Effects of an n-3-deficient diet on brain, retina, and liver fatty acyl composition in artificially reared rats

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Abstract **Rat pups born to dams fed a diet with 3.1% of total fatty acids as α-linolenic acid (LNA) were fed, using an artificial rearing system, either an n-3-deficient (n-3-Def) or an n-3-adequate (n-3-Adq) diet. Both diets contained 17.1% linoleic acid, but the n-3-Adq diet also contained 3.1% LNA. The percentage of brain docosahexaenoic acid (DHA) continuously decreased (71%) with time over the 29 days of the experiment, with concomitant increases in docosapentaenoic acid (DPAn-6). In the retina, the percentage of DHA rose in the n-3-Adq group, with an apparent increased rate around the time of eye opening. However, there was a flat curve for the percentage of DHA in the n-3-Def group and a rising DPAn-6 with time. Liver DHA was highest at the time of birth in the n-3-Adq group but fell off somewhat over the course of 29 days. This decrease was more pronounced in the n-3-Def group, and the DPAn-6 rose considerably during the second half of the experiment. This method presents a first-generation model for n-3 deficiency that is more similar to the case of human nutrition than is the commonly employed two-generation model.**—Moriguchi, T., S-Y. Lim, R. Greiner, B. Lefkowitz, J. Loewke, J. Hoshiba, and N. Salem, Jr. **Effects of an n-3-deficient diet on brain, retina, and liver fatty acyl composition in artificially reared rats.** ***J. Lipid Res.*** **2004.** 45: **1437–1445.**

Supplementary key words artificial rearing • docosahexaenoic acid • docosapentaenoic acid • fatty acid composition

There is much interest in the lipid and essential fatty acid community in the interactions of essential fatty acids with measures of nervous system function. Moreover, in the wider field of nutrition, intensive efforts are under way to determine the effects of various nutrients on growth and development. Much of this work employs rats or other rodents as the experimental animal. However, in many studies that begin at weaning or afterwards, it has been found that much of the formative period of development is past. Thus, methods have been developed that make possible the artificial feeding of rat pups at the earlier stages of life. For example, intragastric infusion has been used to control the essential fatty acid composition of artificial rat milk in order to study n-3 fatty acid deficiency (1, 2) or supplementation (3). This method was at least partially successful in producing animals with a loss in nervous system docosahexaenoic acid (DHA), inasmuch as there was ~50% loss in the first generation (1). However, this method has some disadvantages, including the stress of surgery and forced feeding, and the absence of social interaction and suckling and swallowing behavior. But perhaps its greatest limitation is that this method cannot begin until day 4–5 (1, 3, 4).

Hoshiba (5, 6) developed a completely new system for the artificial rearing of rat pups based on the use of natural suckling behavior together with a bottle-nipple system devised to control the resistance required for milk flow. He has shown that this system can be used to feed rats an artificial milk beginning within 12 h of birth (6). This system has recently been adapted to the study of essential fatty acids by Lim et al. (7). In this work, Lim et al. demonstrated that rat pups fed on an n-3-deficient (n-3-Def) diet from postnatal day 2 to adulthood had spatial learning deficits. These pups were artificially reared using artificial milk modeled on that used by Kanno et al. (4) but modified so as to control the n-3 fatty acid content. The objective of the present study was to demonstrate that a marked level of DHA deficiency could be achieved in the nervous system by the use of this system without resorting to the

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Abbreviations: AA, arachidonic acid (20:4n6); DHA, docosahexaenoic acid (22:6n3); DPAn-6, docosapentaenoic acid (22:5n6); EPA, eicosapentaenoic acid (20:5n3); LA, linoleic acid, (18:2n6); LNA, linolenic acid (18:3n3); n-3 Adq, n-3 adequate; n-3 Def, n-3 deficient.

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multiple generation approach that has heretofore been standard. This paper describes the time course of tissue fatty acid responses during n-3-Def or n-3-adequate (n-3-Adq) diets. In this model, a diet that contains α-linolenic acid (LNA) is fed to the dam so that n-3 deficiency is induced only after birth. An experiment was performed to determine whether the controlled feeding of the maternal diet could be shortened so that it began only after conception. In this way, first generation n-3-Def rat pups could be generated in a relatively short period of time.

MATERIALS AND METHODS

Animals and study design

The general scheme for the study design is presented in **Fig. 1**. Female Long-Evans rats were obtained at 3 weeks of age from Charles River Laboratories (Portage, MI) and were maintained within the National Institutes on Alcohol Abuse and Alcoholism animal facility under conventional conditions with controlled temperature (23 ± 1°C) and a 12 h light cycle (6 AM–6 PM). Upon arrival, the rats were immediately placed on a semisynthetic diet that was based on the AIN-93G standard (8) and contained 3% LNA (see n-3-Adq diet below). At 11 weeks of age, they were mated with 12-week-old males of the same strain and separated from the males after 3 days. For some experiments, time-pregnant rats were obtained on gestational day 3 (Charles River Laboratories) and immediately switched to the n-3-Adq diet. Pregnant rats were removed to individual cages at 17 days after mating and observed for delivery twice a day. Newborn male pups were separated from each dam within 12 h of birth such that each artificially reared group contained only one pup from each dam. This was done to produce experimental groups that were internally more diverse. Also, the various experimental groups were more comparable, because they contained siblings of the other groups. The groups received either an n-3-Adq or an n-3-Def artificial rat milk diet (see below). The remainder of the pups were kept with their dams after the group was culled to 10 pups/litter ("dam's milk group"). One male pup from each litter was selected at birth and designated for the dam's milk group measurements, thus providing for a group containing the siblings of the other experimental groups for better controlled comparisons. The pups for each experiment were selected on a random basis, with the constraint that the mean body weights were not significantly different. The dams were maintained on a pelleted version of the same n-3-Adq diet throughout the experiment. The artificial rearing system was used for pup feeding until day 17. On days 17–29, artificial rat milk was fed in a 50 ml water bottle. The pups fed artificial rat milk from four separate experiments were sacrificed by decapitation at various ages between 0 and 29 days and their brains, retinas, and livers collected for lipid analysis. Stomach contents of dam-reared pups were collected to estimate fatty acid levels in dam's milk when the pups were 0, 10, and 20 days old. The tissues and stomach contents were stored in an ultracold freezer at −80°C until removal for lipid analysis. All animal procedures were approved by the National Institute on Alcohol Abuse Alcoholism Animal Care and Use Committee.

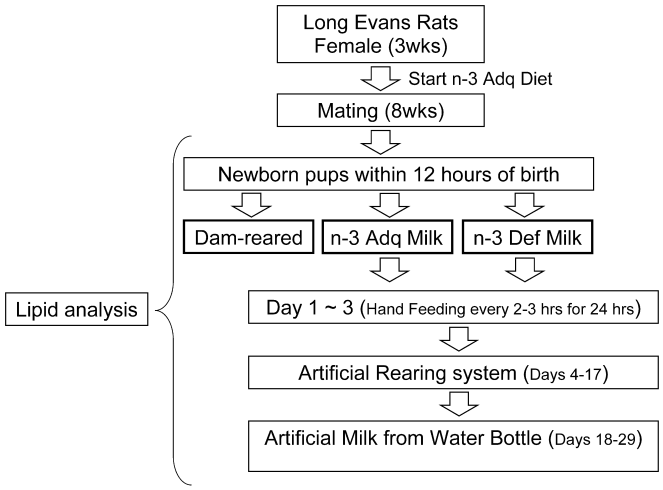

Fig. 1. Flow diagram illustrates study design.

Diets and artificial milk formula

The pelleted diet used for the dams was modified from the AIN-93G rodent diet (8). The maternal diet was an n-3-Adq diet (**Table 1**) containing safflower oil as a source of linoleic acid (LA; 18:2n6) and flaxseed oil as a source of LNA, (18:3n-3). The levels of LA and LNA, expressed as a percentage of total fatty acids, were 15.3 and 3.1%, respectively.

Preparation of the artificial rat milk was modeled after the method of Kanno et al. (4) with modification of the lipid and protein sources in order to minimize sources of n-3 fatty acids in the basal diet (**Table 2**). The only protein sources added to the milk were casein (Alacid 710) and whey protein isolate (Alacen 895, NZMP North America, Inc., Santa Rosa, CA). The fat

TABLE 1. Composition of maternal pelleted diet

	n-3-Adequate
	g/100 g diet
Ingredient	
Casein, vitamin-free[a]	20
Carbohydrate	60
Cornstarch	15
Sucrose	10
Dextrose	20
Maltose-dextrin	15
Cellulose	5
Mineral and salt mix[a]	3.5
Vitamin mix[a]	1
L-cystine	0.3
Choline bitartrate	0.25
TBHQ	0.002
Fat source	
Hydrogenated coconut oil	7.75
Safflower oil	1.77
Flaxseed oil	0.48
Fatty acid composition[b]	
Saturates	77.2
Monounsaturates	4.3
18:2n-6	15.3
18:3n-3	3.1
20:4n-6	0.02
22:6n-3	ND
n-6/n-3	4.91
18:2n-6/18:3n-3	4.90

ND, not detected; TBHQ, tert-butylhydroquinone.

[a] The casein (catalogue #100625), mineral-salt mix (catalogue #210025) and vitamin mix (catalogue #310025) were from Dyets, Inc.

[b] The 20:5n-3 and 22:5n-3 fatty acids were <0.01%.

TABLE 2. Composition of the artificial rat milk diets[a]

	n-3-Deficient	n-3-Adequate
	g/100 ml milk	
Ingredient		
Protein		
Whey protein isolated (Alacen 895)[b]	4.0	4.0
Acid casein (Alacid 710)[b]	6.3	6.3
Carbohydrate: α-lactose H_2O	1.89	1.89
Vitamin mix	0.87	0.87
Mineral mix	3.13	3.13
Micronutrients	0.1	0.1
Cholesterol	0.04	0.04
Fat source		
MCT oil[c]	1.56	1.56
Coconut oil, hydrogenated	3.24	3.24
Oleate ethyl ester	5.40	5.40
Linoleate ethyl ester	1.80	1.80
Linolenate ethyl ester	—	0.36
Fatty acid composition[d]		
Saturates	32.1	29.3
Monounsaturates	49.4	49.0
18:2n-6	17.1	17.2
18:3n-3	0.002	3.1
20:4n-6	ND[d]	ND[d]
22:6n-3	ND[d]	ND[d]
n-6/n-3	8,550	5.55

MCT, medium-chain triglyceride; ND, not detected.

[a] The two experimental milks, an n-3 fatty acid-adequate diet and an n-3 fatty acid-deficient diet, were based on the Kanno et al. (4) formulation, modified to obtain the extremely low basal level of n-3 fatty acids.

[b] Alacen 895 and Alacid 710 (NZMP, North America Inc., Santa Rosa, CA) included amino acids (g/100 g) alanine (6.0, 3.0), arginine (2.6, 3.7), aspartic acid (12.1, 6.9), cystine/cysteine (3.6, 0.4), glutamic acid (18.0, 20.9), glycine (1.8, 1.8), histidine (2.1, 2.9), isoleucine (5.9, 4.6), leucine (14.4, 9.1), lysine (11.5, 7.7), methionine (2.5, 2.9), phenylalanine (3.9, 5.1), proline (5.2, 10.4), serine (3.6, 5.8), threonine (5.3, 4.3), tryptophan (2.5, 1.2), tyrosine (4.1, 5.5), and valine (5.6, 5.7).

[c] Mead Johnson Nutritionals, Evansville, IN.

[d] Fatty acids were expressed as wt%; ND indicates <0.01%. The 20:5n-3 and 22:5n-3 fatty acids were less than 0.01%.

sources used in the basal artificial milk were medium-chain triglyceride oil and hydrogenated coconut oil. Unsaturated fatty acids were added as purified ethyl ester compounds (Nu-Chek Prep, Elysian, MN) including oleate, LA, and LNA ethyl ester. The n-3 fatty acid level in the n-3-Def milk was less than 0.002% of total fatty acids. The n-3 fatty acids in the n-3-Adq milk were 3.1% in the form of LNA. The fatty acid data in Tables 1 and 2 represent actual gas-liquid chromatography (GC) analyses of the entire diet (and not theoretical values or analyses of just the lipid mixtures).

Artificial rearing procedure

The artificial rearing procedure was modified from a method first reported by Hoshiba (6). Briefly, this system made use of an artificial milk flow unit (milk-pumping system) that includes six nursing bottles and a rearing box maintained at 33°C by a warm-water circulation pump (**Fig. 2**). Up to five rat pups can be accommodated in each rearing box, and two to four boxes were used per experiment. The nursing bottle has three tubes (fill tube, outlet tube, and vent tube) and nipples, which are made of a silicone rubber. The nipple consists of inner and outer parts and a stopper devised to control pressure as well as to avoid the leaking of milk. The newborn male rats began the artificial feeding regime within 12 h of birth. There was an initial training period, during which they were hand fed via nursing bottles every 2–3 h for the first 3 days. The system was designed so that rats could use their natural feeding behavior, i.e., they lay on their backs and drew the milk in from the nipple thru suction. They were allowed as much milk as they wanted in each feeding session. Between feedings, they were placed in the rearing box fitted with six nursing bottles in order to become acclimated to the nursing bottles as a source of milk. Each pup was stimulated on the abdomen and the anal region in order to induce excretion prior to each feeding. This procedure was repeated every 3 h for 12 h (9 AM–9 PM) after the training period.

Fresh milk was exchanged for the milk in the nursing bottles by pumping for 2 min every half-hour. After the training period, nearly all of the pups acquired the method of drinking artificial milk from nursing bottles in the rearing box. Nipple sizes of nursing bottles were increased as the pups grew (four sizes were used). Pelleted diets were introduced about the time that teeth began to appear (14–17 days); these pellets maintained the n-3-Adq or n-3-Def diet fatty acyl distributions of the milk as previously described (7).

Lipid extraction, transmethylation, and gas chromatography

Tissue samples were thawed, weighed, and homogenized in methanol-hexane, and methylated in acetyl chloride according to the method of Lepage and Roy (9). Varying amounts of methyl docosatrienoate (22:3n-3) for brain, retina, and liver, and methyl heneicosanoate (21:0) for the stomach contents of dam-reared pups were added as internal standards to each sample to compensate for differences in tissue weight and lipid concentration. As an aid to avoiding lipid oxidation during the procedures, 50 μg/ml butylated hydroxytoluene was added to the methanol-hexane homogenizing solvent. The hexane extracts were concentrated to a small volume with a stream of nitrogen and transferred to microvials for GC injection.

Fatty acid methyl esters were analyzed with an HP-5890B gas chromatograph equipped with a flame ionization detector (Agilent, Palo Alto, CA) and a fused silica capillary column (DB-FFAP, 30 m × 0.25 mm inner diameter x 0.25 μm film thickness; J and W Scientific, Folsom, CA). The detector and injector temperatures were set to 250°C. The oven temperature program began at 130°C and increased to 175°C at 4°C/min, then increased at 1°C/min to 210°C, and finally increased at 30°C/min to 245°C, with a final hold for 15 min. Hydrogen was used as carrier gas at a linear velocity of 50 cm/sec. A custom-mixed, 30 component, quantitative methyl ester standard containing 10–24 carbons and 0–6 double bonds was used for assignment of retention times and to ensure accurate quantification (Nu-Chek Prep 462, Elysian, MN). Fatty acid data were expressed as percent of total peak area, which corresponded to weight % to within 5%, as demonstrated by quantitative standard mixtures. Internal standards were used to calculate tissue fatty acid concentrations.

Analysis of data

The time course curves for DHA and docosapentaenoic acid (DPAn-6) data from each tissue were fitted by regression analysis using the Sigma Plot program (SPSS Science Inc., Chicago, IL). Data on the stomach contents of dam-reared pups are expressed as mean ± SEM. These fatty acid data were analyzed by one-way ANOVA using Statistica (Statsoft, Tulsa, OK). Body weight differences between the artificially reared and the dam-reared groups were compared at discrete time points using Student's *t*-test.

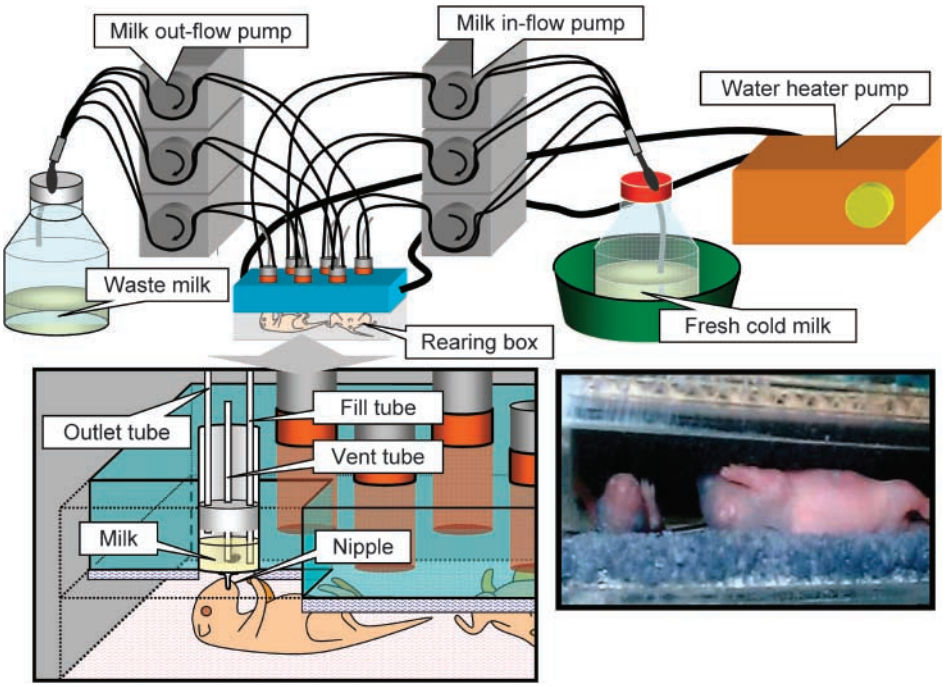

Fig. 2. The artificial rearing method. The artificial rearing procedure was modified from a method by Hoshiba (5, 6).

RESULTS

Maternal diet experiment

In the past, two-generation models have generally involved an extended feeding period for the dam, often extending back to weaning (10–14). It would greatly shorten the experiment if the controlled feeding could begin after conception, so that time-pregnant dams could be obtained commercially. Thus, an initial experiment was performed to determine whether the length of the feeding of our controlled diet to the dam resulted in changes to the dam milk or pup brain fatty acid composition. Generally, in these experiments, females have been obtained at 3 weeks of age and fed the n-3-Adq diet throughout their growth to adulthood so as to include mating, pregnancy, and lactational periods. As an alternative, in order to shorten this long and expensive process, time-pregnant dams were obtained as early as possible from a commercial supplier, on day 3 of gestation, and immediately switched to the n-3-Adq diet. At birth, the milk of dams prepared by these two different methods was compared by collecting the stomach contents of the pups. This revealed a slight increase in DHA (23%, $P < 0.05$) and arachidonic acid (AA) (15%, $P < 0.05$) in the pup stomach contents from the time-pregnant dams. There were no significant changes in the brain fatty acid composition of newborn pups from dams with differing lengths of controlled diet intake. By 10 days of age, there were only very minor differences in the milk fatty acyl content of pup stomach contents from the two sets of dams. However, the pup brains of the time-pregnant group at 10 days exhibited increased percentages of AA (7%, $P < 0.01$), docosatetraenoic acid (13%, $P < 0.01$), and DHA (14%, $P < 0.01$). In order to explain these small but significant differences, the commercial diet fed the time-pregnant dams was analyzed. It was observed that the chow diet used (Purina 5L79) contained fish meal and significant percentages of AA (0.16%), eicosapentaenoic acid (EPA) (1.03%), and DHA (0.53%) expressed as the percentage of total fatty acids. Thus, the history of the maternal feeding could be demonstrated to have a measurable effect under these particular circumstances; however, the differences were minimal.

Artificial rearing

The body weights of the artificially reared rats were generally less than those of the dam-reared animals during the initial milk-feeding phase. For example, at day 20, the body weights of the artificially reared animals were 43.4 ± 1.8 g and those of the dam-reared were 60.6 ± 3.5 g ($t(5) = 2.94$, $P < 0.05$). However, after 29 days, there was no difference in body weight between the dam-reared and the artificially reared pups (dam-reared, 109.7 ± 3.9 g; artificially reared, 106.1 ± 0.3 g; $t(5) = 0.56$, $P = 0.60$). There were no differences in body weight between the two artificially reared groups related to the n-3 content of the diet. Artificial rearing led to some difficulties with intestinal bloating that led to death in some cases. This difficulty was believed to be related to excessive air intake; the bacterial milieu of the milk and the rat tissues was tested but revealed no unusual flora. The artificial rat milk diets employed ethyl esters of unsaturated fatty acids so as to maintain a very low level of n-3 fatty acids (Table 2). Also, sources of proteins were tested, and those with the lowest

TABLE 3. Fatty acyl composition of the brain, retina and liver in newborn rats[a]

Fatty Acid	Brain (n = 8)	Retina (n = 6)	Liver (n = 8)
Saturated			
10:0	0.09 ± 0.01	0.24 ± 0.09	0.19 ± 0.02
12:0	0.17 ± 0.02	0.78 ± 0.13	3.27 ± 0.21
14:0	1.83 ± 0.04	3.01 ± 0.27	4.23 ± 0.15
16:0 DMA	1.91 ± 0.02	1.13 ± 0.32	0.16 ± 0.01
16:0	26.43 ± 0.10	22.80 ± 0.68	24.10 ± 0.56
18:0 DMA	1.54 ± 0.04	0.63 ± 0.14	0.13 ± 0.01
18:0	15.78 ± 0.07	15.02 ± 0.66	8.17 ± 0.39
20:0	0.13 ± 0.003	0.44 ± 0.06	0.07 ± 0.01
22:0	0.09 ± 0.01	0.37 ± 0.08	0.14 ± 0.01
24:0	0.12 ± 0.01	0.44 ± 0.08	0.20 ± 0.01
Total saturated	48.10 ± 0.14	45.06 ± 0.83	40.87 ± 0.55
Monounsaturated			
16:1n-7	1.74 ± 0.03	4.29 ± 0.34	2.56 ± 0.10
18:1 DMA	0.36 ± 0.01	1.18 ± 0.44	0.06 ± 0.004
18:1n-9	10.55 ± 0.08	15.57 ± 0.66	15.65 ± 0.71
18:1n-7	2.87 ± 0.04	4.35 ± 0.43	3.23 ± 0.09
20:1n-9	0.25 ± 0.01	0.47 ± 0.05	0.34 ± 0.01
22:1n-9	0.03 ± 0.01	ND	0.04 ± 0.003
24:1n-9	0.21 ± 0.01	0.75 ± 0.10	0.12 ± 0.02
Total monounsaturated	16.01 ± 0.08	26.20 ± 0.67	22.00 ± 0.84
n-6			
18:2n-6	0.50 ± 0.01	2.23 ± 0.88	6.51 ± 0.13
18:3n-6	ND	0.28 ± 0.05	0.49 ± 0.06
20:2n-6	0.07 ± 0.003	0.13 ± 0.02	0.34 ± 0.02
20:3n-6	0.28 ± 0.01	0.27 ± 0.02	0.60 ± 0.03
20:4n-6	11.08 ± 0.13	7.96 ± 1.03	11.05 ± 0.54
22:2n-6	0.07 ± 0.01	0.42 ± 0.05	ND
22:4n-6	2.87 ± 0.12	1.82 ± 0.20	2.03 ± 0.12
22:5n-6	2.98 ± 0.15	1.63 ± 0.25	1.42 ± 0.11
Total n-6	17.85 ± 0.15	14.63 ± 0.71	22.44 ± 0.72
n-3			
18:3n-3	ND	ND	0.24 ± 0.01
20:5n-3	ND	ND	0.83 ± 0.05
22:5n-3	0.22 ± 0.01	0.30 ± 0.04	1.38 ± 0.05
22:6n-3	10.45 ± 0.24	4.71 ± 0.57	7.62 ± 0.36
Total n-3	10.96 ± 0.23	5.26 ± 0.57	10.06 ± 0.39
22:5n-6/22:6n-3	0.29 ± 0.01	0.29 ± 0.04	0.19 ± 0.01
22:5n-6+22:6n-3	13.42 ± 0.34	5.06 ± 1.09	9.03 ± 0.42
n-6 + n-3	28.81 ± 0.28	18.04 ± 1.22	32.50 ± 1.09
Total fatty acids (μg/mg)	17.29 ± 0.42	9.06 ± 1.53	50.35 ± 3.14

ND, not detected (i.e., <0.01%). Each parameter is presented as the mean ± SEM.
[a]Wt% of total fatty acids.

levels of n-3 polyunsaturates were used. Although the pelleted diet did not employ ethyl ester sources of unsaturated fatty acids, the n-3 level was also very low, and this diet was used only for the last 9 days.

Brain

The pup brain DHA and DPAn-6 levels at the time of birth were 10.45 ± 0.24% and 2.98 ± 0.15%, respectively (**Table 3**). Thereafter, the DHA level in the dam-reared group increased gradually and reached 11.88 ± 0.32% (n = 5) at 29 days of age. The time course curve for the brain DHA in the n-3-Adq group was very similar to that for the dam-reared group, with its level rising to ~12% (**Fig. 3**). On the other hand, the brain DHA level of the n-3-Def group decreased rapidly from about day 10 to day 17 and then continued to gradually decrease percentagewise to a level of 3.4% at the end of the experiment. The brain DPAn-6 of the dam-reared and n-3-Adq groups decreased gradually to less than 1% by 29 days of age. For example, the brain DPAn-6 in the dam-reared group at 29 days was 0.86 ± 0.02% (n = 5). The level of brain DPAn-6 in the n-3-Def group showed a rapid increase after 5 days of age and was ~9.0% at 29 days (Fig. 3).

Retina

The pattern of DHA development in the retina was interesting, and distinct from that seen in the brain. Whereas the brain began postnatal life with a nearly adult percentage of DHA, the retina at birth contained only 4.71 ± 0.57% of DHA (Table 3). In the dam-reared group, there appeared to be a linear increase in DHA until ~10 days of age (**Fig. 4**). Eye opening at ~12–14 days of age was accompanied by a jump in the DHA level in the 10–14 day period, with the level increasing by 15 percentage points within 5 days. The increase in the percentage of retinal DHA appeared to be sigmoid, reaching its adult level by the end of the experiment at 29 days. There was a slight lag in the DHA increase in the n-3-Adq group, but the same level was reached by 29 days, and the curves were

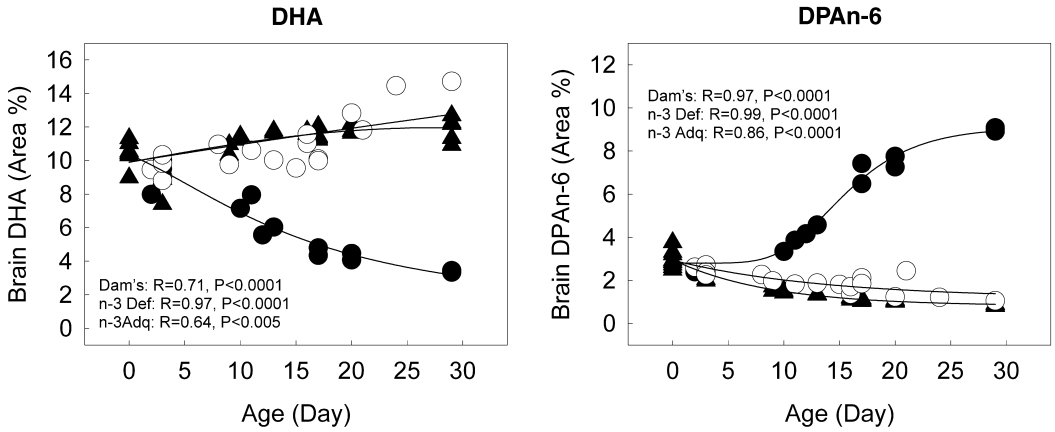

Fig. 3. Alteration of the docosahexaenoic acid (DHA) and the docosapentaenoic acid (DPAn-6) in brain during the artificial rearing. Newborn male rats were given n-3-deficient (closed circles) and n-3-adequate (open circles) artificial formulas within 12 h of birth. The closed triangles represent the dam's milk group as reference points. The *R* values represent the correlation coefficients for the fitted curves.

similar. The DPAn-6 in these two groups began low at birth (1.63 ± 0.25%) and remained constant. In the n-3-Def group, there was also a slight increase in DHA up to ~10 days of age; however, in the period of eye opening and afterwards, there was no further increase in the retinal DHA. After day 13, the DPAn-6 in the n-3-Def group rose quickly to 28.4% at day 29.

Liver

The liver DHA in the dam-reared group was 7.62 ± 0.36% at birth (Table 3) but had decreased to ~4% of fatty acids by day 20 (**Fig. 5**). There was more biological variability in the liver; for example, between days 5 and 15, values in the n-3-Adq group ranged from ~4% to 11%. Thereafter, there appeared to be a slight decline in the DHA level, ending at 4.4% at 29 days. A smooth curve was obtained for the liver DHA in the n-3-Def group, which exhibited a decline by day 10 to ~1%. The curve asymptotically declined thereafter, falling to ~0.3% at day 29. The DPAn-6 in the dam-reared and n-3-Adq groups gradually and linearly declined from a low initial level (1.42 ± 0.11%). However, the liver DPAn-6 in the n-3-Def group followed a time course curve much like that of the retina, inasmuch as it increased linearly at first and then with increasing slope.

Stomach contents in dam-reared group

An effort was made to assess the diet of the dam-reared pups by collecting the curdled contents of their stomachs. Rat pups were sacrificed in the morning after suckling from their dams during the night. The stomach contents on the first day after birth contained very high levels of the n-6 polyunsaturates AA, docosatetraenoic acid, and DPAn-6, as well as significant elevations in the n-3 polyunsaturates EPA and DHA (**Table 4**). The AA and DHA percentages in the stomach contents were 3.7% and 1.2% of total fatty acids on the first day of life, respectively. By day 10, however, the long-chain polyunsaturated content had

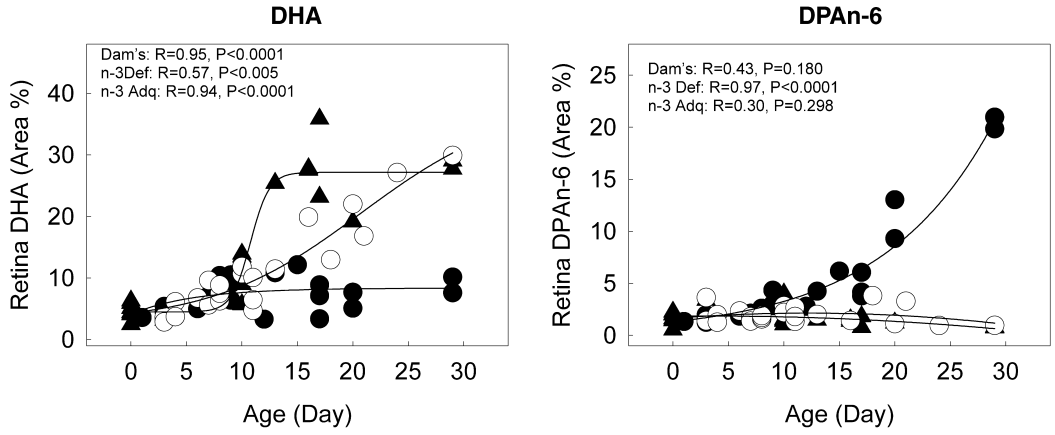

Fig. 4. Alteration of DHA and DPAn-6 in retina during artificial rearing. Newborn male rats were given n-3-deficient (closed circles) and n-3-adequate (open circles) artificial formulas within 12 h of birth. The closed triangles represent the dam's milk group as reference points. The *R* values represent the correlation coefficients for the fitted curves.

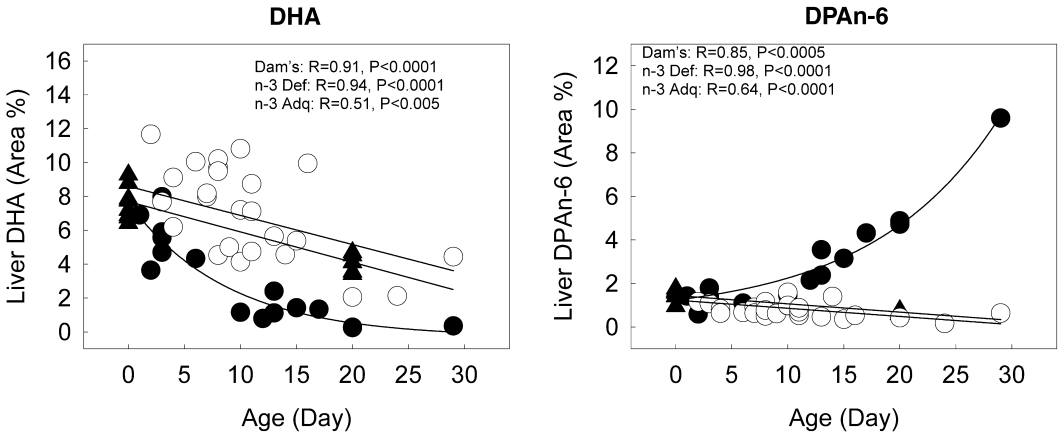

Fig. 5. Alteration of DHA and DPAn-6 in liver during artificial rearing. Newborn male rats were given n-3-deficient (closed circles) and n-3-adequate (open circles) artificial formulas within 12 h of birth. The closed triangles represent the dam's milk group as reference points. The *R* values represent the correlation coefficients for the fitted curves.

fallen precipitously, with AA, DPAn-6, and DHA all falling to ~20% or less of their values on day 1. LA was constant, but LNA increased significantly. This pattern continued in the day 20 stomach content samples, with long-chain polyunsaturates decreasing further and LNA increasing further. The percentage of monounsaturated fatty acid fell, whereas the saturates increased significantly between days 0 and 10. It should be noted that the absolute concentration of total fatty acids was falling precipitously, inasmuch as it fell to about half of the birth level by 20 days of age.

DISCUSSION

In the present experiment, rat pups were successfully reared on artificial milk formula from the first day of life. Pups fed an n-3-Def formula exhibited a loss of more than 70% of their brain DHA by 29 days of age, replacing it primarily with DPAn-6. Similarly, the retinal level of DHA was ~69% lower in the pups fed the n-3-Def milk formula relative to those fed the same formula to which 3.1% LNA had been added. If this diet were to be continued, these low levels of nervous system DHA could be maintained into adulthood.

TABLE 4. Fatty acid composition of dam-reared pup stomach contents at 0, 10, and 20 days after birth (wt% of total fatty acids)[a]

	Pups' Stomach Contents		
Fatty Acids	Day 0 (n = 4)	Day 10 (n = 4)	Day 20 (n = 5)
Total saturates	49.32 ± 0.94	70.00 ± 0.24[b]	66.19 ± 2.05[b]
Total monounsaturates	29.90 ± 1.03	17.75 ± 0.19[b]	18.41 ± 2.42[b]
n-6			
18:2n-6 (LA)	6.44 ± 0.19	6.86 ± 0.05	7.86 ± 0.38[a]
20:4n-6 (AA)	3.73 ± 0.14	0.72 ± 0.01[c]	0.43 ± 0.03[c]
22:4n-6 (DTA)	1.59 ± 0.14	0.26 ± 0.003[c]	0.09 ± 0.01[c]
22:5n-6 (DPAn-6)	0.39 ± 0.06	0.04 ± 0.001[c]	0.04 ± 0.003[c]
Total n-6	13.92 ± 0.31	8.72 ± 0.07[c]	8.77 ± 0.38[c]
n-3			
18:3n-3 (LNA)	0.48 ± 0.02	0.90 ± 0.003[c,e]	1.31 ± 0.07[b]
20:5n-3 (EPA)	0.55 ± 0.03	0.18 ± 0.003[c,d]	0.10 ± 0.01[c]
22:6n-3 (DHA)	1.21 ± 0.04	0.24 ± 0.003[c]	0.16 ± 0.01[c]
Total n-3	2.99 ± 0.07	1.58 ± 0.01[c]	1.68 ± 0.07[c]
n-6/n-3	4.66 ± 0.04	5.51 ± 0.02[c]	5.22 ± 0.11[b]
DPAn-6/DHA	0.32 ± 0.04	0.15 ± 0.001[b]	0.22 ± 0.01[a]
Total fatty acids (μg/mg)	413.5 ± 97.3	286.86 ± 7.4	197.24 ± 23.8

AA, arachidonic acid; DHA, docosahexaenoic acid; DPAn-6, docosapentaenoic acid; DTA, docosatetraenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; LNA, linolenic acid. Each parameter is presented as the mean ± SEM for 4–5 pups from different dams.

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$ compared with day 0 group.
[d] $P < 0.05$.
[e] $P < 0.001$ compared with day 20 group (Tukey HSD test after one-way ANOVA).

It was of interest that the dam-reared animals were receiving preformed DHA from their dams (as evidenced by their stomach contents) even though the dams had been fed a diet with LNA as the sole source of n-3 fatty acids for an extended period. Thus, the dam-reared pups received DHA, whereas the artificially reared pups maintained on the n-3-Adq diet did not. However, the level of LNA in the stomach contents of the dam-reared pups (0.5–1.3%) was lower than that fed to the n-3-Adq pups (3.1%). These factors may in some manner counterbalance each other. Still, the initially slightly higher levels of DHA observed in the nervous system in the dam-reared pups relative to the n-3-Adq pups were likely due to the presence of preformed DHA in their diets.

An important issue is whether this "first generation" model can generate large enough losses in nervous system DHA so that functional changes can be observed. Few such studies are available, but Lim et al. (7) have recently shown, in an experiment similar in design to the present one, that adult rats with a 70% loss in brain DHA performed more poorly in spatial tasks. The degree of n-3 deficiency here can be compared with other two- or three-generational studies in which functional effects were observed. For example, Weisinger et al. (13) were able to show that there was a loss in retinal sensitivity and b-wave implicit times in rats after three generations of n-3 deficiency. However, these rats had only a 55% loss of retinal DHA, probably owing to their age and the presumed slow accretion of n-3 fatty acids from the periphery, and showed no differences in a-wave amplitudes or in most of the phototransduction parameters measured. Wainwright et al. (14, 15) reported that rats with ~40–50% loss of brain DHA had no alteration in spatial task performance. Also, Moriguchi and Salem (10) have recently demonstrated, in a DHA repletion paradigm, that spatial task performance is altered when the brain level of DHA declines by 40% or more. It thus appears that many of the available measures of retinal physiology and brain behavior generally require a 50% decrease or more in brain or retinal DHA to observe a loss in function. The artificial rearing method presented here achieves a somewhat greater loss in DHA than this and so may be expected to produce animals that exhibit functional alterations of the nervous system.

It was of great interest that the percentage of brain DHA at birth was high whereas the retinal value was relatively low (cf. Figs. 3, 4). Thus, the time courses for these two nervous system organs with respect to DHA content are quite distinct. The "normal" retinal development entails a steady accretion of DHA, as reflected by the steady rise in the percentage of DHA. In the case of n-3 deficiency, however, retinal DHA remains near the low level characteristic at birth. The "normal" pattern of brain accretion of DHA, however, is one in which there is only a slight increase in the percentage of DHA during the first 4 weeks of development. When n-3 fatty acids are not fed, there is a rapid diminution in the percentage of brain DHA in the first 3 weeks of life.

It must be noted here that the percentages of DHA in various organs at birth has little meaning without reference to the maternal diet during gestation. One example of this was shown in the higher levels of milk AA and DHA on the first day of birth in pups of time-pregnant dams who had been fed EPA and DHA prior to pregnancy. This is evidence that the maternal body complement of these long-chain polyunsaturates influences the dam's ability to output AA and DHA in milk. The controlled maternal diet employed in this study was the n-3-Adq diet that contained 3.1% of fatty acids as LNA (and no EPA or DHA). This diet was chosen to provide an ample source of n-3 fatty acids during fetal development so that pups would be born with a supply of tissue DHA and other n-3 fatty acids. This situation models fairly well that of humans in the Western world, where most of the n-3 fatty acids are supplied by LNA. It would thus be expected that human infants who receive vegetable oil-based formulas in which the essential fatty acids are supplied by corn oil or safflower oil would exhibit a marked decline in brain (16–18) and retinal DHA within the first months of life, similar to that observed for the n-3-Def rats in the present study.

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