liver was collected, stored in cryotubes and snap-frozen by submerging in liquid nitrogen. Total RNA was isolated from the liver samples using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA was purified using RNeasy columns and on-column RNase-free DNase treatment (Qiagen, Germany), following the manufacturer's protocol. The absence of RNA degradation was checked on a 1 % Tris–borate-EDTA buffer/agarose gel analyzing 28S and 18S rRNA subunits, after electrophoresis in 80 V during 1.5 h. The purity and concentration were measured with an UV spectrophotometer (UV Mini 1240, Shimadzu). The A260/A280 ratio was used as an indication of RNA quality. Reverse transcription was carried out with 1 μg of RNA using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, USA) in a 10 μL volume. The reaction was performed in a thermo cycler (MyCycleTM Thermo Cycler, Bio Rad) using the following temperatures: 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min. The cDNA was then diluted to 5 ng/μL.

Real-time quantitative PCR using SYBR Green dye was used to evaluate Ppara, Rxra, Cpt1a, Acox1, Acadvl, Lxra, Srebf1, Fasn, Acaca and Ncor1 gene expression. Actin beta (Actb), Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and 18S ribosomal RNA (Rn18 s) were used as internal controls and the geometric mean calculated and used to normalize data. The primer sequences and references are shown in Table 2. qPCR was performed using 4 μL diluted cDNA, 5 μL of 1× SYBR Green master mix (Applied Biosystems, USA), 0.4 μL of each of 10 μM forward and reverse primers, and 0.2 μL of DNase- and RNase-free water in MicroAmpTM Optical 96-Well Reaction Plate (Applied Biosystems, USA). Each sample was run in triplicate, and a 6-point relative standard curve was generated using serial dilutions of cDNA prepared from liver samples to calculate efficiency of each primer pair. Each assay plate included a negative control in triplicate. The CV was below 10 % for all the primer pairs used. The reactions were performed in an ABI Prism 7500-Fast instrument (Applied Biosystems, USA) using the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C, a final extension for 5 min followed for a melting curve stage (15 s at 95 °C, 1 min at 60°c, 30 s at 95 °C and 15 s at 60 °C). mRNA abundance (A) was calculated as $A = 1/\text{efficiency}^{\Delta CT}$, where $\Delta CT = \text{sample CT} - \text{geometric mean of the 3 housekeeping genes}$ (Bionaz et al. 2012).

Statistical analyses

The results are presented as mean ± SEM. All the statistical analyses were performed using SAS (SAS Institute Inc, Cary, NC, USA). Data (mRNA abundance) were log transformed prior to statistical analysis. The pregnancy rate was compared among groups and generations by Chi-square. Diet consumption, diet fatty acid profile, number and weight of pups were compared by one-way ANOVA and Tukey test. For biochemical parameters and mRNA abundance the Mixed Model procedure was used, considering the effects of diet, generation, and its interactions. A value of $P < 0.05$ was considered statistically significant. To the groups H/C (F1) and H/C/C (F2), the H group (G0) was taken as the founder generation, as they were derived from it (see Fig. 1).

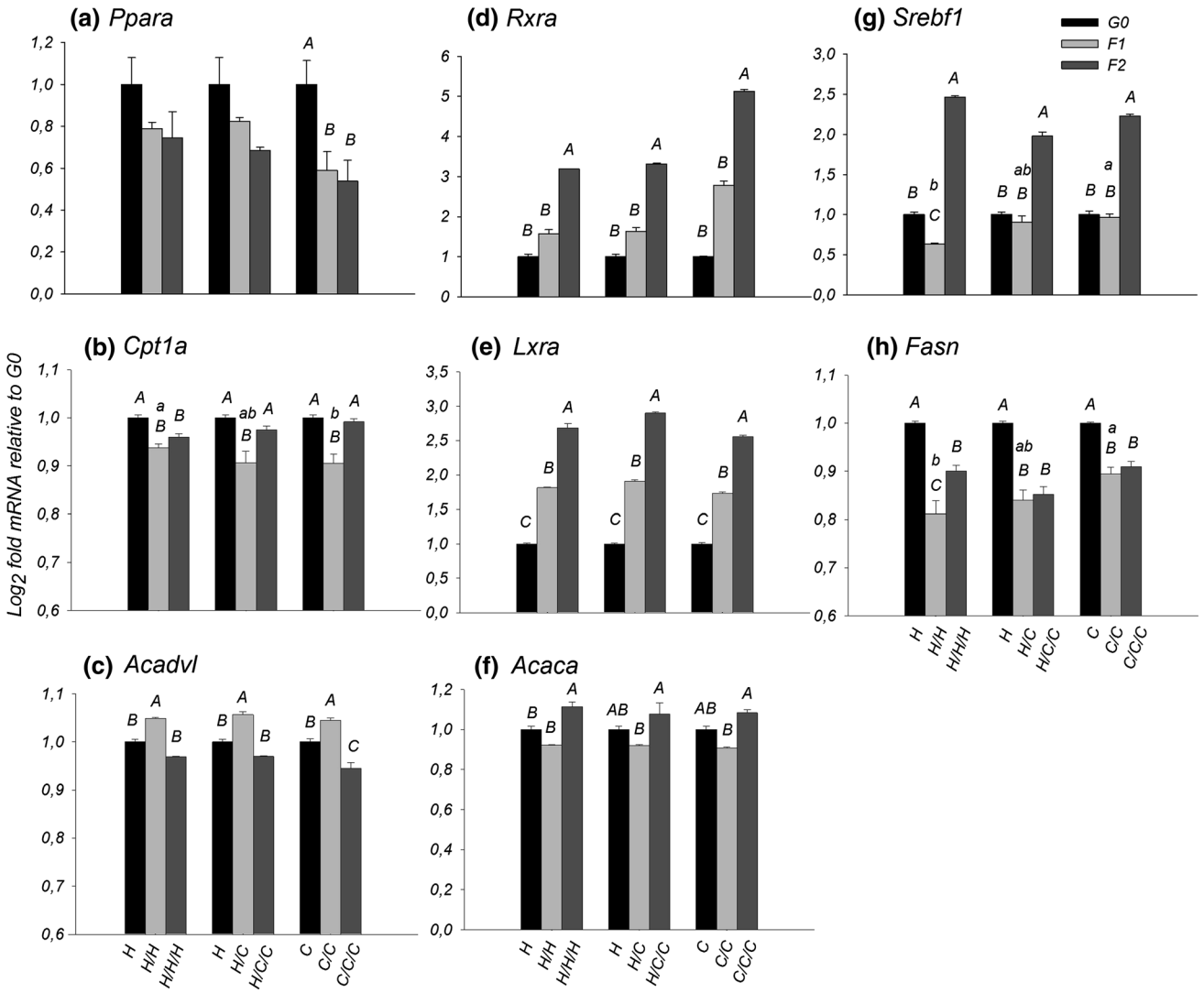

Fig. 2 mRNA fold change of the H, H/C and C groups, in F1 and F2 relative to the G0 for **a** Ppara, **b** Cpt1a, **c** Acadvl, **d** Rxra, **e** Lxra, **f** Acaca, **g** Srebf1, **h** Fasn. *Capital letters* indicate differences between generations in the same group ($P < 0.05$). *Lowercase letters* indicate differences between groups in the same generation ($P < 0.05$)

Results

Fatty acid oxidation genes

Diet did not elicit a difference in Ppara ($P > 0.05$) and Rxra expression ($P > 0.05$). Regarding Ppara target genes Cpt1a, Acadvl and Acox1, a diet effect was only observed in the F1 generation for Cpt1a, where the H/H group had greater expression ($P = 0.011$) than the C/C group (Fig. 2b).

The generational effect in Ppara expression was observed in the C group, with a reduction from G0 to F1 ($P = 0.05$) and G0 to F2 ($P = 0.03$) (Fig. 2a). For Rxra, all groups did not have difference from G0 to F1, but had a significant increase in the F2 expression, H (G0–F2: $P < 0.001$ and F1–F2: $P = 0.044$), H/C (G0–F2: $P < 0.001$ and F1–F2: $P = 0.009$) and C group (G0–F2: $P < 0.001$ and F1–F2: $P = 0.011$) (Fig. 2d). Along generations the expression of Acadvl was opposite to Cpt1a, while for Cpt1a there was a decrease from G0 to F1(H, $P = 0.003$; H/C, $P < 0.001$; C, $P < 0.001$) followed by an increase from F1 to F2 (H/C, $P < 0.001$; C, $P < 0.001$) (Fig. 2b), for Acadvl there was an increase from G0 to F1 (H, $P = 0.020$; H/C, $P = 0.003$; C, $P = 0.034$) and then a decrease from F1 to F2 ($P < 0.001$ for all groups) (Fig. 2c). No generational effect was observed in Acox1 expression ($P > 0.05$—see supplemental Fig. 1).

Lipogenic genes

A difference of Srebf1 mRNA expression between groups can be observed only in the F1 generation, where C/C group had a greater expression than the H/H group

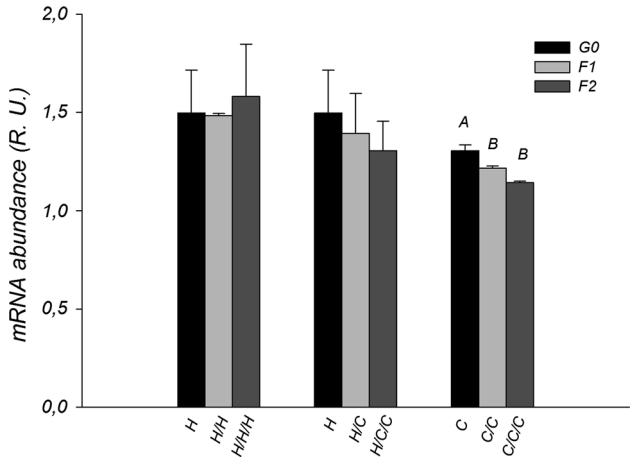

Fig. 3 NCor1 mRNA abundance (relative units) of the H, H/C and C groups along generations. *Capital letters* indicate differences between generations in the same group ($P < 0.05$)

($P = 0.019$) (Fig. 2g). Regarding the target genes, differences between groups were observed only for Fasn, in the F1 generation, where the C/C group had greater ($P = 0.006$) expression than the H/H group (Fig. 2h).

The generational effect could be observed in the increasing expression of Lxra in all groups, H (G0–F1: $P < 0.001$, G0–F2: $P < 0.001$ and F1–F2: $P = 0.023$), H/C (G0–F1: $P < 0.001$, G0–F2: $P < 0.001$ and F1–F2: $P = 0.001$) and C (G0–F1: $P < 0.001$, G0–F2: $P < 0.001$ and F1–F2: $P = 0.002$) (Fig. 2e). The hepatic expression of Srebf1 of the H group decreased between G0 to F1 ($P = 0.035$) followed by an increase between G0 to F2 ($P < 0.001$) and F1 to F2 ($P < 0.001$). The groups H/C and C had no differences between G0 to F1; however, the expression of Srebf1 increased significantly in F2 generation compared to F1 and G0 generations ($P < 0.001$ for all comparisons) (Fig. 2g).

For Fasn, all the groups had a very similar profile, a decrease between G0 to F1 ($P < 0.001$ for all groups) and also G0 to F2 (H, $P < 0.001$; H/C, $P < 0.001$; C, $P = 0.004$). Only in the H group expression increased ($P = 0.002$) from F1 to F2 (Fig. 2h). For Acaca, an increase (H, $P = 0.001$; H/C, $P = 0.002$; C, $P < 0.001$) was observed in all groups between F1 to F2 (Fig. 2i).

Nuclear co-repressor expression

No diet effect was observed in the regulation of NCor1 ($P > 0.05$). Along generations only the C group was affected, demonstrating a decrease in the expression between G0 and the other generations (F1: $P = 0.027$ and F2: $P < 0.001$) (Fig. 3).

Table 3 Fatty acid profile of the experimental diets (percentage of each fatty acid)

Fatty acids	Diets	
	H (flaxseed oil)	C (soybean oil)
ω-3:ω-6 ratio	2.44/1[b]	0.07/1[a]
Polyunsaturated	61.11 ± 0.742[a]	49.52 ± 0.791[b]
Linoleic ($C_{18:2}$)	17.75 ± 1.180[b]	45.99 ± 0.540[a]
α-Linolenic ($C_{18:3}$)	43.26 ± 1.849[a]	3.39 ± 0.384[b]
Eicosadienoic ($C_{20:2}$)	0.04 ± 0.005[b]	0.07 ± 0.013[a]
Eicosatrienoic ($C_{20:3}$)	0.04 ± 0.003	0.04 ± 0.010
Eicosapentaenoic ($C_{20:5}$)	0.02 ± 0.011	0.03 ± 0.021
Monounsaturated	20.68 ± 0.486[b]	27.74 ± 0.582[a]
Palmitoleic ($C_{16:1}$)	0.18 ± 0.003[a]	0.16 ± 0.004[b]
Cis-10-heptadecanoic ($C_{17:1}$)	0.03 ± 0.006	0.04 ± 0.006
Oleic ($C_{18:1}$)	20.33 ± 0.477[b]	27.14 ± 0.571[a]
Eicosenoic ($C_{20:1}$)	0.14 ± 0.022[b]	0.41 ± 0.061[a]
Saturated	14.87 ± 0.299[b]	17.83 ± 0.298[a]
Caproic ($C_{6:0}$)	0.08 ± 0.054	0.03 ± 0.009
Caprylic ($C_{8:0}$)	0.04 ± 0.015	0.02 ± 0.003
Capric ($C_{10:0}$)	0.06 ± 0.002[a]	0.05 ± 0.002[b]
Lauric ($C_{12:0}$)	0.06 ± 0.002[a]	0.05 ± 0.002[b]
Myristic ($C_{14:0}$)	0.37 ± 0.006[a]	0.33 ± 0.010[b]
Pentadecanoate ($C_{15:0}$)	0.08 ± 0.001[a]	0.06 ± 0.001[b]
Palmitic ($C_{16:0}$)	7.69 ± 0.185[b]	11.00 ± 0.060[a]
Heptadecanoic ($C_{17:0}$)	0.10 ± 0.001[b]	0.11 ± 0.001[a]
Stearic ($C_{18:0}$)	5.58 ± 0.123[a]	4.57 ± 0.162[b]
Arachidic ($C_{20:0}$)	0.40 ± 0.105[b]	0.80 ± 0.256[a]
Behenic ($C_{22:0}$)	0.24 ± 0.017[b]	0.56 ± 0.012[a]
Tricosanoic ($C_{23:0}$)	0.05 ± 0.002[b]	0.06 ± 0.007[a]
Lignoceric ($C_{24:0}$)	0.12 ± 0.015	0.16 ± 0.024
Heneicosanoic ($C_{21:0}$)	0.01 ± 0.002[b]	0.03 ± 0.003[a]

[a,b] Significative differences of fatty acid proportion between diets ($P < 0.05$)

Dietary fatty acid profile and intake

The ratio ω3/ω6 was 2.44/1 in the H diet and 0.07/1 in the C diet. Saturated and monosaturated fatty acid percentage was greater ($P < 0.05$) in the C diet, while the PUFAs was greater ($P < 0.05$) in the H diet. The α-linolenic acid (ALA) was almost 13 times greater in the H diet, as the linoleic acid (LA) was more than double in the C diet (Table 3). Every day the diet consumption was recorded, and we could confirm that the diet fatty acid profile did not affect food intake ($P > 0.05$). The mean daily food intake for G0 (adaptation period until weaning) was 20.0 ± 1.0 g for H group and 20.5 ± 1.0 g for the C group; the F1 (from its weaning until the F2 weaning) intake was 16.1 ± 0.8 g for the H/H group, 15.4 ± 0.8 g for the H/C group and 16.9 ± 0.7 g for the C/C group; and the F2 (from its weaning until the F3 weaning) was 18.0 ± 1.0 g for the

Table 4 Blood serum concentration (mmol/L) of glucose, triacylglycerol (TAG) and non-esterified fatty acids (NEFA)

Generation	Group	Glucose	TAG	NEFA
G0	H	129.15 ± 9.47	71.75 ± 7.03	0.325 ± 0.021
	C	138.75 ± 8.63	80.21 ± 13.39^{B}	0.343 ± 0.031^{B}
F1	H/H	128.68 ± 2.98	49.89 ± 16.02	0.327 ± 0.025
	H/C	119.96 ± 2.34	89.04 ± 21.79	0.268 ± 0.026
	C/C	130.85 ± 10.98	60.41 ± 11.78^{C}	0.258 ± 0035^{B}
F2	H/H/H	141.054 ± 4.51	57.93 ± 8.56^{b}	0.329 ± 0.035^{b}
	H/C/C	166.83 ± 12.11	86.80 ± 11.60^{ab}	0.220 ± 0.007^{c}
	C/C/C	144.18 ± 4.43	109.25 ± 16.01^{Aa}	0.417 ± 0.034^{Aa}

Capital letters indicate differences between generations in the same group ($P < 0.05$). Lowercase letters indicate differences between groups in the same generation ($P < 0.05$)

H/H/H group, 19.7 ± 0.7 g for the H/C/C group and 17.6 ± 0.9 g for the C/C/C group.

Blood metabolites

The blood biochemical analyses indicated that the fatty acid profile of the diet did not influence the glucose levels, as they were similar among the groups and generations, showing normoglycemic levels. The omega-3 rich diet decreased TAG in the F2 ($P = 0.04$) compared with the females that were receiving the C diet in F2 generation. A generation effect was observed for TAG concentrations in the C group, and there was a reduction comparing G0 to F1 ($P < 0.0001$) and an increase in F2 (G0–F2: $P < 0.01$, F1–F2: $P = 0.02$), while the other groups maintained constant levels. Regarding NEFA, differences between groups were also only observed in the F2 generation, where the C/C/C group had greater NEFA concentration than the H/H/H group ($P = 0.02$), which in turn had greater concentration than the H/C/C group ($P = 0.005$). We must highlight that although differences were observed between generations, the animals that received the H diet maintained a constant concentration of NEFA, while the C group had a decrease in concentration from G0 to F1 ($P = 0.04$) and increased from F1 to F2 ($P = 0.005$). These parameters are shown in Table 4.

Reproductive parameters and body weight

The pregnancy rate was similar between the groups throughout the generations (G0, F1 and F2—$P > 0.05$) and were in the acceptable range of 62.5–87.5 %. There was no effect of dietary treatment on the number of pups per litter ($P > 0.05$), but in the F1 generation the pup mean weight at birth was greater in the H/H group (7.33 ± 0.21 g) than the C/C group (6.55 ± 0.13 g) ($P = 0.01$). Considering the C group throughout the generations, we observed a reduction ($P = 0.01$) in the pup mean weight from G0 (7.39 ± 0.22 g) to F1 (6.55 ± 0.13 g) that was maintained in the F2 (6.88 ± 0.41 g).

Discussion

Over the last three decades, research on fatty acid metabolism, specially PUFAs, have exposed the benefits of these molecules on normal health and chronic diseases, through the regulation of lipid metabolism (Soulimane-Mokhtari et al. 2005; Kagohashi et al. 2010; Makni et al. 2011), cardiovascular (Adkins and Kelley 2010; Tousoulis et al. 2014; de Oliveira Otto et al. 2013) and immune function (Tapia et al. 2014; Yates et al. 2014; Liu et al. 2013). All fetal supply of ω-3 and ω-6 comes from the mother, crossing the placenta, either in the form of the essential fatty acid, or their long-chain PUFAs derivatives (Herrera 2002). The experimental diets offered to the animals in this study contained high amounts of PUFAs, almost 50 % in the C diet, and more than 60 % in the H diet. The consumption of food was not affected by the fatty acid profile in accordance with Rice and Corwin (Rice and Corwin 2002). The essential fatty acids offered through the diet were converted to other metabolites and incorporated in the liver in different proportions. The H group had more ALA and its metabolites, eicosapentaenoic acid (EPA) and docosapentaenoic acid (DHA) than the C group; while the animals that received the C diet had greater concentrations of LA and its metabolite arachidonic acid (AA) (data not shown), indicating activity of desaturases and elongases.

Studies demonstrated the existence of an interrelationship between Ppars, Srebf1 and Lxr. More than 10 years ago, in vitro and in vivo experiments proved that PUFAs suppressed Srebf1 activity by inhibition the Lxr/Rxr binding to LXREs (Yoshikawa et al. 2002, 2003). In the present study, only the C group decreased Ppara mRNA abundance from G0 to F1 and F2 generations, the other groups maintained their expression levels (Fig. 2a). In

contrast, the expression of Srebf1 increased from G0 through F2 regardless of experimental group. A similar profile was observed for Lxra and Rxra expression, concerning with the studies cited above, providing evidence of the regulation of Srebf1 via Lxra activation. As the level of Ppara did not increase significantly, and Srebf1 did not decrease with a high PUFA diet, we suggest the existence of a post-transcriptional regulatory mechanism controlling fatty acid oxidation and lipogenic function potentially through proteolytic processing and autoloop regulatory circuit (Takeuchi et al. 2010).

In the liver, Ppara activation represses Srebf1 expression (Fernandez-Alvarez et al. 2011) and the decrease in Ppara coupled with the increase in Srebf1 expression across generations seems to support the mechanistic regulation between these transcription factors. Other factors, such as mammalian target of rapamycin complex 1 (mTOC1) (Lewis et al. 2011; Bakan and Laplante 2012; Takashima et al. 2009), protein kinase Akt (Yecies et al. 2011), fibroblast growth factor 21 (FGF21) (Zhang et al. 2011), S6 kinase 1 (S6K1) (Li et al. 2011) and adiponectin (Awazawa et al. 2009) could regulate Srebf1 transcription. Although the insulin response was not evaluated in this study, a higher insulin sensitivity across generations could be acting on the activation of Srebf1 promoter, primarily by increasing the activity of Lxrs or their heterodimerizing partner Rxrs (Chen et al. 2004). In accordance with this, a recent study demonstrated the increase in insulin sensitivity in the second generation of rats exposed to diets with high PUFAs content (Hirabara et al. 2013). The Rxra is intimately linked with Ppara, since its heterodimerization is necessary to activate and to promote the binding to PPRE in target gene promoters (Pegorier et al. 2004), and its increase during the experimental period might have been a response to Lxra and Srebf1.

The observed reduction in Fasn expression over the three generations, even with enhanced Srebf1 expression, seems opposite to the well-known effect of Srebf1 on Fasn expression (Teran-Garcia et al. 2007), suggesting that other factors may be regulating Fasn. One study with canola oil as PUFA source did not report a suppression of lipogenic gene expression, suggesting that diet did not induce liver lipid catabolism nor suppressed lipid synthesis, while a diet with highly unsaturated fatty acids from a fish/fungal source strongly suppressed both pathways (Sealls et al. 2008). Other factors such as growth hormone (GH), prolactin (PRL), STAT5, glucagon and insulin may also participate in the regulation of Fasn expression (Hogan and Stephens 2005; Moon et al. 2002).

Another interesting finding of this study was the opposite behavior of the Cpt1a and Acadvl genes. We speculate that the decrease from G0 to F1 observed in Cpt1a expression, when there was an increase at the same time point in Acadvl, was a cellular mechanism of regulation in the equilibrium between fatty acid transfer from cytoplasm to the mitochondria and β-oxidation, a manner to increase fatty acid entrance to supply the reduction in fatty acid oxidation. Related to the expression control exerted by co-repressors, PPAR and the evaluated target genes must be under influence of others main co-regulators, as SRC-1 (Dasgupta et al. 2014) and SMRT (Watson et al. 2012), since NCor1 expression level was almost constant. As what was observed with Acox1, a lack of diet and/generational effect on the regulation of their expression.

DNA methylation is the major epigenetic modification that controls gene expression in physiologic and pathologic states (Barres and Zierath 2011). As reviewed by Holness et al., nutrients can cause acute and long-term modifications at the gene expression level and lead to epigenetic modifications (Holness et al. 2010) and a recent study has already indicated epigenetic regulation induced by ALA (Niculescu et al. 2013). In this context, the dietary PUFA may act at the "end" of the process, or by controlling the activity of the nuclear receptors, not their abundance. This phenomenon must be evaluated in the future, so the observed changes in mRNA abundance from this study can be better understood.

The experimental diets did not alter the plasma glucose concentration, in accordance with other recent studies and indicating that chronic feeding of diets rich in PUFAs do not alter the glucose metabolism and its plasma concentration (Rice and Corwin 2002; Mellouk et al. 2012). It has already been shown that feeding animals with ω-3 and ω-6 diets is effective in lowering plasma triacylglycerol levels (de Assis et al. 2012; Harris 1989), as we observed in this experiment. We must highlight the apparent regulation of gene expression and blood metabolites across generations induced in the C group, indicating that diets with a high concentration of PUFAs, independently of the ω-3/ω-6 ratio, can induce metabolic regulation across generations.

About the reproductive parameters, the lack of difference in pregnancy rate between the groups and generations suggests that the ratio between ω-6 and ω-3 did not influence female fertility, similar to a study feeding flaxseed (Leal Soares et al. 2010). The greater pup mean weight of the F1 generation in the group fed with the omega-3 rich diet than controls, which were fed with the omega-6 rich diet, demonstrates that maternal consumption of omega-3 PUFAs during pregnancy had an effect on fetal and offspring growth and development. The reduction in pup mean weight in the control group between G0 to F1, and its maintenance in F2, indicated that more attention should be given to the ω-3/ω-6 ratio during the perinatal period, especially to ω-3 excess and its deleterious effect (Church et al. 2008). Again these results suggest the effects of maternal diet on offspring development.

In conclusion, feeding animals with PUFAs allowed for an initial study of the transgenerational control of hepatic lipogenesis and metabolism. There is clear evidence of cumulative effects throughout generations, independent of the PUFAs source, at least in part via up and down regulation of lipid metabolism-related genes.

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Conflict of interest Carolina B. Jacometo, Eduardo Schmitt, Luiz F. M. Pfeifer, Augusto Schneider, Francielle Bado, Fernanda T. da Rosa, Simone Halfen, Francisco A. B. Del Pino, Juan J. Loor, Marcio N. Corrêa and Nelson J. L. Dionello declare that they have no conflict of interest.

Ethical standard All institutional and national guidelines for care and use of laboratory animals were followed and the experimental protocol was approved by the Animal Welfare Committee from Federal University of Pelotas (Permit number: 0976).

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