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Dietary fish oil and flaxseed for rabbit does: fatty acids distribution and Δ6-desaturase enzyme expression of different tissues

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Standard feeds are imbalanced in term of n-6/n-3 polyunsaturated fatty acids (PUFA) ratio, with a low proportion of the latter. The reproductive system appears to be strongly affected by administration of n-3 PUFA, and ingredients rich in α-linolenic acid (ALA; i.e. vegetable sources) or EPA and DHA acids (i.e. fish oil) can be included in animal diets to balance PUFA intake. The aim of this study was to evaluate the effect of dietary supplementation with flaxseed (ALA) or fish oil (EPA and DHA) on PUFA metabolism in rabbit does. A total of 60 New Zealand White female rabbits were assigned to three experimental groups: control group, FLAX group fed 10% extruded flaxseed and FISH group fed 3% fish oil. Blood, milk, liver and ovaries were collected from the does to assess the lipid composition; furthermore, FADS2 *gene expression was assessed in liver and ovary tissues. Reproductive performance of does was also recorded. The fertility rate and number of weaned rabbits improved with n-3 dietary supplementation: does at first parity showed the lowest reproductive results, but the administration of n-3 reduced the gap between primiparous and multiparous does. Feed consumption and milk production were not affected by the feeding regime. The fatty acid composition of milk, plasma, liver and ovaries were widely influenced by diet, showing higher concentrations of n-3 long-chain PUFA (LCP) in does fed with n-3 enriched diets. FISH diet resulted in the highest n-3 LCP enrichment, whereas in the FLAX group, this increase was lower. Blood and milk showed low levels of LCP, whereas liver and ovaries were the main sites of n-3 LCP synthesis and accumulation. Accordingly, although the liver is the main metabolic centre for LCP synthesis, ovaries also have a prominent role in LCP generation.* FADS2 *expression in liver and ovary tissue was downregulated by FISH administration. In conclusion, the enrichment of diets with n-3 PUFA could be an effective strategy for improving the reproductive performance of does.*

Keywords: rabbit, n-3 long-chain fatty acids, *FADS2*, liver, ovary

Implications

The role of n-3 fatty acids is relevant for many physiology activities; however, well-defined dietary recommendations for animals are not yet provided. Dietary n-3 could be supplied by precursors (e.g. vegetal source of α-linolenic acid (ALA)) and/or by long-chain polyunsaturated fatty acids (LCP) (fish oil rich in EPA, dosapentaenoic acid (DPA) and DHA). This paper clearly shows that the enrichment of diets with n-3 PUFA (both as precursor or LCP), improves the reproductive performance of rabbit does mainly during critical phase of production (primiparous). This better performance is obtained without hormonal treatment or intensification of reproductive rhythm, thus consistent with the general principles of animal welfare.

Introduction

The extent of linoleic acid (LA, C18:2n-6) and ALA (C18:3n-3) conversion into LCP (⩾20 carbons) greatly depends on expression and activity of desaturase and elongase enzymes, which are the same for both n-3 and n-6 fatty acid series (Barceló-Coblijn and Murphy, 2009; Gregory *et al.*, 2011). Elongase (2 and 5) lengthens the carbon chain, whereas delta 6- (Δ6d) and delta 5-desaturase (Δ5d) introduce double bonds and are encoded by *FADS2* and *FADS1* genes, respectively (Nakamura and Nara, 2004). The Δ6d is considered to be the rate-limiting enzyme in the synthesis of

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arachidonic acid (AA; 20:4n-6), EPA (20:5n-3) and DHA (22:6 n-3) acids from their precursors (Cho *et al.*, 1999). The ability to convert precursors into LCP also depends on animal species (Dal Bosco *et al.*, 2004; Oliveira *et al.*, 2010) and, within the same species, it is affected by sex, hormonal status, intestinal biota, genetic strain and feed (De Meester *et al.*, 2012).

Several studies have demonstrated that many animal species are rather poor EPA and DHA synthesisers (Muskiet *et al.*, 2004), despite LCP being especially abundant in the brain, retina, reproductive tissues and gametes and having a relevant role in many physiological pathways and pathological disorders, such as cardiovascular disease, depression and reproductive dysfunction (Simopoulos, 1991). In particular, high levels of dietary n-3 LCP during critical periods, that is, fecundation and the first phase of pregnancy, has positive effects on fertilisation and early embryo development, probably by modulating the main hormonal pathways (Diskin and Morris, 2008). In rabbit does, Rebollar *et al.* (2014) showed that dietary enrichment with n-3 LCP improves the endocrine function of the corpora lutea, increasing weight and size of kits and reducing mortality rate. The same dietary protocol increased plasma leptin and oestradiol in primiparous does (Rodríguez *et al.*, 2017), implying better body condition and sexual receptivity.

Standard feeds are imbalanced in terms of the n-6/n-3 ratio, with a low proportion of the latter (De Meester *et al.*, 2012). Polyunsaturated fatty acid are relevant constituent of the reproductive tissues, then the fatty acid profile appear to be strongly affected by ingredients rich in ALA (vegetables sources), EPA and DHA (fish oil), that can be included in animal diets to balance dietary PUFA (Maranesi *et al.*, 2015). However, the ALA effect has been poorly investigated (Rosero *et al.*, 2016), and no results are available on the rabbit reproductive system. Moreover, it is supposed that the LCP synthesis (including enzyme activity, messenger RNA (mRNA) expression) is tissue-specific (Broughton *et al.*, 2010) and much of their activity depends on the metabolic destination (e.g. liver, reproductive organs). Based on these findings, this study analysed the effect of n-3 fatty acids (provided by flaxseed or fish oil dietary supplementation) in rabbit does and, in particular, their distribution in milk, plasma, liver and ovaries, and the expression of Δ6 desaturase in liver and ovarian tissue.

Material and methods

Materials

Deoxyribonuclease I (DNAase I Amp. Grade), Superscript III Reverse Transcriptase (Superscript III First-Strand Synthesis System) and DNA ladders were purchased from Life Technologies Italia (Monza, Monza Brianza, Italy). Reagents for the isolation and purification of total RNA (TRIzol), Taq DNA polymerase (Platinum), RNAse-free tubes, deionised water, deoxyNTPs, primers for 18S RNA, corresponding competimers (QuantumRNA 18S Internal Standards) and *FADS2* were also acquired from Life Technologies. NucleoSpin Gel and PCR cleanup were from Macherey-Nagel Inc. (Bethlehem, PA, USA). Chloroform, methanol and ethanol were purchased from Carlo Erba (Milan, Italy).

Polyunsaturated fatty acid-2 fatty acid methyl ester standards were purchased by Matreya (Pleasant Gap, PA, USA). Enicosenoic acid methyl esters (C21:0) was purchased by Sigma-Chemical Co. (St. Louis, MO, USA).

Animals and diets

The experimental protocol for this trial was carried out according to the Spanish Policy for Animal Protection D53/2013 and assessed and approved by the Animal Ethics Committee of the Community of Madrid (Ref. PROEX 302/15). Sixty nulliparous New Zealand White does were followed for four consecutive reproductive cycles and the performance traits were recorded. Rabbits were housed in the experimental farm of the Polytechnic University of Madrid. Temperature in the building was in the range 18°C to 23°C, air circulation (15 air changes/h) and humidity ($50 \pm 5\%$); these conditions were maintained throughout the trial. Each treatment group consisted of 20 rabbits with each group receiving a different diet (Table 1) as follows:

- control group was fed *ad libitum* with the control diet containing soya bean oil as the main source of fat;
- FLAX group was fed the control diet, substituting 10% of the soya bean oil with extruded flaxseed;
- FISH group was fed the control diet, substituting 3% of the soya bean oil with fish oil (NORDIC NATURALS omega-3®, Watsonville, CA, USA).

The dietary formulation was established according to previous experiments (Dal Bosco *et al.*, 2004; Rebollar *et al.*, 2009 and 2014) and in respect of rabbit requirements. To avoid PUFA peroxidation, all diets were supplemented with 200 mg/kg α-tocopheryl-acetate.

Sexual receptivity was recorded at artificial insemination (AI) by examining the vulva (Castellini and Lattaioli, 1999); in particular, does with a red and turgescent vulva were considered to be sexually receptive. No oestrus synchronisation was performed and the reproductive rhythm was 42 days, with AI taking place at 11 days after kindling. Ovulation was induced by intravaginal inoculation with 10 μg of GnRH (Receptal®, Intervet International B.V., AN Boxmeer, Netherlands). Non-pregnant does were re-inseminated 21 days after the previous AI, according to the dual band management system. On the day of AI, all does were submitted to ultrasound scanning (ALOKA model SSD-500; Hitachi Europe, Milano, Italy) of the perirenal area (close to the 2nd to 3rd lumbar vertebrae) after shaving the area with a hair trimmer. Scapular fat thickness was measured on the left and right side. Perirenal fat (g) was estimated using a regression curve, as reported in a previous paper (Dal Bosco *et al.*, 2003). The number of live-born pups and weaned rabbits were also registered, and no adoption was performed. Feed intake of

Table 1 *Formulation, chemical composition (g/kg) and nutritional value of rabbit diets*

Ingredients	Diets[1]		
	C	FLAX	FISH
Barley meal	290	240	270
Wheat bran	125	125	125
Dehydrated alfalfa meal	400	400	400
Soya bean meal 44% CP	130	90	130
Soya bean oil	10	–	–
Extruded linseed	–	100	–
NORDIC NATURALS omega-3®[2]	–	–	30
Beet molasse	10	10	10
Calcium diphosphate	10	10	10
Vitamin–mineral premix[3]	12	12	12
Limestone	7	7	7
Salt	6	6	6
DL-methionine	0.1	0.1	0.1
CP	173.1	174.4	172.1
Ether extract	30.3	56.0	39.9
Crude fibre	144.0	145.8	144.4
Ash	82.1	87.8	85.9
NDF	283.7	283.4	279.1
ADF	170.4	178.6	170.0
ADL	35.8	40.9	40.3
Estimated digestible energy (MJ/kg)[4]	10.39	10.57	10.50

[1]C = control diet containing soya bean oil as the main source of fat; FLAX = control diet substituting 10% of the soya bean oil with extruded flaxseed; FISH = control diet, substituting 3% of the soya bean oil with fish oil (NORDIC NATURALS omega-3®).
[2]NORDIC NATURALS omega-3® = purified deep sea fish oil (from anchovies and sardines) containing EPA 330 mg/100 g, DHA 220 mg/100 g, other n-3 long-chain polyunsaturated fatty acids 140 mg/100 g + α-tocopherol for preservation.
[3]Amount per kilogram: vitamin A 11.000 IU; vitamin D_3 2.000 IU; vitamin B_1 2.5 mg; vitamin B_2 4 mg; vitamin B_6 1.25 mg; vitamin B_{12} 0.01 mg; vitamin E 200 mg; biotine 0.06 mg; vitamin K 2.5 mg; niacine 15 mg; folic acid 0.30 mg; D-pantotenic acid 10 mg; coline 600 mg; Mn 60 mg; Cu 3 mg; Fe 50 mg; Zn 15 mg; I 0.5 mg; Co 0.5 mg.
[4]According to Maertens *et al.* (1988).

pups (until weaning) and pre-weaning mortality and milk production (difference in weight of does before and after lactation) was evaluated daily; whereas the litter weight was recorded weekly. After 21 days of lactation, milk samples were manually collected from the mothers, using a moulded glass tube, after an injection of 2 IU of oxytocin (Schley, 1975).

Blood samples were taken from the ear-vein and stored in tubes contained Na_2-EDTA; plasma was obtained after centrifugation at 3000 × **g** for 15 min. At the end of the experiment, 10 rabbit does ($n = 10$/group) were sacrificed in accordance with the guidelines and principles on the care and use of research animals; specifically, animals were euthanised with an overdose of barbiturate (30 mg/kg of pentothal; Dolethal, Lab, Vetoquinol, Madrid, Spain). The ovaries and the liver were then promptly removed and thoroughly washed with saline. Within a few minutes, tissue samples were cut into appropriately sized pieces (30 mg) and either rinsed with RNAse-free water and frozen at −80°C for subsequent evaluation of gene expression (Maranesi *et al.*, 2015) or stored at −20°C for subsequent analysis of moisture, protein, total lipid, ash and fatty acid composition.

Chemical analysis and fatty acid profile

The chemical composition of the diets was determined in triplicate, according to the methods of the Association of Official Analytical Chemists (1995). Lipid extraction of feed, milk, plasma, liver and ovary tissues was performed according to Folch *et al.* (1957) and esterification was performed according to Christie (1982). The trans-metilation procedure was conducted using enicosenoic acid methyl esters (Sigma Chemical Co.) as the internal standard. The recovery rates of internal standard were 92 ± 3%, 98 ± 2%, 89 ± 4%, 75 ± 3% and 63 ± 2% in feed, milk, plasma, liver and ovary tissues, respectively. The fatty acid composition was determined using a Varian gas-chromatograph (CP-3800) equipped with a flame ionisation detector and a capillary column of 100 m length × 0.25 mm × 0.2 μm film (Supelco, Bellefonte, PA, USA). Helium was used as the carrier gas with a flow of 2 ml/min. The split ratio was 1 : 80. The oven temperature was programmed at 40°C and held for 1 min, then increased up to 163°C at a rate of 2°C/min, held for 10 min, increased up to 180°C at a rate of 1.5°C/min, held for 7 min, increased up to 187°C at a rate of 2°C/min held for 2 min, and then increased up to 230°C at a rate of 3°C/min and held for 25 min. The injector and detector temperatures were set at 270°C and 300°C, respectively. Individual FAME were identified by comparing the relative retention times of peaks in the sample with those of standard mixture (FAME Mix Supelco; 4 : 0 to 24 : 0) plus *cis*-9, *cis*-12 C18:2; *cis*-9 *cis*-12 *cis*-15 C18:3; *cis*-9 *cis*-12 *cis*-15 C18:3 (all from Sigma-Aldrich). Fatty acids were expressed as % of total fatty acids. The average amount of each fatty acid was used to calculate the sum of the total saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and PUFA.

RNA extraction and expression analysis

Total RNA was extracted from ovary and liver samples from each rabbit ($n = 5$) as described by Maranesi *et al.* (2015). Five micrograms of total RNA were reverse transcribed in 20 μl of Superscript III First-Strand Synthesis System using random hexamer according to the protocol provided by the manufacturer. Genomic DNA contamination was checked by developing the PCR without reverse transcriptase. The multiplex PCR amplification was performed as previously described (Maranesi *et al.*, 2015) with the use of 1 μl of complementary DNA as a template for *FADS2* primers (Supplementary Table S1).

Cycling conditions consisted of an initial denaturising cycle at 94°C for 75 s, followed by 30 cycles for each target gene at 94°C for 15 s, 60°C for 30 s, 72°C for 45 s and a final extension step at 72°C for 10 min. Within each experiment, the complete set of samples was processed in parallel in two different PCR (2 samples/group in the first, 3 samples/group in the second), using aliquots of the same PCR master mix. The amplified PCR-generated products (18 of 25 μl total reaction volume) were analysed by electrophoresis on 2%

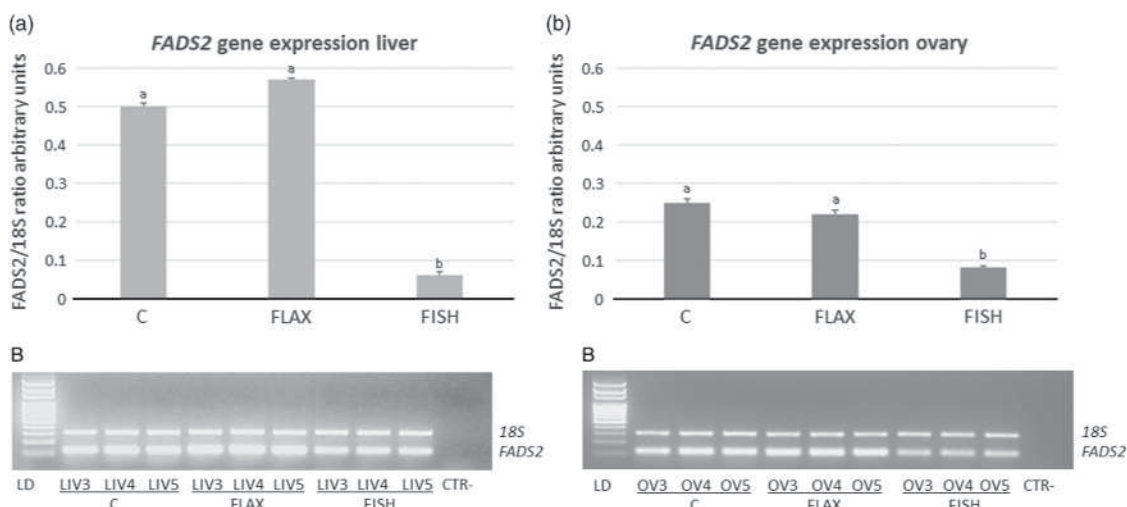

Figure 1 Expression of *FADS2* messenger RNAs (mRNAs) in liver (a) and ovary (b) collected from rabbits belonging to control (C), flaxseed (FLAX) and fish oil (FISH) groups. The lower panels (B) show a representative photograph of a 2% agarose ethidium bromide-stained gel used to analyse the PCR products (three of the five animals per group). The sizes of the amplified products are shown on the right of the gel. LD is the 100-bp DNA ladder and CTR− is the negative control. The other lanes identify the corresponding organs: LIV = liver, OV = ovary. The upper panels (a) shows the data derived from densitometric analyses of the gels. For each organ, the values (means ± SD) combine the results from five different rabbits and are reported in arbitrary units of *FADS2* mRNA relative to that of 18S used as internal standard. [a,b]Different letters above the bars indicate significantly different values ($P < 0.01$).

agarose gel using ethidium bromide staining (Figure 1a and b).

Analysis of amplification products was carried out as reported elsewhere (Maranesi *et al*., 2015). The amount of the *FADS2* mRNA was expressed in arbitrary units as the ratio of each PCR product for target gene normalised against the 18S coamplified product.

The amplified products, collected from the agarose gel after electrophoresis, were purified with NucleoSpin Gel and PCR cleanup, and their identity confirmed by DNA sequencing with Sanger's method.

Statistical analysis

Statistical analysis of reproductive data (sexual receptivity, live born, weaned rabbits, litter weight, milk production, feed intake, perirenal fat) was performed using a mixed model (SAS, 1990) with the fixed effect of parity, dietary group (Control, FLAX and FISH) and the interaction (parity × dietary group). According to preliminary analysis, the parity was grouped into two classes (primiparous: $n = 1$; and pluriparous: $2 \leqslant n \leqslant 4$). The same effects (parity and diet) on fertility and mortality rate (categorical data) was analysed by means a χ^2 test (proc CATMOD). The chemical composition of milk, the fatty acids profile of feed, milk and tissues (plasma, liver and ovaries) and gene expression were analysed at the end of experiment with a one-way linear model (ANOVA) and the significance was assessed by Bonferroni's *t*-test. Data are reported as Least Square mean and the level of statistical significance was set at $P \leqslant 0.05$.

Results

The fatty acid composition of diets (Table 2) showed lower SFA, MUFA and n-6 PUFA (C18:2 n-6) content in the n-3 enriched groups. Both n-3 enriched diets showed higher total n-3 fatty acid values compared to the control diet (30.89% and 32.69% *v.* 22.50% in FLAX and FISH *v.* Control groups, respectively); accordingly, the n-3/n-6 ratio was also higher.

Compared to the control diet, flaxseed supplementation mainly increased ALA (31.08% *v.* 22.19%) and the n-3/n-6

Table 2 *Main fatty acids (% of total fatty acids) of experimental diets administered to the rabbits*

	Diets[1]				
Fatty acids	C	FLAX	FISH	DSE	*P* value
SFA	19.27[b]	18.01[a]	18.70[ab]	1.95	0.042
MUFA	18.11[b]	16.74[a]	17.95[b]	1.52	0.034
C18:2cis n-6, LA	39.03[b]	33.47[a]	32.28[a]	2.41	0.013
C18:3 n-3, ALA	22.19[a]	31.08[c]	27.18[b]	1.97	0.029
C20:3n-6	0.14	0.05	0.04	0.02	0.564
C20:4n-6, AA	0.20	0.10	0.11	0.01	0.657
C20:5n-3, EPA	0.18[a]	0.20[a]	1.01[b]	0.07	0.026
C22:5n-3, DPA	0.02[a]	0.08[a]	0.34[b]	0.02	0.033
C22:6n-3, DHA	0.11[a]	0.09[a]	2.16[b]	0.08	0.012
PUFA	61.89[a]	65.09[b]	63.23[ab]	4.55	0.047
LCPn-3	0.31[a]	0.37[a]	3.51[b]	0.94	0.028
Σn-3	22.50[a]	30.89[b]	32.69[b]	2.41	0.034
Σn-6	39.27[b]	33.62[a]	32.43[a]	3.05	0.035
n-3/n-6	0.57[a]	0.93[b]	0.95[b]	0.08	0.016

DSE = difference of standard error; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; LA = linolenic acid; ALA = α-linolenic acid; AA = arachidonic acid; DPA = docosapentaenoic acid; PUFA = polyunsaturated fatty acids; LCP = long-chain polyunsaturated fatty acids (⩾20C).
Each value is the mean of three replicates.
[1]C = control diet containing soya bean oil as the main source of fat; FLAX = control diet substituting 10% of the soya bean oil with extruded flaxseed; FISH = control diet, substituting 3% of the soya bean oil with fish oil (NORDIC NATURALS omega-3®).
[a,b,c]Means in the same row with different letters differ significantly ($P < 0.05$).

Table 3 *Effect of different dietary supplementations on productive and reproductive performance of does and mortality of young rabbits (*n *= 20/ group)*

Diet[1]		C		FLAX		FISH			
Parity		I	II to IV	I	II to IV	I	II to IV	Pooled SE (χ^2)	*P* value
Sexual receptivity	%	63.0[a]	75.0[b]	75.0[b]	85.0[c]	70.0[ab]	84.0[c]	6.3	0.024
Fertility rate	%	57.7[a]	68.9[b]	73.3[b]	84.4[c]	73.3[b]	80.0[bc]	*8.4	0.034
Live born	N	8.9[a]	10.2[b]	9.3[a]	10.8[b]	9.2[a]	10.8[b]	0.7	0.045
Weaned rabbits	N	7.6[a]	8.6[a]	7.9[a]	8.9[b]	7.9[a]	8.9[b]	0.6	0.012
Mortality rate	%	14.6	15.7	15.0	18.0	14.1	17.5	*2.2	0.678
Litter weight	g	4 575[a]	5 454[b]	4 962[a]	5 939[b]	5 001[a]	6 000[b]	371	0.048
Milk production	g/day	167[a]	205[b]	169[a]	210[b]	172[a]	218[b]	14.8	0.021
Feed intake (0 to 30 days)	g/day	246[a]	340[b]	256[a]	345[b]	260[a]	338[b]	11.6	0.019
Estimated perirenal fat	g/doe	19.8[a]	48.7[b]	20.2[a]	51.0[b]	21.1[a]	52.8[b]	1.2	0.023

[1]C = control diet containing soya bean oil as the main source of fat; FLAX = control diet substituting 10% of the soya bean oil with extruded flaxseed; FISH = control diet, substituting 3% of the soya bean oil with fish oil (NORDIC NATURALS omega-3®).
χ^2 value for non-parametric traits, where $P < 0.05$.
[a,b,c]Values within a row with different superscripts differ significantly at $P < 0.05$.

Table 4 *Effect of different dietary supplementations on fatty acid composition of blood plasma (% of total fatty acids) in rabbit does (*n *= 10/group)*

	Diets[1]				
Fatty acids	C	FLAX	FISH	Pooled SE	*P* value
SFA	52.76	52.63	52.20	3.76	0.765
MUFA	20.45	20.22	19.83	2.59	0.832
C18:2n-6	19.58	18.45	17.02	1.05	0.654
C18:4 n-6	0.25	0.23	0.19	0.03	0.067
C20:4n-6, AA	4.09[b]	3.24[a]	3.18[a]	0.30	0.034
C22:5n-6	0.16	0.23	1.11	0.08	0.065
C18:3 n-3, ALA	2.05[a]	3.86[b]	2.63[a]	0.58	0.024
C20:5n-3, EPA	0.11[a]	0.20[a]	1.24[b]	0.09	0.023
C22:5n-3, DPA	0.19[a]	0.22[a]	0.71[b]	0.04	0.045
C22:6n-3, DHA	0.32[a]	0.34[a]	1.81[b]	0.02	0.035
PUFA	26.79	27.15	27.97	2.02	0.765
Σn-3	2.67[a]	4.58[b]	6.39[c]	1.98	0.041
LCPn-3	0.62[a]	0.72[a]	3.76[b]	0.79	0.033
Σn-6	24.08[b]	22.30[a]	21.49[a]	1.86	0.042
n-3/n-6	0.11[a]	0.20[b]	0.29[b]	0.09	0.011

SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; LA = linolenic acid; ALA = α-linolenic acid; AA = arachidonic acid; DPA = docosapentaenoic acid; PUFA = polyunsaturated fatty acids; LCP = long-chain polyunsaturated fatty acids (⩾20 C).
[1]C = control diet containing soya bean oil as the main source of fat; FLAX = control diet substituting 10% of the soya bean oil with extruded flaxseed; FISH = control diet, substituting 3% of the soya bean oil with fish oil (NORDIC NATURALS omega-3®).
[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

ratio (0.93 *v.* 0.57). As expected, the percentage of EPA and DHA and total n-3 LCP were higher in the FISH group.

The reproductive performance of does is reported in Table 3. The whole group of nulliparous does had the same high fertility (95%; data not shown). Many of these traits were affected by the dietary treatments and parity order. In particular, the traits of economic relevance (fertility rate and number of weaned rabbits) improved with n-3 dietary supplementation. Feed consumption and milk production were not affected by the feeding regime.

As expected, does at first parity showed the lowest reproductive results, but the administration of n-3 reduced the gap between primiparous and multiparous does. Primiparous does fed n-3-enriched diets showed the same perirenal fat depots, which increased in the following kindling.

Blood (Table 4) and milk (Table 5) showed low levels of n-3 LCP, whereas liver (Table 6) and ovaries (Table 7) were the main sites of n-3 accumulation. The FISH group generally had the highest values of n-3 LCP, whereas the FLAX group showed lower values, suggesting that endogenous synthesis was low. Compared to the control group, blood plasma of the FLAX group showed higher values of ALA, total n-3 and the n-3/n-6 ratio, whereas AA and total n-6 were lower. The FISH group showed the highest amount of n-3 LCP (e.g. EPA, DHA and DPA) in blood plasma.

The fatty acid profile of the liver showed significant changes, mainly in PUFA and SFA profiles, with a trend similar to that already observed in blood plasma. Flax administration increased ALA content and, to a lesser extent, n-3 LCP (EPA and DPA). Administration of fish oil dramatically increased the concentration of n-3 LCP in the liver (24.33% *v.* 2.11% and 0.39% in FISH, FLAX and control groups, respectively), mainly of DHA.

Experimental diets did not affect the chemical composition of milk, whereas major changes occurred in the PUFA profile. Flax administration mainly improved ALA and reduced LA content, whereas fish oil enriched n-3 LCP levels (about 20 times higher than in control).

Dietary supplementation with n-3 also modified the FA profile of ovarian tissue. In the FLAX group, the amount of ALA in ovaries was enormously increased (approximately 10 times higher than control and FISH groups) and, to a lesser extent, n-3 LCP; at the same time, LA and AA decreased (0.13 and 0.24 times lower than in the control and FISH groups, respectively). The FISH group had about five times more n-3 LCP in the ovaries than the control group, particularly DPA and DHA.

Table 5 *Effect of different dietary supplementations on proximate composition (%) and fatty acid profile of milk (% of total fatty acids) in rabbit does (n = 10/group)*

	Diets[1]				
	Control	FLAX	FISH	Pooled SE	*P* value
Composition					
Moisture	72.15	71.84	71.99	2.56	0.359
Protein	12.41	12.58	12.50	0.87	0.198
Lipid	9.99	10.25	10.09	1.54	0.236
Lactose	3.04	2.79	3.03	0.25	0.154
Ash	2.40	2.54	2.39	0.14	0.078
Fatty acids					
SFA	66.57	66.02	66.50	1.02	0.087
MUFA	15.73	16.76	15.07	1.25	0.356
C18:2cis n-6, LA	12.99[b]	11.42[a]	11.45[a]	0.23	0.112
C18:4 n-6	0.17	0.10	0.13	0.08	0.089
C20:4n-6, AA	0.65	0.50	0.47	0.08	0.257
C22:5n-6	0.17[a]	0.21[a]	0.42[b]	0.05	0.049
C18:3 n-3, ALA	2.79[a]	4.43[b]	2.38[a]	0.89	0.033
C20:5n-3, EPA	0.05[a]	0.06[a]	1.16[b]	0.19	0.045
C22:5n-3, DPA	0.03[a]	0.05[a]	0.39[b]	0.07	0.037
C22:6n-3-DHA	0.03[a]	0.07[a]	0.91[b]	0.31	0.047
PUFA	17.45	17.22	18.43	1.06	0.130
LCPn-3	0.11[a]	0.12[a]	2.46[b]	0.79	0.037
Σn-3	2.90[a]	4.55[b]	4.86[b]	1.98	0.041
Σn-6	13.96[b]	12.32[a]	12.57[a]	0.47	0.023
n-3/n-6	0.20[b]	0.36[a]	0.39[a]	0.08	0.035

SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; LA = linolenic acid; ALA = α-linolenic acid; AA = arachidonic acid; DPA = docosapentaenoic acid; PUFA = polyunsaturated fatty acids; LCP = long-chain polyunsaturated fatty acids (⩾20C).
[1]FLAX = control diet substituting 10% of the soya bean oil with extruded flaxseed; FISH = control diet, substituting 3% of the soya bean oil with fish oil (NORDIC NATURALS omega-3®).
[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

Table 6 *Effect of different dietary supplementations on fatty acid composition of liver (% of total fatty acids) in rabbit does (n = 10/group)*

	Diets[1]				
Fatty acids	Control	FLAX	FISH	Pooled SE	*P* value
SFA	40.93[b]	37.87[ab]	35.20[a]	3.42	0.032
MUFA	13.80	14.61	12.94	1.25	0.089
C18:2 n-6, LA	35.03[c]	27.45[b]	18.19[a]	2.24	0.012
C18:3 n-6	0.12	0.08	0.09	0.01	0.126
C18:4 n-6	0.23	0.10	0.70	0.05	0.201
CLA*c*9*t*11	0.03	0.05	0.18	0.02	0.139
CLA*t*10*c*11	0.02	0.02	0.06	0.01	0.151
C20:2cis11,14 n-6	0.24	0.24	0.04	0.04	0.123
C20:4n-6, AA	6.57[c]	2.46[a]	5.00[b]	0.58	0.044
C22:4n-6	1.10[b]	0.10[a]	0.10[a]	0.05	0.030
C22:5n-6	0.25	0.20	0.03	0.11	0.129
C18:3 n-3, ALA	1.09[a]	14.69[b]	1.49[a]	2.50	0.038
C20:5n-3, EPA	0.08[a]	0.70[b]	5.07[c]	0.55	0.040
C22:5n-3, DPA	0.28[a]	0.66[b]	1.73[c]	0.20	0.036
C22:6n-3, DHA	0.03[a]	0.75[b]	17.60[c]	2.00	0.035
PUFA	44.96[a]	45.68[ab]	51.31[b]	3.55	0.024
LCPn-3	0.39[a]	2.11[b]	24.33[c]	1.01	0.034
Σn-3	1.48[a]	16.80[b]	25.82[c]	1.88	0.040
Σn-6	43.08[b]	30.61[a]	25.30[a]	2.24	0.023
n-3/n-6	0.03[a]	0.54[b]	1.02[c]	0.21	0.046

SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; LA = linolenic acid; ALA = α-linolenic acid; AA = arachidonic acid; DPA = docosapentaenoic acid; PUFA = polyunsaturated fatty acids; LCP = long-chain polyunsaturated fatty acids (⩾20C).
[1]FLAX = control diet substituting 10% of the soya bean oil with extruded flaxseed; FISH = control diet, substituting 3% of the soya bean oil with fish oil (NORDIC NATURALS omega-3®).
SE: standard error.
[a,b,c]Values within a row with different superscripts differ significantly at $P < 0.05$.

RNA expression of *FADS2* encoding Δ6d (Figure 1) in the liver and ovaries showed a downregulation with fish oil supplementation, whereas in the FLAX group ($P < 0.05$) this was slightly lower than in the controls. Messenger RNA for *FADS2* was expressed in all the tissues examined, with the same trend in the experimental groups. The highest levels of *FADS2* mRNA were detected in the liver (Figure 1a) and ovaries (Figure 1b) of control and FLAX groups, with no significant differences between the two groups. Fish oil supplementation downregulated *FADS2* mRNA expression in both the liver and ovary, with a fivefold ($P < 0.01$) decrease in the former and a twofold ($P < 0.01$) decrease in the latter compared to control and FLAX groups.

Discussion

The main finding of this study was that dietary supplementation with n-3 fatty acids, either as a precursor (FLAX) or derivative (FISH), affected fatty acid metabolism in rabbit does and improved their reproductive performance. Although the higher accumulation of n-3 LCP in different body sites was recorded when they were directly administered (FISH group), flaxseed produced an increased ratio of n-3 PUFA reducing AA and total n-6 in all the sites examined. In rats, ALA-enriched diets increased ALA, EPA, and DPA content in plasma, liver, heart and brain (Ayalew-Pervanchon *et al*., 2007).

Of particular interest was the fatty acid profile of ovarian tissue; flax administration increased the amount of ALA in the ovaries 10 times and also, but to a lesser extent, the amount of n-3 LCP. The FISH group had about five times more n-3 LCP than control, mainly DPA and DHA.

However, the present study showed that the effect of precursors, compared with derivatives, on LCP synthesis was not particularly high and that dietary n-3 supplementation modulated *FADS2* mRNA expression. In particular, administration of fish oil significantly reduced *FADS2* mRNA expression, whereas flax induced a slight downregulation ($P > 0.01$).

Published literature confirms an inhibitory effect of EPA and DHA, and possibly ALA (Cho *et al*., 1999; Nakamura and Nara, 2004) on ALA conversion, which may be due to the downregulation of Δ6d activity (Tang *et al*., 2003). The same authors showed that PUFA lower the hepatic abundance of *FADS2* mRNA by inhibiting the rate of its gene transcription

Table 7 *Effect of different dietary supplementations on fatty acid composition of ovaries (% of total fatty acids) in rabbit does (n = 5/group)*

	Diets[1]				
Fatty acids	Control	FLAX	FISH	Pooled SE	*P* value
SFA	22.36[a]	26.45[b]	25.18[a]	2.07	0.023
MUFA	21.36[a]	26.82[b]	26.27[b]	2.20	0.011
C18:2n-6, LA	13.15[b]	7.02[a]	8.50[a]	1.01	0.030
C18:4 n-6	1.66	0.89	1.00	0.05	0.107
CLA*c*9*t*11	1.00	0.87	0.87	0.08	0.068
CLA*t*10*c*11	0.08	0.40	0.58	0.01	0.083
C20:2*c*11,14 n-6	3.12[c]	0.95[a]	1.84[b]	0.14	0.038
C20:4n-6, AA	15.03[b]	2.91[a]	3.95[b]	2.80	0.014
C22:4n-6	6.41[b]	0.18[a]	0.75[a]	0.15	0.035
C22:5n-6	3.05	2.11	1.98	0.07	0.091
C18:3 n-6	1.01	0.87	0.50	0.42	0.111
C18:3 n-3, ALA	2.19[a]	20.95[b]	2.89[a]	1.14	0.087
C20:5n-3, EPA	0.50[a]	1.99[ab]	3.76[b]	0.28	0.021
C22:5n-3, DPA	1.63[a]	1.60[a]	5.80[b]	0.04	0.032
C22:6n-3, DHA	2.06[a]	2.30[a]	13.25[b]	0.80	0.050
PUFA	51.92[b]	47.01[a]	47.49[ab]	3.80	0.047
LCPn-3	4.78[a]	5.89[b]	22.70[c]	2.02	0.018
Σn-3	6.97[a]	27.84[b]	25.65[b]	3.54	0.020
Σn-6	42.50[c]	15.31[a]	19.49[b]	4.51	0.024
n-3/n-6	0.16[a]	1.81[b]	1.31[b]	0.81	0.030

SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; LA = linolenic acid; ALA = α-linolenic acid; AA = arachidonic acid; DPA = docosapentaenoic acid; PUFA = polyunsaturated fatty acids; LCP = long-chain polyunsaturated fatty acids (⩾20C).
[1]FLAX = control diet substituting 10% of the soya bean oil with extruded flaxseed; FISH = control diet, substituting 3% of the soya bean oil with fish oil (NORDIC NATURALS omega-3®).
[a,b,c]Values within a row with different superscripts differ significantly at $P < 0.05$.

and they proposed a molecular mechanism by which dietary n-3 fatty acid modifies ALA desaturation and elongation.

To our knowledge, this is the first study that has demonstrated Δ6d expression in rabbit ovarian tissue; the presence of *FADS2* mRNA expression in the ovary confirms its role in LCP metabolism. Indeed, the eicosanoids derivatives of LCP (mainly prostaglandin E2, PGE2) have an active role in modulating reproduction, regulating the ovulation and fertilisation processes (Weems *et al.*, 2006).

Δ6d metabolism is widely modified by feed, substrate competition, body tissue and genetic strain. Indeed, although ALA is the preferred substrate for Δ6d (Portolesi *et al.*, 2007) with an affinity two to three times greater than for LA, the excess LA, which is typical of standard animal diets, leads to its certain conversion into AA. Further, it has been reported that Δ6d affinity for different precursors is also dependent on genetic strain, with fast-growing rabbit strains having a higher preference for n-6 PUFA than n-3 (Castellini *et al.*, 2016).

Nevertheless, independent of dietary n-3 supplementation, a better reproductive performance (Table 3), particularly in primiparous does, was shown. Primiparous does generally have low reproductive performance, partly due to poor body condition during lactation (Rebollar *et al.*, 2009). Indeed, during the first kindling, the rabbit doe has a low feed intake which does not compensate for the high-energy output of milk production, resulting in a severe energy deficit (Xiccato *et al.*, 1999). It is widely known that this imbalance affects the nutritional and hormonal status of females (Fortun-Lamothe, 2006).

The reason for the improvement in sexual receptivity, fertility rate and numbers of live-born pups is not clear, and it is probably related to an improved hormonal status. There is extensive evidence that dietary PUFA influence biosynthetic pathways involved in prostaglandin synthesis and steroidogenesis that have multiple roles in the regulation of reproductive function. Rodríguez *et al.* (2017) reported that dietary administration of n-3 increased oestradiol and leptin throughout lactation and progesterone during the pre- (Rebollar *et al.,* 2014) and post-implantation phases of gestation (Rodríguez *et al.*, 2018). This higher progesterone concentration could improve implantation and placentation, the survival of foetuses and consequently the reproductive efficiency in primiparous female rabbits.

Dietary PUFA affect the blood concentrations of their metabolites and modulate growth factors in the ovaries, follicular fluid, oviduct and uterus (Webb *et al.,* 2007). In laying hens, Eilati *et al.* (2013) showed that dietary administration of fish or flax oils significantly reduced *COX1* and *COX2* and, consequently, PGE2 and prostaglandin F2α (PGF2α).

Prostaglandin F2α and PGE2 have a crucial role in the control of female reproductive processes, including follicular development, ovulation, lifespan of corpora lutea, pregnancy and parturition (Weems *et al.*, 2006). In particular, the reduction of PGE2, the most pro-inflammatory

prostaglandin, reduces the inflammation associated with ovulation, whereas the inhibition of endometrial PGF2α results in an anti-luteolytic effect and enhances embryo survival (Mattos *et al.*, 2003; Wathes *et al.*, 2007).

According to the available information, the improved reproductive performance of does in n-3 supplemented groups could be explained as follows:

- the reduction of AA, which is partly due to downregulation of *FADS2* and stronger competition ALA/LA, which in turn is the precursor of inflammatory eicosanoids;
- the probable inhibition by AA of PGE2 (by downregulation of *COX1* and *COX2*);
- the increase in steroidogenesis, which improves ovarian cell functions.

The second way in which mammals can benefit from n-3 PUFA supplementation of the mothers is when the litters consume milk containing high concentrations of n-3 LCP (Lin *et al.*, 1991). Our results showed that the dietary treatments modified the PUFA profile of milk (Table 5), whereas MUFA and SFA were similar in all experimental groups. The n-3 supplemented does had n-6 and n-3 levels in milk that were lower or higher, respectively, than the Control animals. Flax administration mainly improved ALA content of milk, while fish oil greatly increased the n-3 LCP levels.

Other studies reported similar changes in the PUFA profile of rabbit milk. Rodríguez *et al.* (2018) showed that the amount of n-3 LCP in milk, such as EPA, DPA and DHA, increased when the diet was enriched with fish oil.

However, in our study, the improvement of milk profile did not significantly affect the performance of litters at weaning. In this regard, further studies on the possible epigenetic effect of n-3 addition in pups would be desirable.

Conclusions

Based on the results of this study, dietary n-3 PUFA enrichment could be considered as a good strategy to physiologically improve the reproductive performance of rabbit does. In the present study, improved performance was obtained without exogenous treatment (hormones) or any intensification of reproductive rhythm, thus consistent with the principles of animal welfare. Furthermore, although the liver is confirmed to be the main metabolic centre for LCP synthesis, reproductive tissues (i.e. ovaries) have a prominent role in LCP generation, which is probably linked to ovary own requirement. Further studies are needed to evaluate in detail the effects of n-3 dietary administration on the hormonal status of does and, accordingly, of rabbit offspring (e.g. epigenetic modifications).

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Declaration of interest

The authors declare no potential conflicts of interest associated with this research.

Ethics statement

The work carried out for this research was according to the Spanish Policy for Animal Protection D53/2013 and assessed and approved by the Animal Ethics Committee of the Community of Madrid (Ref. PROEX 302/15).

Software and data repository resources

None of the data were deposited in an official repository.

Supplementary materials

To view supplementary material for this article, please visit https://doi.org/10.1017/S175173111900020X

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