Effects of dietary omega-3 PUFAs on growth and development: somatic, neurobiological and reproductive functions in a murine model.

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Abstract

Omega-3 polyunsaturated fatty acids (ω-3 PUFAs) are relevant to fetal and infant growth and development. Objective: to assess whether long-term exposure to dietary ω-3 PUFA imbalance alters pre- and/or postnatal pups' development and reproductive function later in life. Mice dams were fed with ω-3 PUFA Control (soybean oil, 7%), Deficient (sunflower oil, 7%) or Excess (blend oil; 4.2% cod-liver + 2.8% soybean) diet before conception and throughout gestation-lactation and later on, their pups received the same diet from weaning to adulthood. Offspring somatic, neurobiological and reproductive parameters were evaluated. Excess pups were lighter during the preweaning period and shorter in length from postnatal day (PND) 7 to 49, compared to Control pups ($P<.05$). On PND14, the percentage of pups with eye opening in Excess group was lower than those from Control and Deficient groups ($P<.05$). In Excess female offspring, puberty onset (vaginal opening and first estrus) occurred significantly later and the percentage of parthenogenetic oocytes on PND63 was higher than Control and Deficient ones ($P<.05$). Deficient pups were shorter in length (males: on PND14, 21, 35 and 49; females: on PND14, 21 and 42) compared with Control pups ($P<.05$). Deficient offspring exhibited higher percentage of bending spermatozoa compared to Control and Excess offspring ($P<.05$). These results show that either an excessively high or insufficient ω-3 PUFA consumption prior to conception until adulthood, seems inadvisable because of the potential risks of short-term adverse effects on growth and development of the progeny or long-lasting effects on their reproductive maturation and function.

Keywords: ω-3 PUFA; perinatal development; neurobehavior; reproductive function; fish oil; sunflower oil.

1. Introduction

The interaction of genetics and environment, nature, and nurture is the foundation for health and disease. Nutrition is an environmental factor of major importance. Currently, the type and amount of polyunsaturated fatty acids (PUFAs) in the diets are being intensely studied because most of the Western countries have diminished their consumption, leading to a dietary ω-6/ω-3 ratio higher than that one on which humans evolved and for which their genetic patrons were established [1].

Linoleic acid (LA) and α-linolenic acid (ALA) are precursors of the omega-6 (ω-6) and omega-3 (ω-3) PUFA family, respectively. In mammals, LA and ALA cannot be synthesized *de novo* and therefore, these compounds have to be obtained from the diet [2]. Members from both ω-6 and ω-3 series are not interchangeable and compete for the same enzymatic system to provide longer and more unsaturated products [1,2]. Therefore, a balanced ω-6/ω-3 ratio is crucial for homeostasis and normal development throughout the whole life cycle [1,3]. For rodents, an optimal ratio between 1 and 6 has been suggested [4].

Studies in mice have provided strong evidence about the effects of prenatal exposure to environmental factors on postnatal phenotypes [5]. The greatest epigenetic plasticity takes place during gamete maturation and embryogenesis, and the consequences can last for part or the whole life of the exposed generation, and even be transmitted to subsequent generations [6,7]. The Barker's or "Fetal Programming" hypothesis suggests that fetal and neonatal conditions may program organ growth and favor diseases later in life [8].

In this context, it is well known that ω-3 PUFAs are relevant for growth, development and health during pregnancy, lactation and infancy [9,10]. There is a relationship between maternal dietary ω-3 consumption with gestation length and birth

weight [11,12], and several animal and human studies have suggested that ω-3 PUFA intake has a significant impact on growth, vision and brain functions [13-17]. For example, the ω-3 PUFA content of preterm and term infant diets has been associated with improved cognitive capability [15,18] and visual outcome [17,19].

Regarding the reproductive processes, ω-3 PUFAs can modify the biosynthetic pathways involved in both prostaglandin (PG) synthesis and steroidogenesis [2,20,21]. Furthermore, the PUFA composition of the membrane may affect cellular responses through changes in membrane fluidity, receptor binding characteristics or downstream activation [21]. Studies in men [22] and boars [23] have demonstrated the benefits of ω-3 PUFA consumption on male reproductive capacity; yet, other studies in different species have reported no effects [24-26]. Studies conducted in female rats have also highlighted the positive effects of diets rich in ω-3 PUFAs on gestational performance [27]. Fish oils, rich in ω-3 PUFAs, may also benefit fertility in cattle and reduce the risk of preterm labor in women [21,28]; however, in both cases, current evidence to support these observations is inconclusive. Differential effects of ω-3 and ω-6 PUFAs on ovarian function and oocyte quality have also been reported [29], yet the literature to date has been inconsistent.

Maternal ω-3 PUFA deficiency could adversely affect fetal and postnatal development. Conversely, an increased maternal intake could minimize such risks [30, 31]. On the other hand, excessive intake of all essential dietary nutrients are associated with adverse effects, but in the case of ω-3 PUFAs, few health risks are ascribed to this condition and its long-term consequences remain unclear [32-35]. As insufficient data is available to establish an upper level where the toxic effects of ω-3 PUFAs might be observed, the practice has been deemed as safe [35].

The present study was designed to assess whether long-term exposure to variable ω-3 PUFA dietary contents, alters pre- and/or postnatal pups' development and their

reproductive function later in life. We hypothesized that excessive or deficient consumption of ω-3 PUFAs by mice dams before conception, during pregnancy and lactation, and subsequently by these pups from weaning until adulthood could modify the offspring's somatic, neurobiological and reproductive development and function.

2. Materials and methods

2.1. Animals and study design

Sexually mature albino Swiss mice (N:NIH) were housed in standard opaque cages with wood shavings as substrate. The animals were maintained under a 14/10 h light/dark photoperiod, room controlled temperature (24±2°C) and free access to food and water. The protocol and animal treatments used in this study were approved by the Animal Care and Use Committee of the Facultad de Ciencias Médicas, Universidad Nacional de Córdoba (protocol number 37/17, Committee UNC-RHCS 674/09). The timeline of experiments is shown in Figure 1.

Female mice (10 weeks old, 24-26 g body weight), were stratified by body weight and randomly assigned to one of the three diets using a random number generator, two weeks prior to copulation. After this acclimatization period, females were individually paired with a male and monitored daily to detect the vaginal plug; once it was observed (considered as gestational day 0 [GD0]), females were relocated in individual cages.

Dams continued receiving the same diet throughout the entire pregnancy and lactation periods. Maternal body weight was determined on GD1, 7, 14 and 17 and once a week during lactation. Delivery day was recorded as postnatal day 0 (PND0). The number of pups per litter was registered on this day by simple observation without handling the pups. On PND1, pups were weighed, sexed and each litter was reduced to eight pups (four males and four females, when possible) to avoid the potential confounding effect of litter

size. Physical, behavioral and reproductive preweaning development was assessed as described by Santillán et al. (2010) [9]. After weaning (three weeks old), the pups were assigned to the same diet as their mother until adulthood (PND63). In this period, physical characteristics and reproductive functions were evaluated in each offspring. When the variables were not sex-dependent, the experimental unit considered was the entire litter.

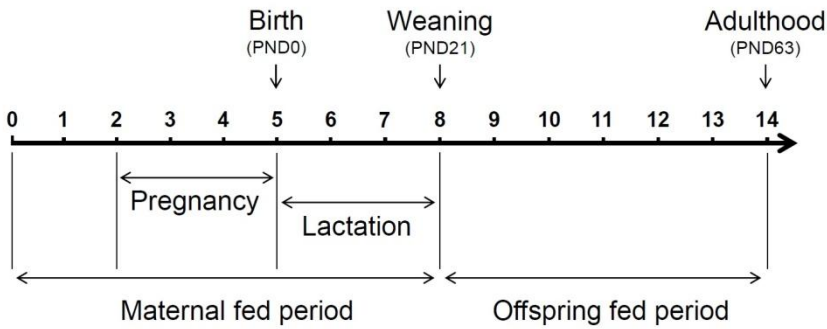

Figure 1. Timeline (weeks) of diet exposure. 0-2 weeks: Diet acclimatization. The day after birth, (postnatal day 1, PND1) pups were weighed and sexed and the litter was reduced to eight animals each. During lactation, physical, neurobiological and reproductive milestones were registered. After this period, assessment of growth and reproductive function was performed. At adulthood, ovulation rate and oocyte quality were evaluated in half of the female offspring, and plasma progesterone concentration was evaluated in the other half (all litters were represented in each parameter). At the same time, semen characteristics and plasma testosterone concentration were evaluated in the male offspring.

2.2. Diets

The composition of the Control diet, a modification of the AIN-93G purified diet [4], as well as the Deficient and Excess ω-3 PUFA conditions, are presented in Table 1. Total dietary energy and percentage of kilocalories as fat in each diet are also presented in Table 1. All three diets were produced in-house using raw components purchased from

local markets. AIN-93G standards have determined the ideal fatty acid (FA) composition and ω-6/ω-3 ratio for growth, pregnancy and lactational phases in rodents. The Control diet was made with soybean oil (7% w/w), whereas the Deficient ω-3 PUFA diet contained sunflower oil. The Excess ω-3 PUFA diet contained a blend oil (4.2% cod-liver + 2.8% soybean= 7% in total). Soybean and sunflower oils were supplied by Aceitera General Deheza (Córdoba, Argentina) and cod- liver oil by Parafarm (Buenos Aires, Argentina). The FA composition of each diet was determined using gas chromatography and is provided in Table 2. We used the naturally occurring FA profiles of fish, soybean and sunflower oils. The oils used in this study were selected because they are usually consumed by humans, especially soybean and sunflower oils, and they are also used in clinical and animal studies. All three diets contained butylated hydroxytoluene (200 mg/kg of oil) to prevent oxidation. Diets were stored at 4°C and to further protect against oxidation, a fresh amount was provided three times a week.

Table 1
Composition of the ω-3 fatty acid Control, Deficient and Excess diet (g/kg of food)

Ingredient	Control	Deficient ω-3	Excess ω-3
Fat			
Soybean oil	70.0		28.0
Sunflower oil		70.0	
Cod-liver oil			42.0
Protein			
Casein	160.0	160.0	160.0
Carbohydrate			
Cornstarch	382.5	382.5	382.5
Sucrose	320.0	320.0	320.0
Fiber	20.0	20.0	20.0
Vitamin			
AIN-93 vitamin mix	10.0	10.0	10.0
Mineral			
AIN-93 mineral mix	35.0	35.0	35.0
Choline bitartrate	2.5	2.5	2.5
Total energy, kcal	4080.0	4080.0	4080.0
% as fat	15.4	15.4	15.4
% as protein	15.7	15.7	15.7
% as carbohydrate	68.9	68.9	68.9

Table 2
Fatty acid composition of treatment diets (g/100 g of food)

Fatty acid	Control	Deficient ω-3	Excess ω-3
Saturated			
14:0	-	-	0.21
16:0	0.80	0.52	0.99
18:0	0.21	0.25	1.31
20:0	0.06	-	-
22:0	0.05	0.09	-
Others		0.09	0.07
Total	**1.12**	**0.94**	**2.58**
Monounsaturated			
16:1	-	-	0.30
18:1	2.03	2.58	0.93
20:1	-	-	0.13
22:1	-	-	0.06
Total	**2.03**	**2.58**	**1.42**
Polyunsaturated			
18:2ω-6	3.27	3.48	1.58
18:3ω-3	0.57	-	0.31
18:4ω-3	-	-	0.09
20:5ω-3	-	-	0.43
22:5ω-3	-	-	0.09
22:6ω-3	-	-	0.32
Others	-	-	0.18
Total	**3.85**	**3.48**	**3.00**
Total ω-6 PUFAs	3.27	3.48	1.61
Total ω-3 PUFAs	0.57	-	1.25
ω-6/ω-3 ratio	5.70	-	1.29

Fat sources: Control diet, 7% soybean oil; Deficient ω-3 diet, 7% sunflower oil; Excess ω-3 diet, 7% blend oil (60% cod-liver + 40% soybean).
Rodent requirements of: 18:2ω-6 (linoleic acid), 1.2%; 18:3ω-3 (alpha-linolenic acid), 0.2-0.4%; ω-6/ω-3 ratio, 1-6.
Fatty acid accounting for less than 0.05% are not shown but included in "others".

2.3. Physical parameters

Pups' body weight (g) and length (cm, from the middle of the head to the base of the tail) were weekly measured from PND7 to PND63. Other physical parameters monitored were: fur appearance (the emergence of immature hair); pinna detachment (the bilateral unfolding of external ear); lower incisor eruption (emergence from the gingiva) and eye opening (separation of the upper and lower eyelid in both eyes) [9,36]. In agreement with Santillán et al. [9], to determine the appropriate day to evaluate these parameters and to avoid excessive animal handling, a preliminary study was performed in six litters without any treatment. The day on which approximately 75% of the animals acquired the parameter was defined as the reference day for such parameter.

172 *2.4. Neurobiological tests*

173 Three tests were performed to assess the neurobiological development during the
174 lactation phase. To reduce bias, data were recorded by two different operators at the same
175 time (to avoid excessive animal handling and training influence) and the average data were
176 reported. The reference day was determined as previously described for the physical
177 parameters.

178 *2.4.1. Surface righting reflex:* on PND7, each pup was placed on its back over a flat
179 surface during four seconds and then released. The time required to repose all four paws in
180 contact with the surface was recorded with a stopwatch. The number of animals with
181 successful response in less than two seconds was registered [9,36].

182 *2.4.2. Cliff avoidance:* on PND7, each pup was placed over a top box surface with the
183 forepaws and nose over the edge (20 cm height). The time required to complete backing
184 and turning away from the edge was recorded and the number of animals with successful
185 response within 30 seconds was registered [9,36].

186 *2.4.3. Negative geotaxis:* on PND8, each pup was placed in a head-down position on a 45-
187 degree inclined cardboard surface. The time taken to complete a 180-degree turn was
188 recorded, and the number of animals with successful response in less than 30 seconds was
189 registered [9,36].

190

191 *2.5. Male puberty onset*

192 Male pups were observed daily from PND17 and puberty onset was determined as
193 the day when both testes were descended into the scrotum [9].

194

195 *2.6. Female puberty onset and characteristics of the estrous cycle*

196 Female pups were inspected daily for vaginal opening starting on PND21.
197 Thereafter, vaginal cytology was daily examined to detect the first estrus and to assess the
198 sexual cycle length as well as the duration of each phase. Five complete cycles were
199 studied in each animal [37].
200
201 *2.7. Ovulation rate and oocyte quality (nuclear maturity, parthenogenetic activation and*
202 *degeneration)*
203 Half of the adult female offspring (PND63; all litters represented) were induced to
204 superovulate using 5 IU of pregnant mare serum gonadotropin (i.p.) followed, 48 h later,
205 by 10 IU of human chorionic gonadotropin (hCG; i.p.) [20]. Animals were euthanized by
206 cervical dislocation 17-18 h after hCG administration. The cumulus-oocyte complexes
207 were collected by puncturing both oviduct ampullae, placed into center-well dishes with 1
208 ml of modified Tyrode's solution [38] and counted to determine the ovulation rate. One
209 third of the oocytes harvested from each animal were used to integrate a pool per treatment
210 to evaluate maturity. Cumulus complexes were removed with hyaluronidase and the
211 absence of the germinal vesicle (GV), as a sign of oocyte maturity, was assessed under an
212 inverted microscope at 200x [36]. The other two thirds of the cumulus-oocyte complexes
213 harvested from each animal were kept on separate center-well dishes (one per animal),
214 incubated at 37°C (5% CO_2; 95% air) for 24 h and finally evaluated under inverted
215 microscope at 200x to determine the degenerated or activated ova percentage.
216 Parthenogenetic oocytes were classified as pronuclear stage, two cells or more than two
217 cells [20].
218
219 *2.8. Hormonal determinations*

Animals were euthanized by decapitation and blood was collected in heparinized tubes and centrifuged at 150 g for 30 min. The supernatant was separated and stored at -20°C until processing [20]. Progesterone concentration was determined in the metestrus phase in the other half of the adult female offspring (not induced to superovulate; all litters represented) and testosterone concentration was determined in the male offspring.

Progesterone determinations were performed using a commercial ^{125}I-progesterone radioimmunoassay kit (Coat-A-Count Progesterone, Siemens). The antiserum had less than 3.5% cross-reactivity with other steroids, except for 5-α-pregnan-3,20-dione (9%) (data provided by the company). The assay sensitivity was 0.1-40 ng/ml. Intra-assay coefficient of variation was less than 10%. All samples were assayed on the same day in order to avoid inter-assay variation.

Testosterone concentration was determined by enzyme immunoassay using a polyclonal anti-testosterone antibody, testosterone standard and their corresponding horseradish peroxidase conjugate (testosterone R156/7, Department of Population Health and Reproduction, C. Munro, UC Davis, CA, USA). Briefly, flat bottom microtiter plates (Nunc Maxisorp, VWR, Mississauga, ON, Canada) were first coated with 50 µl of the anti-testosterone antibody diluted in coating buffer (50mM bicarbonate buffer, pH 9.6; 1:10500), covered with acetate sealers to prevent evaporation and incubated overnight at 4°C. After 16-24 h, plates were washed to remove any unbound antibody with 0.02% Tween 20 solution using a Bio-Tek ELx 405VR microplate washer (Bio-Tek Instruments). Immediately after washing, 50 µl of plasma samples, standards, and controls were added in duplicates, followed by 50 µl of horseradish peroxidase conjugate diluted in EIA buffer (1:20000). Plates were then covered and incubated at room temperature for 2 h. Following incubation, the plates were washed and blotted dry, and 100 µl of substrate solution (50mM citrate, 1.6mM hydrogen peroxide, and 0.4mM 2,20-azino-di-(3-ethylbenz-

thiazoline sulfonic acid) diammonium salt, pH 4.0) were added to each well [39,40]. Absorbance was measured at 405 nm using a microplate reader (Thermo Electron Corporation, USA). The assay sensitivity was 0.047 ng/ml. Intra-assay and inter-assay coefficient of variation were less than 10% and 15%, respectively. Cross-reactivity values were: 5-α-dihydrotestosterone (57.4%), androstenedione (0.27%), androsterone (0.04 %), cholesterol (0.03%) and <0.02 % with all other steroids tested.

2.9. Semen characteristics and testicular weight

In a subgroup of adult male offspring (all litters represented), both testicles were dissected and weighed [41]. The cauda epididymis was removed and sperm samples were assessed for concentration, motility, maturity, viability, response to hypoosmotic shock and acrosomal integrity as previously described in Puechagut et al. (2012) [41].

2.10. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM), as the median (quartile 1 and 3; Q1-Q3), or as percentage, as appropriate. Dams' and pups' body weight and length were analyzed by two-way repeated-measures ANOVA followed by LSD *post hoc* test. Physical and neurobiological parameters, puberty onset, characteristics of the estrous cycle, ovulation rate, nuclear oocyte maturity, testicular weight, semen characteristics and plasma hormone levels were analyzed by one-way ANOVA followed by LSD *post hoc* analysis or nonparametric Kruskal-Wallis test, as appropriate. The following data were log transformed before applying the ANOVA model: male/female ratio at birth, vaginal opening, non-progressive sperm, immotile sperm, bending forms and sperm with both signs of immaturity, and plasma progesterone concentration. The effect of dietary treatment on oocyte parthenogenesis and degeneration was assessed using the Chi-

square test. Statistics Graph Pad Prism 5.0 (Graph Pad Software, Inc., San Diego, CA, USA) and Dell Statistica 13, Dell Inc. (2015) were used to perform graphical and statistical analysis, respectively. *P* values <.05 were considered statistically significant.

3. Results

3.1. Maternal body weight and gestational outcomes

There were no significant differences in dams' body weight during gestation and lactation in any of the treated groups (Figure 2), except on GD17, in which the Excess group dams were heavier than the Deficient ones (*P*<.05). As expected, a progressive increase in maternal body weight was observed throughout gestation (F(3,63)=410.87, *P*<.05) and a time effect was also detected throughout lactation (F(3,63)=13.99, *P*<.05). However, neither a diet effect nor a diet×time interaction were detected in both periods (*P*>.05). No group differences in gestational length, litter size, sex ratio or pups' weight at birth were observed (Table 3).

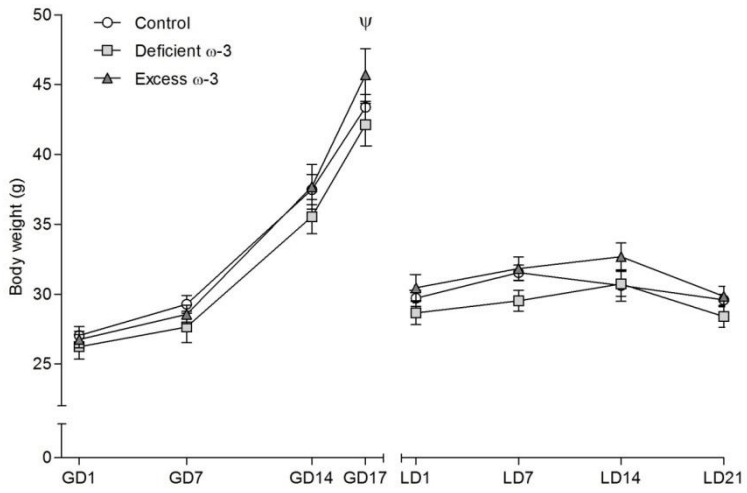

Figure 2. Body weight during gestation or lactation in murine dams fed with variable levels of ω-3 PUFAs. GD, gestation day; LD, lactation day. Results are expressed as mean ± SEM. n=8 dams per diet.

ψ $P<.05$ versus Deficient ω-3 in a two-way ANOVA for repeated measures followed by a LSD *post hoc* analysis.

Table 3
Maternal and birthing outcomes of mice fed with a Control, Deficient or Excess ω-3 fatty acid diet

	Control	Deficient ω-3	Excess ω-3
Gestational length (d)	19.00 (19.00-19.00)	19.00 (19.00-19.00)	19.00 (19.00-19.00)
Number of pups/litter	10.25 ± 0.31	10.00 ± 0.76	10.88 ± 0.55
Male/female ratio at birth	1.12 ± 0.23	1.14 ± 0.32	1.18 ± 0.20
Litter weight at birth (g)	15.62 ± 0.56	15.16 ± 0.94	16.52 ± 0.81
Pup body weight at birth (g)	1.52 ± 0.03	1.53 ± 0.04	1.52 ± 0.03

Results are expressed as median (Q1-Q3; quartile 1 and 3 respectively) or as mean ± SEM. n=8 dams per diet.

3.2. Offspring's physical and neurobiological milestones

The Excess diet induced a reduction in the offspring body weight during the pre-weaning period, compared with the other two groups ($P<.05$), and an increase in this variable from PND49 to PND56 in pups of both sexes, compared with the Deficient group ($P<.05$) (Figures 3 and 4). On PND63, this difference was not evident in female pups but remained in the males.

Pups in the Excess group were shorter in length than those under Control diet from PND7 to PND49 in both sexes ($P<.05$) while Deficient offspring were shorter than Control ones on PND14 and PND21 ($P<.05$) (Figures 3 and 4).

From this time onwards, there were some differences according to gender: Deficient males on PND35 and PND49 and females on PND42 were shorter than those under the Control diet ($P<.05$). Despite these differences during the postweaning period, all groups reached similar values on PND56. Growth was observed over time (female offspring weight $F(8,712)=6103.03$, $P<.05$; female offspring length $F(8,712)=5095.13$, $P<.05$; male offspring weight $F(8,728)=5595.16$, $P<.05$; male offspring length $F(8,728)=5427.75$, $P<.05$) and a diet×time interaction was detected (female offspring weight $F(16,712)=7.72$, $P<.05$; female offspring length $F(16,712)=6.64$, $P<.05$; male offspring weight $F(16,728)=5.22$, $P<.05$; male offspring length $F(16,728)=6.94$, $P<.05$).

The percentage of eye opening in PND14 was lower in the Excess group compared with the other two groups (P<.05). There were no significant differences in the acquisition of the remaining physical or neurobiological parameters evaluated (Table 4).

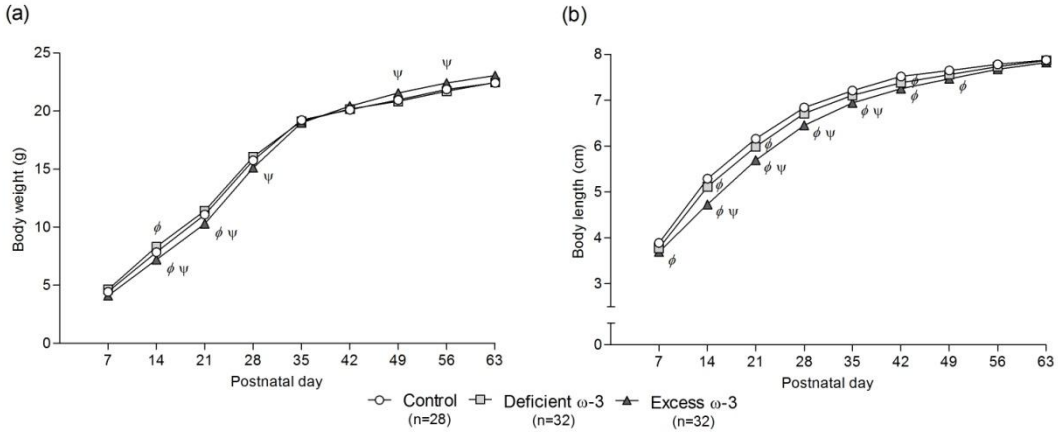

Figure 3. Changes in body weight (a) and length (b) of female albino Swiss offspring exposed to different dietary levels of ω-3 PUFAs prior to gestation until adulthood. n=number of animals. Results are expressed as mean ± SEM. Two-way ANOVA for repeated measures followed by a LSD *post hoc* analysis was used to compare group and time effects.

ϕ P<.05 versus Control.

ψ P<.05 versus Deficient ω-3.

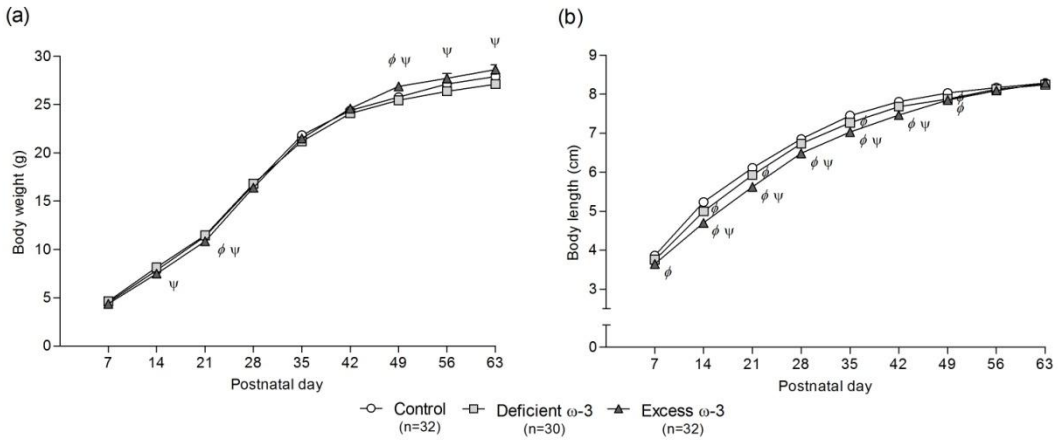

Figure 4. Changes in body weight (a) and length (b) of male albino Swiss offspring exposed to different dietary levels of ω-3 PUFAs prior to gestation until adulthood. n=number of animals. Results are expressed as mean ± SEM. Two-way ANOVA for repeated measures followed by a LSD *post hoc* analysis was used to compare group and time effects.

ϕ $P<.05$ versus Control.

ψ $P<.05$ versus Deficient ω-3.

Table 4
Physical and behavioral development in albino Swiss offspring from dams exposed to Control, Deficient ω-3 or Excess ω-3 diet

Parameter	Control	Deficient ω-3	Excess ω-3	
Physical parameters (%)				
Fur appearance (PND2)	100.00 (75.00-100.00)	50.00 (0.00-100.00)	100.00 (0.00-100.00)	
Pinna detachment (PND4)	100.00 (100.00-100.00)	100.00 (100.00-100.00)	100.00 (100.00-100.00)	
Incisor eruption (PND11)	100.00 (87.50-100.00)	100.00 (87.50-100.00)	100.00 (100.00-100.00)	
Eye opening (PND14)	100.00 (75.00-100.00)	100.00 (87.50-100.00)	62.50 (50.00-75.00)	ϕ ψ
Behavioral tests (%)				
Surface righting reflex (PND7)	82.81 ± 5.25	76.56 ± 6.44	68.75 ± 7.09	
Cliff avoidance (PND7)	87.50 (75.00-100.00)	87.50 (87.50-100.00)	100.00 (62.50-100.00)	
Negative geotaxis (PND8)	93.75 (62.50-100.00)	81.25 (62.50-87.50)	75.00 (50.00-87.50)	

Postnatal day (PND) in parentheses is the reference day for that parameter. Results are expressed as median (Q1-Q3; quartile 1 and 3 respectively) or as mean ± SEM. n=8 litters per diet; 8 pups each.
ϕ $P<.05$ versus Control in a Kruskal-Wallis test.
ψ $P<.05$ versus Deficient ω-3 in a Kruskal-Wallis test.

3.3. Puberty onset and characteristics of the estrous cycles

Descent of both testes occurred on PND19 in all groups. Vaginal opening and first estrus in the Excess group occurred later than in the other two groups ($P<.05$). The female sexual maturation had a negative correlation with the pup's body weight on PND7, 14, 21 and 28. When considering the length of the estrous cycle and each phase separately, the analysis showed no significant differences. However, the Excess condition induced higher percentage of the diestrus length out of the whole cycle compared to the other dietary groups ($P<.05$) (Table 5).

Table 5
Puberty onset and characteristics of estrous cycles in albino Swiss pups exposed to variable dietary levels of ω-3 fatty acids

Parameter	Control	Deficient ω-3	Excess ω-3	
Number of animals	28 (8 litters)	32 (8 litters)	31* (8 litters)	
Vaginal opening (PND)[1]	29.86 ± 0.45	29.94 ± 0.44	32.74 ± 0.64	ϕ ψ
First estrus (PND)[2]	30.00 (29.00-33.00)	30.50 (29.00-33.00)	34.00 (32.00-38.00)	ϕ ψ
Length of estrous cycles (days)[2]	5.00 (4.80-5.20)	5.00 (4.80-5.20)	5.00 (4.80-5.40)	
Length of estrus phase (days)[2]	1.20 (1.00-1.20)	1.20 (1.00-1.20)	1.20 (1.00-1.20)	
Length of diestrus phase (days)[2]	2.40 (2.20-2.60)	2.40 (2.00-2.60)	2.80 (2.20-3.00)	
Length of estrus/length of cycle (%)[2]	22.41 (20.69-24.00)	20.83 (19.23-23.08)	20.87 (20.00-23.81)	
Length of diestrus/length of cycle (%)[1]	46.95 ± 1.05	47.67 ± 1.03	52.19 ± 1.39	ϕ ψ

PND, postnatal day. Results are expressed as median (Q1-Q3; quartile 1 and 3 respectively) or as mean ± SEM.
Comparisons between groups were made by one-way ANOVA ([1]) or Kruskal-Wallis test ([2]). A total of 5 cycles were evaluated in each mouse after vaginal opening. * There was one Excess female not included due to lack of estrous cyclicity.
ϕ *P*<.05 versus Control.
ψ *P*<.05 versus Deficient ω-3.

337

338 *3.4. Ovulation rate and oocyte quality*

339 There were no significant differences among groups in terms of ovulation rate and
340 percentages of mature oocytes and degenerated forms. After 24 h of cumulus-enclosed
341 oocytes incubation, the percentage of spontaneous parthenogenetic activation was higher in
342 the Excess group compared with the other two groups (*P*<.05) (Table 6).

Table 6
Ovulation rate and oocyte quality in albino Swiss offspring exposed to variable dietary levels of ω-3 fatty acids from conception to adulthood

Parameter	Control	Deficient ω-3	Excess ω-3	
Number of animals	13*	15	13*	
Ovulation rate[1]	30.62 ± 2.88	26.19 ± 3.07	31.86 ± 3.33	
Maturity (%)[2]	100,00 (100,00-100,00)	100,00 (100,00-100,00)	100,00 (95,00-100,00)	
Number of incubated oocytes (24h)	269	271	300	
Spontaneous activation (%)[3]	34.36	33.71	43.20	ϕ ψ
Pronuclear stage (%)[4]	3.37	1.12	1.57	
2 cells (%)[4]	62.92	61.80	48.82	ϕ
More than 2 cells (%)[4]	33.71	37.08	49.61	ϕ
Degenerated (%)[5]	3.72	2.58	2.00	

[1] Ovulation rate: number of oocytes recovered from both oviductal ampullae. Results are expressed as mean ± SEM and comparisons between groups were performed with one-way ANOVA. [2] Results are expressed as median (Q1-Q3; quartile 1 and 3, respectively) and comparisons between groups were made by Kruskal-Wallis test. [3] Percentage of incubated oocytes excluding degenerated oocytes. [4] Percentage of activated oocytes. [5] Percentage of incubated oocytes. [3, 4, 5] Data were analyzed by Chi-square test. * There were two Control and two Excess females not included because of oocytes absence in the ampullae after hormonal induction.
ϕ *P*<.05 versus Control.
ψ *P*<.05 versus Deficient ω-3.

343

344 *3.5. Plasma progesterone concentration*

345 There were no significant differences in plasma progesterone concentration (ng/ml)
346 among groups, despite an observed trend towards decreased progesterone level in the

Excess group: Control 11.08 ± 2.11 (n=10); Deficient 13.35 ± 1.63 (n=14); Excess 8.65 ± 0.88 (n=16); *P*=.07. The number of plasma samples used for progesterone measurements was lower than the number of females not induced to ovulate in the Control and Deficient groups (n=13 and n=17, respectively). This is because plasma samples from all three dietary groups were used to perform two hormonal determinations and in the cases in which the volume was not enough to perform both, corticosterone was prioritized (results not included in this report).

3.6. Plasma testosterone concentration

No significant differences were observed in testosterone levels (ng/ml) between any of the groups: Control 0.49 ± 0.13 (n=19); Deficient 0.32 ± 0.10 (n=23); Excess 0.23 ± 0.15 (n=10).

3.7. Semen characteristics and testicular weight

There were no significant differences in sperm concentration and motility among groups. However, the Deficient group exhibited a higher percentage of bending immature gametes compared to the Control group (*P*<.05). No significant differences were observed in sperm viability, tail swelling after hypoosmotic shock, acrosomal integrity or testicular weight between any dietary groups (Table 7).

Table 7
Functional activity of caudal epididymal sperm and testicular weight from albino Swiss offspring exposed to variable levels of ω-3 fatty acids from conception to adulthood

Parameter	Control	Deficient ω-3	Excess ω-3
Number of animals	15	19	15
Sperm concentration (x 10^6/mL)	26.18 ± 2.07	21.51 ± 1.87	26.72 ± 2.98
Motile (%)	84.00 (74.00-89.00)	84.00 (78.00-89.00)	79.00 (73.00-88.00)
Progressive (%)	84.00 (74.00-88.00)	80.00 (78.00-89.00)	75.00 (73.00-84.00)
Non-progressive (%)	0.77 ± 0.48	3.11 ± 1.82	2.77 ± 1.48
Immotile (%)	18.00 ± 1.90	18.08 ± 2.30	0.27 ± 2.35
Immature features			
Bending (%)	3.93 ± 0.81	8.11 ± 1.36 ϕ	5.87 ± 0.93
Cytoplasmic drop (%)	14.07 ± 2.34	14.55 ± 2.26	15.00 ± 3.17
Viable spermatozoa (%)	86.00 (79.00-80.00)	81.00 (73.00-85.00)	81.00 (71.00-86.00)
Hypoosmotic tail swelling (%)	76.87 ± 2.14	77.37 ± 1.25	75.13 ± 2.84
Acrosomal integrity (%)	90.00 (84.00-92.00)	90.00 (80.00-93.00)	86.00 (78.00-92.00)
Testicular weight (g)	0.18 ± 0.003	0.17 ± 0.003	0.17 ± 0.010

Results are expressed as mean ± SEM or as median (Q1-Q3; quartile 1 and 3 respectively).
ϕ P<.05 versus Control in a one-way ANOVA followed by LSD *post hoc* analysis.

4. Discussion

The aim of this study was to assess the effects of long-term exposure to dietary ω-3 PUFA imbalance before conception to adulthood, on the somatic, neurobiological and reproductive development and function of mice. Our results show that an excess in ω-3 PUFAs leads to the impairment of several physical developmental parameters in the lactating offspring and delays growth in length over the lactation and postweaning periods, in addition to female reproductive development. On the other hand, the ω-3 PUFA deficiency also delays growth in length during the lactation and postweaning periods and produces a higher count of bending spermatozoa (a sign of gamete immaturity).

In lactating pups from dams fed with the Excess diet, we observed lower body weight and length and delayed eye opening, but no differences were observed in body weight at birth. These results are in accordance with previous studies reporting adverse consequences in postnatal growth and development after maternal dietary excess of ω-3 PUFAs during pregnancy and lactation [42-47]. Nevertheless, some of these studies cannot rule out if the adverse effects are due to very low level of ω-6 PUFAs or elevated level of

ω-3 PUFAs, since they used fish oil (very rich in ω-3 PUFAs but extremely poor in ω-6 PUFAs) as the only lipid source to create the excessive condition [46,47].

Lactation could be considered a more susceptible period to the harmful effects of an increased maternal consumption of ω-3 PUFAs [44,47], probably because changes induced by diet in the FA profile of maternal milk are less compensated in the Excess than in the Deficient situation, in which the mobilization from maternal fat stores could maintain milk FA composition [44,47]. Moreover, Excess diet is able to inhibit the development of mammary tissue and thus milk production by reducing LA to arachidonic acid (AA) conversion [48]. In rodent studies, intrauterine exposure to excessive ω-3 PUFAs affected brain myelination and neurobehavioral function in the pups [47,49]. Therefore, the acquisition of the suckling reflex and the pups' ability to feed could be affected. Nevertheless, the neurobiological evaluation performed later did not show any significant change.

In rat offspring exposed to ω-3 PUFA excess through their mothers, a delay in pinna detachment was observed [46,47]. In the present study, in lactating mice exposed to the Excess diet, eye opening was delayed. These results suggest that ω-3 PUFA excess may lead to alterations in tissue maturation. Oppositely, when the same parameters were evaluated in offspring exposed to ω-6 excess diet, there was an advancement [9], suggesting that ω-6 net content and ω-6/ω-3 ratio on diet are relevant for these developmental milestones.

The recovery in body weight observed in the Excess pups after weaning may be attributable to their own access to the dam food, as suggested by Wainwright et al. [43]. Interestingly, in the late phase of the evaluated period, the Excess group reached a higher body weight than the Deficient group.

In mice offspring exposed to ω-3 PUFA excess, body length recovery followed a slower rhythm after weaning. This suggests the persistence of some mechanisms of nutritional toxicity on longitudinal growth speed [47,50], at least until PND56, when this differences disappeared. In pups exposed to Deficient diet, a reduction in body length was observed but the postweaning recovery was faster than in the Excess condition. One work has suggested that offspring from dams fed with high or low ω-3 PUFA diets may have altered skeletal growth [46]. In a previous study, we observed a reduction in the pups' length at weaning after maternal exposure to a high ω-6/low ω-3 PUFA diet [9]. Taken together, these results highlight the relevance of ω-3 PUFA content on dam diet to the progeny growth.

Neither the Excess nor the Deficient conditions had significant effects on maternal body weight during gestation and lactation, gestational length, litter size, sex ratio, birth weight, or the developmental and neurobehavioral milestones of fur appearance, pinna detachment, teeth eruption, surface righting reflex, cliff avoidance and negative geotaxis. These findings are consistent with previous studies on maternal deficiency and excess of dietary ω-3 PUFAs [42,46,47,51-54] and contrast with those reporting prolonged gestational age [55] or reduced birth weight [47,55] after the exposure to high ω-3 PUFA levels.

The female mice exposed to excessive ω-3 PUFAs had delayed puberty onset. Smith et al. reported immaturity in hypothalamic and ovarian components of the reproductive axis due to LA deficiency and the subsequent reduced availability of AA for synthesis of bioactive metabolites, especially PGE_2 [56]. The ω-3 PUFA excess may displace and reduce the tissular bioavailability of AA. Moreover, AA and its derived PG depletion can inhibit the steroidogenic acute regulatory protein and sexual steroid synthesis, which are necessary to the maturation of internal and external genital organs

[21,57]. The low body weight of Excess female pups during lactation is also able to delay the timing of puberty [58]. Correlation analysis showed that the lower body weight, the later sexual maturation.

The length of the estrous cycle as well as each composing phase was not modified by dietary ω-3 PUFA levels. These may respond to an adequate provision of LA by all the diets [56]. In offspring exposed to the Excess diet, a relative prolongation of diestrus at the expense of proestrus and/or metestrus was registered. Metestrus depends on the corpus luteum activity and excessive ω-3 PUFAs may reduce luteotrophic PG, shortening this phase [59]. In reference to ovulation rate, we found no differences between our dietary groups, in accordance with other investigations [60,61]. Higher PUFA concentrations than those used in the present study are necessary to produce variations in this parameter [62].

Cumulus-enclosed oocytes from mice exposed to the Excess diet showed lower quality than other dietary groups, since they were spontaneously activated and expressed advanced transition timing between activation stages [63]. Cumulus cells play an important role in oocyte nutrition and activation process [63,64]. An increasing proportion of ω-3 PUFAs in the diet and later in the cells may reduce PGE synthesis by the mouse oocyte-cumulus complex, and this reduction has been associated with high parthenogenetic activation rates [63,65]. In relation to the other oocyte quality parameters evaluated in this study, diets did not modify neither the percentage of oocyte maturity (GV stage absence) nor the percentage of degenerated ova. Another work studied the oocyte quality using other methods and, similarly, did not found any effect after the administration of linseed oil (56% ALA) [60].

The adult male mice exposed to ω-3 PUFA deficient diet had higher percentages of bending spermatozoa in samples obtained from the cauda epididymis. This alteration may hinder the normal sperm migration through the female tract [66]. It is well known that

PUFA levels are relevant to membrane constitution and maturation during spermatogenesis [67,68] and epididymal transit. During the latter, PUFA to saturated FA ratio increases [69]. Another characteristic of mature cauda spermatozoa is the higher presence of disulfide bonds in proteins and the reduction of this feature is associated with an increased count of bending gametes [69]. The PUFA content in the sperm membrane modifies redox status [21] and in turn, could alter the production of double bonds by glutathione peroxidase [70]. Further studies are necessary to determine the impact of ω-3 PUFA deficiency on sperm redox status and disulfide-bridging events.

Diets did not induce modifications neither in female progesterone nor in male testosterone plasmatic concentrations. Likewise, other studies showed no variations due to dietary ω-3 PUFA level on progesterone [60,71] or testosterone concentrations [72,73].

The supplementation and fortification of foods is an attractive strategy to increase ω-3 PUFA intake [21] and this is considered to be a safe practice [35]; however, there are still some controversies regarding the effects of ω-3 PUFA supplementation on the inhibition of ω-6 PUFA metabolic pathways and on postnatal development [74]. Our observations are in accordance with the ω-6 displacement hypothesis and show that excess of ω-3 PUFAs in dams diet would be more harmful to the offspring growth and development than deficiency, probably because the mothers may counteract this situation at expense of their own body stores. These findings may have significant implications for nutrient supplement practices during pregnancy and lactation.

In conclusion, these results taken together with those obtained by our group in previous studies using ω-6 PUFA exceeded diets in a similar protocol, show that the consumption of either large or inadequate amounts of ω-3 or ω-6 PUFAs by mothers during pregnancy and lactation, and by their offspring after weaning, seems inadvisable

480 because of the potential adverse effects on growth and development, female sexual
481 maturation, as well as male and female fertility at adulthood.
482
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