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Behavioral Deficits Associated with Dietary Induction of Decreased Brain Docosahexaenoic Acid Concentration

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Abstract: Docosahexaenoic acid (DHA), an n-3 fatty acid, is rapidly deposited during the period of rapid brain development. The influence of n-3 fatty acid deficiency on learning performance in adult rats over two generations was investigated. Rats were fed either an n-3 fatty acid-adequate (n-3 Adq) or -deficient (n-3 Def) diet for three generations (F1–F3). Levels of total brain n-3 fatty acids were reduced in the n-3 Def group by 83 and 87% in the F2 and F3 generations, respectively. In the Morris water maze, the n-3 Def group showed a longer escape latency and delayed acquisition of this task compared with the n-3 Adq group in both generations. The acquisition and memory levels of the n-3 Def group in the F3 generation seemed to be lower than that of the F2 generation. The 22:5n-6/22:6n-3 ratio in the frontal cortex and dams' milk was markedly increased in the n-3 Def group, and this ratio was significantly higher in the F3 generation compared with the F2 generation. These results suggest that learning and cognitive behavior are related to brain DHA status, which, in turn, is related to the levels of the milk/dietary n-3 fatty acids. **Key Words:** n-3 polyunsaturated fatty acid deficiency—Docosahexaenoic acid—Brain fatty acid composition—Morris water maze—Learning ability—Rat.
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Docosahexaenoic acid (DHA; 22:6n-3) is highly concentrated in the adult mammalian nervous system and is rapidly accreted during the brain growth spurt in the perinatal and early postnatal period (Salem et al., 1976; Clandinin et al., 1980; Martinez, 1992; Green et al., 1999). Several randomized, controlled trials, where the key variable is addition of DHA or DHA and arachidonic acid (20:4n-6) to infant formula, indicate that the supplement supports improved performance in visual acuity tasks (Uauy et al., 1990; Birch et al., 1992; Carlson et al., 1993; Makrides et al., 1995), visual recognition memory (Carlson and Werkman, 1996), learning a means-end problem-solving task (Willats et al., 1998), and higher Bayley mental developmental index (Carlson et al., 1994; Birch et al., 2000) or developmental quotient scores (Agostoni et al., 1995). However, other trials have shown no benefit in neural function for long-chain polyunsaturate supplements (Innis et al., 1994; Auestad et al., 1997; Jorgensen et al., 1998; Scott et al., 1998; Makrides et al., 2000). Although arachidonic acid and DHA are always present in human milk (Koletzko et al., 1992; Jensen, 1999), the issue of whether their addition to infant formula is necessary to promote optimal function is still undecided in North America.

Animal models of n-3 fatty acid deficiency can be used to help define the nature and extent of the functional deficits in the nervous system that are associated with this dietary treatment. In these studies, a vegetable oil low in α-linolenic acid such as safflower oil is given to rats for two generations to induce a significant decline in levels of brain and retinal DHA (Tinoco, 1982). In this manner, the variable to be studied may be magnified and behavioral consequences may be more easily discerned. It has previously been observed in such studies that rats on a diet low in n-3 fats perform more poorly in brightness discrimination (Yamamoto et al., 1987, 1988) and shock avoidance tasks (Bourre et al., 1989; Umezawa et al., 1995). Differences in exploratory behavior (Enslen et al., 1991) and elevated plus-maze performance (Nakashima et al., 1993) have also been observed. Some researchers have reported poorer performance in water maze tasks (Coscina et al., 1985; Nakashima et al., 1993; Frances et al., 1996; Jensen et al., 1996; Wainwright et al., 1998), whereas others have found no differences in spatial task performance (Wainwright et al., 1994, 1997). As Wainwright (1992) has pointed out, carefully controlled behavioral experiments are required with adequate numbers of animals. In addition, a carefully controlled dietary regimen is needed involving only one fatty acid substitution, e.g., α-linolenic acid for oleic acid. Furthermore, comparisons of various behavioral

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Abbreviations used: DHA, docosahexaenoic acid; n-3 Adq and n-3 Def, n-3 fatty acid-adequate and -deficient, respectively.

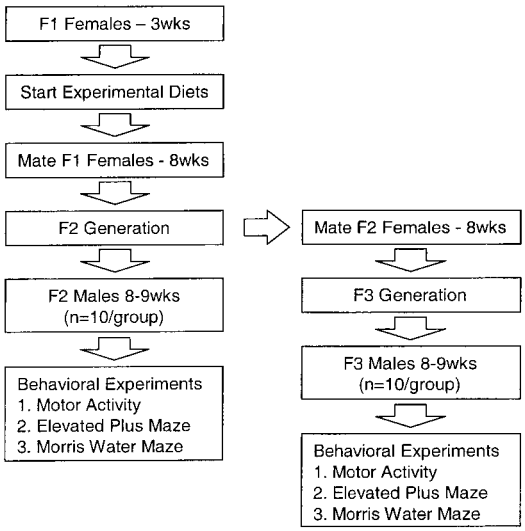

FIG. 1. Flow diagram illustrates study design. The block "Start Experimental Diets" indicates the point at which the dams were separated into two experimental groups, i.e., the n-3 Adq and n-3 Def diet groups. Thereafter, animals were maintained on their respective diets through two generations and tested as indicated.

experiments are complicated by the degree to which brain DHA deficiency is achieved. A persistent but unrecognized problem has been the introduction of n-3 fatty acids in other nonlipid dietary constituents, thus at least partially preventing the brain biochemical alterations to be studied.

In the present experiment, our purpose was to examine the influence of n-3 fatty acid deficiency induced by carefully controlled diet formulations on tasks related to spatial learning and memory in adult rats over two generations. The motor activity test, elevated plus-maze test, and Morris water maze test were used for this purpose.

MATERIALS AND METHODS

Animals and study design

This experimental protocol was approved by the Animal Care and Use Committee of the National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health. Female Long–Evans rats, at 3 weeks of age, were obtained from Charles River (Portage, MI, U.S.A.) and maintained in our animal facility under conventional conditions with controlled temperature (23 ± 1°C) and illumination (12 h; 6:00–18:00).

The flow diagram for our study design is shown in Fig. 1. Weanling females were divided into two groups and placed on one of two experimental diets (see below). Diet and water were provided ad libitum. At 8 weeks of age, these females (F1 generation) were mated with 12-week-old males. Their pups (F2 generation) were weaned to the experimental diet of the dam. When the F2 females were 8 weeks old, they were then mated with males. Their pups (F3 generation) were also weaned to the experimental diet of the dam. Behavioral experiments were conducted when the male rats, in the F2 and F3 generation, were 8–9 weeks of age. Ten males in each group obtained from five or six litters were used for the behavioral experiments.

Experimental diets

The two experimental diets, an n-3 fatty acid-adequate (n-3 Adq) and an n-3 fatty acid-deficient (n-3 Def) diet, were based on the AIN-93 (Reeves et al., 1993) formulation with several modifications to obtain the extremely low n-3 fatty acid level required in this study (Table 1). The custom diets were obtained commercially (Dyets, Bethlehem, PA, U.S.A.). All modifications to the basal diet, such as cornstarch and casein, were made in both dietary formulations. The major difference between the n-3 Def and the n-3 Adq diets was the amount of n-3 fatty acids. This was achieved by adding a small amount of flaxseed oil to the n-3 Adq diet. The fat content in both diets was 10 g/100 g, and the amount of n-3 fatty acids in the n-3 Def and n-3 Adq diets were 0.04 and 3.1% of total fatty acids, respectively (Table 1). There was no difference in the total n-6 fatty acids between the two diets (n-3 Def, 15.1%; n-3 Adq, 15.3%). The rats were maintained on these diets until they were killed.

Behavioral experiments

Male adult rats, in the two groups, were tested in the motor activity test, elevated plus-maze, and Morris water maze; in all cases, the activity of the animals was recorded using a video image analyzer (Videomax V; Columbus Instruments, Columbus, OH, U.S.A.).

Motor activity test. Each rat was individually placed into a cage (25 × 45 × 20 cm), and the ambulatory time and the moving distance were measured for 30 min. Motor activity was measured at the same time of day on every morning for 5 consecutive days.

TABLE 1. *Composition of experimental diets*

Ingredient	Amount (g/100 g of diet)	
	n-3 Def	n-3 Adq
Casein, vitamin-free	20	20
Carbohydrate	60	60
Cornstarch	15	15
Sucrose	10	10
Dextrose	19.9	19.9
Maltose-dextrin	15	15
Cellulose	5	5
Salt mix	3.5	3.5
Vitamin mix	1	1
L-Cystine	0.3	0.3
Choline bitartrate	0.25	0.25
TBHQ	0.002	0.002
Fat	10	10
Hydrogenated coconut oil	8.1	7.75
Safflower oil	1.9	1.77
Flaxseed oil	None	0.48
Fatty acid composition, %[a]		
18:2n-6	15.1	15.3
18:3n-3	0.04	3.1
20:4n-6	ND	0.02
n-6/n-3	378	4.9
18:2n-6/18:3n-3	377	4.9

The two experimental diets—n-3 Adq and n-3 Def—were based on the AIN-93 (Reeves et al., 1993) formulation with several modifications to obtain the extremely low basal level of n-3 fatty acid required in this study. TBHQ, *tert*-butylhydroquinone.

[a] The 20:5n-3, 22:5n-3, and 22:6n-3 fatty acids could not be detected (ND) in these diets, i.e., they were <0.01%.

Elevated plus-maze test. The elevated plus-maze test has been widely used as a measure of anxiety, and the method used was as described elsewhere (File et al., 1993; File and Gonzalez, 1996). The plus-maze consists of two opposite open arms (45 × 15 cm) and two opposite enclosed arms. The arms are connected by a central 15- × 15-cm square, and thus the maze forms a plus shape. The maze was elevated 70 cm above the floor. The animal's path was observed for 5 min after a resting period of 1 min in the central square of the maze. This measurement was repeated for 2 days. The number of entries to the open arms and the visiting time of the open arm were measured.

Morris water maze test. The Morris water maze is used for evaluating the spatial learning performance of rodents (Morris et al., 1982; Morris, 1984). A circular pool (4 feet in diameter and 2 feet deep) containing tap water, filled to within 10 cm from the top, is placed in a setting where various prominent cues, e.g., a metal board on the wall, a prominent door, were arranged. Water temperature was maintained at 20 ± 1°C. The swimming area was arbitrarily divided into four quadrant regions (regions A–D), and two starting points were arranged at the corners of the quadrant rim, which were located diagonally from the platform. To acclimate the rats to the swimming task, they were allowed to swim in the water for 3 min on the day before starting the water maze test. The next day, the rats were subjected to a visible trial to test eyesight. A black (visible) escape platform (10 cm in diameter) was placed in quadrant region A in a circular pool. Its top surface was higher than the water level by 1.0 cm. Each rat was allowed to stay on the platform for 30 s after reaching it (successful rat). If the rat failed to find the platform within 90 s, it was gently placed on the platform for 30 s. On the second day, a transparent platform (hidden platform) was used in place of the black platform, and the height was 1.0 cm below the surface of the water (learning trial). As a result of these two changes, the swimming rat could not see the platform. At the start of the testing period, the rat was placed into the pool from one of the two starting points used in the visible trial. Each rat received two trials per day (session) and was randomly placed at two different starting points with 5-min intervals between each trial. The time required for a rat to reach the hidden platform (escape latency), swimming time, swimming speed, the duration of the immobilized state (resting time), and swimming path were automatically digitized and recorded by computer. Sessions were repeated for four or six consecutive days. On the day following the last session, the platform was removed, and the rat was allowed to search for the platform for 90 s (memory retention trial). The number of crossings of the position where the platform had been placed (quadrant region A) and the number of crossings in the corresponding imaginary positions in the other quadrant regions (regions B–D) were recorded.

Lipid composition

After the behavior experiments, the rats were killed by decapitation. The brains were removed and stored frozen at −80°C. Moreover, at 15 days of life, the extra pups from the same litters as the tested males were used to analyze milk samples from the dams. The milk was collected from the stomachs of these pups and stored at −80°C. The total lipid composition of the frontal cortex in the brain and the total lipid composition of the milk were determined by a modification of the Folch method (Schwertner and Mosser, 1993). The total lipid extracts were transmethylated with 14% BF_3-methanol at 100°C for 60 min by a modification of the method of Morrison and Smith (1959). Methyl esters were then analyzed on a gas chromatograph (model 5890/series II; Hewlett-Packard, Palo Alto, CA, U.S.A.) equipped with a flame ionization detector and fused silica capillary column (DB-FFAP; 30 m × 0.25 mm × 0.25 μm; J&W, Folsom, CA, U.S.A.) with carrier gas (hydrogen) at a linear velocity of 50 cm/s. Injector and detector temperatures were set to 250°C, and the oven temperature program was as follows: 130 to 175°C at 4°C/min, 175 to 210°C at 1°C/min, and then to 245°C at 30°C/min, with a final hold for 15 min. The fatty acid methyl esters from 10:0 to 24:1n-9 were identified by comparison with the retention times of a standard mixture (462; Nu-Chek-Prep, Elysian, MN, U.S.A.). The concentrations of individual and total fatty acids were obtained using an internal standard (frontal cortex, 10 μg of 22:3n-3 free fatty acid; milk, 50 μg of 21:0 free fatty acid).

Statistical analysis

All data were analyzed using Statistica (Statsoft, Tulsa, OK, U.S.A.). The number of successful rats that reached the hidden platform in the visible trial or at least one trial in the first learning trial day were analyzed using Fisher's exact probability test. Other parameters in the behavioral experiments and lipid composition were analyzed by one- and two-way ANOVA or Student's *t* test.

RESULTS

In both the F2 and F3 generations, there were no significant differences in body weight gain between the n-3 Def and n-3 Adq groups during the testing period. For the F2 generation, the mean ± SEM body weights at the beginning of the behavioral testing were 326.6 ± 3.9 g for the n-3 Def and 334.4 ± 3.9 g for the n-3 Adq groups; in the F3 generation groups, the mean ± SEM body weights were 319.4 ± 6.5 g for the n-3 Def and 301.9 ± 3.6 g for the n-3 Adq. There was no significant difference in the body weights for the two dietary groups of the F2 generation [$t(18) = 1.412$, $p = 0.175$]; however, the F3 n-3 Def group weighed significantly more than the n-3 Adq group [$t(18) = 2.337$, $p < 0.05$].

In the motor activity test, the n-3 Adq groups exhibited the typical trait of stable or slightly decreasing mean ambulatory time on successive days. However, the n-3 Def groups in both the F2 and F3 generation exhibited slight increases in ambulatory time with successive trials (Fig. 2). Statistical analyses with the two-way ANOVA indicated no differences between dietary groups for the F2 generation [diet vs. day, $F(4,72) = 0.814$, $p = 0.520$], whereas significance was reached for the F3 generation [diet vs. day, $F(4,72) = 3.932$, $p < 0.01$]. Another closely related measure of motor activity is the moving distance (Fig. 2). Using this measure, similar results were obtained in that there was no significant difference between dietary groups for the F2 generation [diet vs. day, $F(4,72) = 0.328$, $p = 0.858$], but a significant difference was detected for the F3 generation [diet vs. day, $F(4,72) = 4.069$, $p < 0.005$].

In the elevated plus-maze test, there were no significant differences either in the number of entries into the open arms or in the visiting time of the open arm between the n-3 Def and n-3 Adq groups in both generations. It is noted that the absolute number of entries into the open

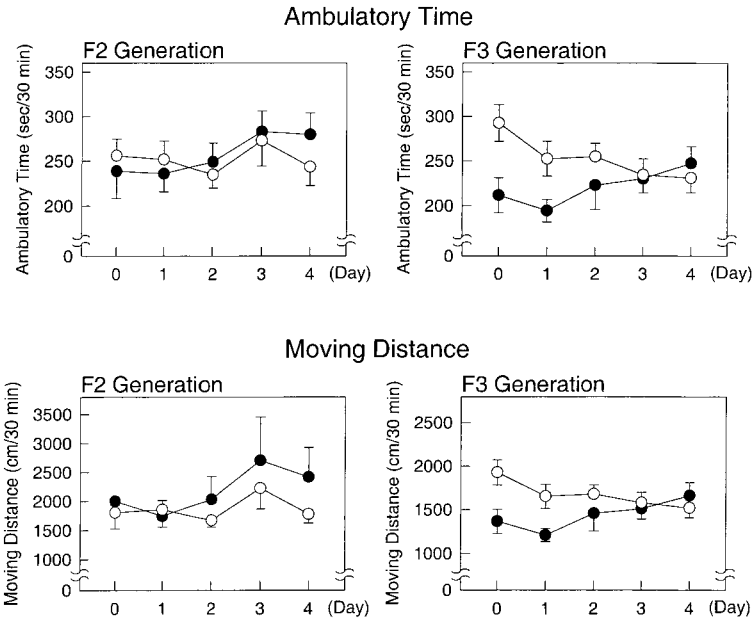

FIG. 2. Effect of n-3 fatty acid deficiency on motor activity. The ambulatory time and moving distance over a 5-day period are given as mean ± SEM (bars) values for 10 rats per group for both the F2 and F3 generations. The n-3 Def group (●) was significantly different in ambulatory time [$F(4,72) = 3.932$, $p < 0.01$] and moving distance [$F(4,72) = 4.069$, $p < 0.005$] compared with the n-3 Adq group (○) in the F3 generation (by two-way ANOVA, diet vs. day interaction).

arm and visiting time was greater in the first set of experiments for the F2 generation in relation to that of the F3 generation. The ratio of day 2/day 1 in the n-3 Def group was marginally higher both in entering the open arm and in the visiting time to the open arm compared with the n-3 Adq group for both generations, but this did not reach statistical significance (Table 2).

In the water maze test, the escape latency was not different between dietary groups or between generations in the visible trial (data not shown); however, the swimming speed in the n-3 Def group of the F2 generation was significantly faster compared with those in the n-3 Adq group [$t(18) = 2.289$, $p < 0.05$; Table 3]. There were no significant differences in the number of successful rats in the visible trial between dietary groups. Although the number of successful rats in the first learning trial was not significantly different between dietary groups in the F2 generation, there was a significant difference in the F3 generation ($p < 0.05$ by Fisher's exact probability test; Table 3). In the learning trials, the escape latency of the n-3 Adq group in the F2 generation gradually decreased over the testing period. In the first experiment, where the F2 generation was used, it was important to provide an adequate number of test days so that it could be demonstrated that n-3 Def rats can fully acquire the spatial task. However, the n-3 Def group showed a significantly longer escape latency than the n-3 Adq group [$F(1,18) = 5.467$, $p < 0.05$] and required five sessions to acquire the task fully (Fig. 3, F2 generation). Only 4 days of testing was used for the F3 generation, however, to assess memory retention without excessive reinforcement and thus increase the likelihood of observing differences between dietary groups. In the F3 generation, the escape latency of the n-3 Adq group was similar to that of the n-3 Adq group in the F2 generation. The n-3 Def group had significantly longer latencies compared with that in the n-3 Adq group [$F(1,18) = 10.632$, $p < 0.005$]; there was a delay of about two sessions to acquire this task (Fig. 3, F3 generation).

During the learning trials, increases in swimming distance were associated with increased escape latency, and this difference was significantly different [F2, $F(1,18)$

TABLE 2. *Effects of n-3 fatty acid deficiency on results of the elevated plus-maze test in rats*

		No. of entries into the open arm			Visiting time of the open arm (s)		
Group	No. of rats	Day 1	Day 2	Day 2/day 1	Day 1	Day 2	Day 2/day 1
F2 generation							
n-3 Def	10	8.5 ± 1.0	7.7 ± 0.9	0.91	102.2 ± 11.2	66.4 ± 12.4	0.65
n-3 Adq	10	9.2 ± 1.2	7.2 ± 0.8	0.78	98.3 ± 17.5	44.8 ± 7.1	0.46
F3 generation							
n-3 Def	10	6.0 ± 0.9	4.9 ± 1.2	0.82	57.7 ± 15.5	45.2 ± 12.6	0.78
n-3 Adq	10	6.6 ± 1.7	3.7 ± 0.4	0.56	53.6 ± 12.7	37.4 ± 7.6	0.70

The number of entries into the open arms and the time spent in the open arms during a 5-min trial period were measured once per day for two days. Data are mean ± SEM values for 10 rats per group.

TABLE 3. *Effects of n-3 fatty acid deficiency on results of the visible trial and the first learning trial in the water maze test*

Group	No. of rats	Swimming speed (cm/s)	No. of successful rats: Visible trial	No. of successful rats: 1st learning trial
F2 generation				
n-3 Def	10	29.8 ± 1.1[a]	0/10	9/10
n-3 Adq	10	26.1 ± 1.2	2/10	10/10
F3 generation				
n-3 Def	10	24.0 ± 1.5	0/10	4/10[b]
n-3 Adq	10	24.5 ± 1.4	3/10	9/10

Swimming speed data are mean ± SEM values for 10 rats per group.
[a] Significant difference from corresponding n-3 Adq value [$t(18) = 2.289$; $p < 0.05$].
[b] The number of successful rats that reached the hidden platform in the visible trial or at least one trial in the first learning trial day were analyzed using Fisher's exact probability test ($p < 0.05$).

$= 10.376$, $p < 0.005$; F3, $F(1,18) = 12.809$, $p < 0.005$ between the two dietary groups; Fig. 4]. The escape latency values were subdivided into swimming time and resting time in both diet groups for each generation. In all groups where the escape latency was increased, the swimming time was also significantly increased. However, the resting times in all groups were similar, and there were no significant differences among the four groups (Fig. 5). Moreover, during the learning trials, the swimming speed of the F2 generation of n-3 Def group

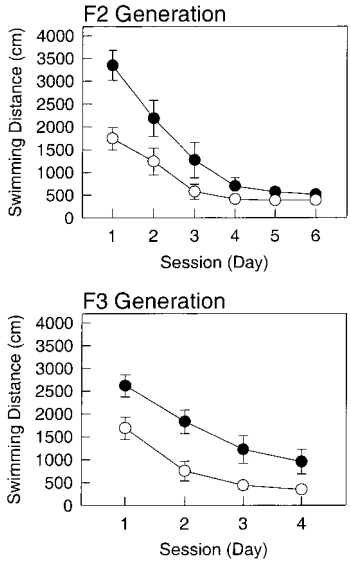

FIG. 4. Effect of n-3 fatty acid deficiency on swimming distance in the Morris water maze. The total distance swum during the learning trials for the n-3 Adq and n-3 Def groups for the F2 and F3 generations is given for 10 rats per group. Data are mean ± SEM (bars) values. The differences between the n-3 Def group (●) and the n-3 Adq group (○) were statistically significant in a two-way ANOVA [F2, $F(1,18) = 10.376$, $p < 0.005$; F3, $F(1,18) = 12.809$, $p < 0.005$].

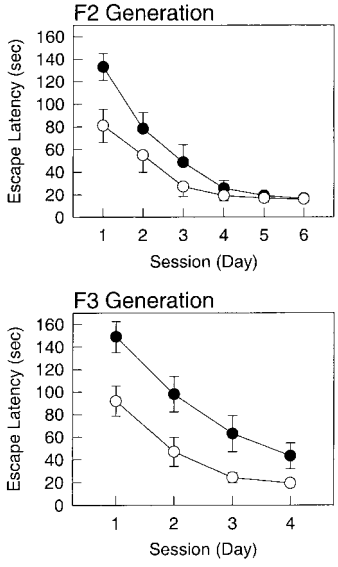

FIG. 3. Effect of n-3 fatty acid deficiency on escape latency in the Morris water maze. The time required for a rat to find and climb onto the hidden platform (escape latency) for the F2 and F3 generations is given for 10 rats per group. Data are mean ± SEM (bars) values. The differences between the n-3 Def group (●) and the n-3 Adq group (○) were statistically significant in a two-way ANOVA [F2, $F(1,18) = 5.467$, $p < 0.05$; F3, $F(1,18) = 10.632$, $p < 0.005$].

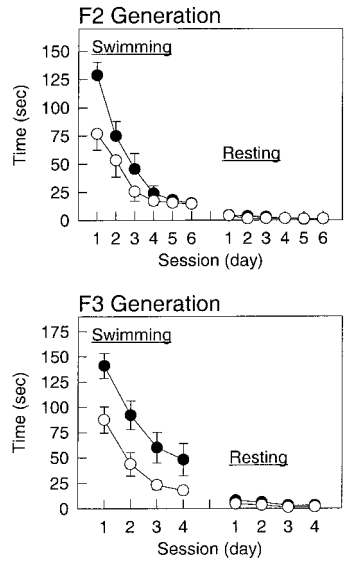

FIG. 5. Fractional analysis of the escape latency in the Morris water maze. The swimming and resting times in the F2 and F3 generations are given as mean ± SEM (bars) values for 10 rats per group. The differences in the swimming time between the n-3 Def group (●) and the n-3 Adq group (○) were statistically significant in a two-way ANOVA [F2, $F(1,18) = 5.981$, $p < 0.05$; F3, $F(1,18) = 11.641$, $p < 0.005$]. The resting time was not statistically different between diet groups in either generation.

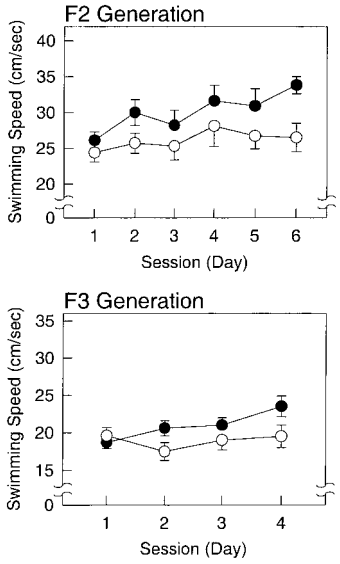

FIG. 6. Effects of n-3 fatty acid deficiency on swimming speed in the Morris water maze. The swimming speed in the learning trials in the F2 and F3 generations is given for 10 rats per group. Data are mean ± SEM (bars) values. The n-3 Adq group (○) was significantly different compared with the n-3 Def group (●) in the F2 generation [two-way ANOVA, $F(1,18) = 4.892$, $p < 0.05$].

was significantly greater than that of the n-3 Adq group [$F(1,18) = 4.892$, $p < 0.05$; Fig. 6]. In the F3 generation, the swimming speed of the n-3 Def group appeared somewhat greater than that of the n-3 Adq group, but this difference did not reach statistical significance [$F(1,18) = 3.241$, $p = 0.089$, Fig. 6].

In the memory retention trial, the number of crossings of the platform position (region A) was significantly greater than those of other regions in each diet group for the F2 generation, but in the F3 generation only the n-3 Adq group showed a significant effect [F2 n-3 Def, $F(3,36) = 5.871$, $p < 0.005$; F2 n-3 Adq, $F(3,36) = 9.568$, $p < 0.0001$; F3 n-3 Adq, $F(3,36) = 8.596$, $p < 0.0005$; Fig. 7]. This preference for the former position of the platform was not observed in the n-3 Def group of the F3 generation [$F(3,36) = 1.367$, $p = 0.269$; Fig. 7, F3 generation]. In the F2 generation, the total number of crossings of the three imaginary positions for the n-3 Def and n-3 Adq groups was 8.5 ± 0.8 and 5.3 ± 0.8, respectively, and was significantly different [$t(18) = 2.887$, $p < 0.01$; Fig. 7, F2 generation]. There was no significant difference in the number of crossings in region A between the n-3 Def group and the n-3 Adq group for the F2 generation ($t(18) = 1.268$, $p = 0.221$; Fig. 7, F2 generation]. This may have been due to overtraining as the F2 generation received 6 days of learning trials, whereas the F3 generation received only 4 days of trials. On the other hand, the number of crossings into region A for the F3 generation was significantly different for the n-3 Def group (2.8 ± 0.5) versus the n-3 Adq group (4.6 ± 0.5) [$t(18) = 2.700$, $p < 0.05$; Fig. 7, F3 generation]. There were no significant differences in the total number of crossings in the three corresponding imaginary positions [$t(18) = 1.051$, $p = 0.307$; Fig. 7, F3 generation].

After the behavioral experiments, the rats were killed, and the lipid composition of the frontal cortex was analyzed (Table 4). There were no striking differences in levels of the saturated or monounsaturated fatty acids or the concentration of total fatty acids between the diet groups or generations. The n-3 Def groups showed a significant increase in four n-6 polyunsaturates (20:3n-6, 20:4n-6, 22:4n-6, and 22:5n-6) and a decrease in the two major n-3 fatty acids (22:5n-3 and 22:6n-3) compared with the n-3 Adq group for both generations. The ratio of 22:5n-6/22:6n-3 in the n-3 Def group markedly increased with a concomitant change in the levels of these n-6 and n-3 fatty acids. In the n-3 Def group of the F3 generation, there was a larger decrease in 22:6n-3 and a greater increase in 22:5n-6, yielding a statistically significant decline in the ratio of these fatty acids in comparison with that of the F2 generation.

The results of the lipid composition of the milk samples collected from the stomachs of the 15-day-old pups are shown in Table 5. The total fatty acid content of the milk samples was ninefold higher than that of the frontal cortex. The total saturated fatty acid content was also higher in the milk sample, but there was less total n-6 and n-3 fatty acid. In both generations, the milk of the n-3 Def group showed a marked decrease in all of the n-3 fatty acids, including 18:3n-3, 20:5n-3, 22:5n-3, and 22:6n-3, all of which were significantly different in com-

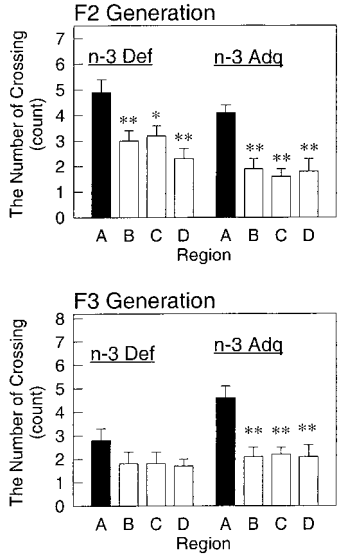

FIG. 7. Effects of n-3 fatty acid deficiency on the memory retention trial. The number of crossings of the platform position (region A; solid column) and the corresponding imaginary positions (regions B–D; open columns) in the F2 and F3 generations is given for 10 rats per group. Data are mean ± SEM (bars) values. Statistical analysis by one-way ANOVA gave the following results: F2 n-3 Def, $F(3,36) = 5.871$, $p < 0.005$; F2 n-3 Adq, $F(3,36) = 9.568$, $p < 0.0001$; F3 n-3 Def, $F(3,36) = 1.367$, $p = 0.269$; F3 n-3 Adq, $F(3,36) = 8.596$, $p < 0.0005$. *$p < 0.05$, **$p < 0.01$ compared with region A by Duncan's multiple range test.

TABLE 4. *Effects of n-3 fatty acid deficiency on total fatty acyl composition of frontal cortex (g/100 g of total fatty acids)*

	F2 generation		F3 generation	
Fatty acid	n-3 Def (n = 7)	n-3 Adq (n = 8)	n-3 Def (n = 10)	n-3 Adq (n = 10)
10:0	0.06 ± 0.02	0.04 ± 0.02	0.03 ± 0.01	0.03 ± 0.02
12:0	0.02 ± 0.004[a]	0.01 ± 0.003	0.01 ± 0.003	0.01 ± 0.003
14:0	0.39 ± 0.05	0.29 ± 0.04	0.44 ± 0.04	0.44 ± 0.02[b]
16:0 DMA	1.24 ± 0.32	1.33 ± 0.19	1.38 ± 0.18	1.68 ± 0.21
16:0	21.20 ± 0.31	21.13 ± 0.17	21.72 ± 0.13	21.77 ± 0.21[c]
18:0 DMA	1.18 ± 0.28	0.69 ± 0.20	1.34 ± 0.18	1.53 ± 0.08[d]
18:0	20.72 ± 0.42	21.22 ± 0.34	20.57 ± 0.20	20.98 ± 0.21
20:0	0.20 ± 0.02	0.21 ± 0.01	0.17 ± 0.01	0.18 ± 0.01
22:0	0.19 ± 0.03	0.17 ± 0.01	0.14 ± 0.01	0.14 ± 0.01
24:0	0.54 ± 0.09	0.47 ± 0.04	0.40 ± 0.03	0.37 ± 0.03[c]
Total saturated	45.81 ± 0.49	45.62 ± 0.42	46.26 ± 0.23	47.19 ± 0.41[c]
14:1	0.16 ± 0.03	0.16 ± 0.05	0.13 ± 0.02	0.14 ± 0.01
16:1n-7	0.38 ± 0.01	0.38 ± 0.01	0.37 ± 0.01	0.37 ± 0.01
18:1n-9	12.23 ± 0.25	12.76 ± 0.26	11.63 ± 0.19[a]	12.24 ± 0.17
18:1n-7	3.34 ± 0.04	3.19 ± 0.05	3.16 ± 0.05[c]	3.04 ± 0.03[c]
20:1n-9	0.54 ± 0.07	0.55 ± 0.04	0.43 ± 0.02	0.47 ± 0.02
22:1n-9	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.002	0.04 ± 0.005
24:1n-9	0.85 ± 0.15	0.90 ± 0.08	0.67 ± 0.04	0.74 ± 0.06
Total monounsaturated	17.54 ± 0.45	18.00 ± 0.38	16.45 ± 0.28	17.06 ± 0.26
18:2n-6	0.56 ± 0.04	0.57 ± 0.01	0.51 ± 0.02	0.50 ± 0.01[b]
18:3n-6	0.04 ± 0.001[a]	0.03 ± 0.001	0.03 ± 0.001[a]	0.03 ± 0.001[c]
20:2n-6	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.003[a]	0.07 ± 0.003[b]
20:3n-6	0.20 ± 0.01[e]	0.28 ± 0.01	0.22 ± 0.01[e]	0.26 ± 0.01
20:4n-6	10.20 ± 0.12[e]	9.42 ± 0.05	10.40 ± 0.16[e]	9.39 ± 0.09
22:4n-6	3.09 ± 0.06[e]	2.32 ± 0.05	3.25 ± 0.11[e]	2.32 ± 0.05
22:5n-6	12.85 ± 0.33[e]	0.65 ± 0.09	13.88 ± 0.13[b,e]	0.65 ± 0.06
Total n-6	27.02 ± 0.47[e]	13.36 ± 0.13	28.37 ± 0.22[c,e]	13.22 ± 0.12
22:5n-3	0.01 ± 0.005[e]	0.11 ± 0.002	0.03 ± 0.003[c,e]	0.11 ± 0.01
22:6n-3	2.88 ± 0.04[e]	16.51 ± 0.36	2.15 ± 0.05[d,e]	16.47 ± 0.16
Total n-3	2.89 ± 0.04[e]	16.62 ± 0.35	2.17 ± 0.05[d,e]	16.57 ± 0.16
22:5n-6/22:6n-3	4.5 ± 0.12[e]	0.04 ± 0.005	6.5 ± 0.10[d,e]	0.04 ± 0.004
22:5n-6 + 22:6n-3	15.7 ± 0.3[a]	17.2 ± 0.4	16.0 ± 0.2[e]	17.1 ± 0.1
n-6 + n-3	29.9 ± 0.5	30.0 ± 0.3	30.6 ± 0.2[a]	29.8 ± 0.2
Total fatty acids (μg/mg of wet tissue)	28.7 ± 0.5	29.3 ± 0.6	28.7 ± 0.4	28.8 ± 0.3

Fatty acid methyl esters from 10:0 to 24:1n-9 were analyzed. 18:3n-3 and 20:5n-3 were not detected, i.e., < 0.01%. Data are mean ± SEM (bars) values for seven to 10 rats.

[a] $p < 0.05$, [e] $p < 0.001$ versus n-3 Adq group; [b] $p < 0.01$, [c] $p < 0.05$, [d] $p < 0.001$ versus same diet group in the F2 generation by Student's *t* test.

parison with those in the n-3 Adq group. The loss of n-3 fatty acids in the milk of n-3 Adq dams led to a marked decline in the brain DHA content of their offspring. The ratio of 22:5n-6/22:6n-3 in the frontal cortex of both generations was significantly higher in the n-3 Def group than in the n-3 Adq group. In the F3 generation, this ratio was significantly higher in the n-3 Def group in comparison with the n-3 Def group of the F2 generation.

DISCUSSION

In the present investigation, there were no consistent differences in the rate of growth between animals in the n-3 Def and the n-3 Adq groups during the testing period. However, in the F3 generation, the deficient group had an ~6% increase in body weight over the adequate animals. This slight increment in body weight in adult animals would not be expected to confer any benefit for the deficient animals. Therefore, the behavioral deficits in the n-3 Def group cannot be ascribed to a difference in developmental stage associated with a difference in body weight.

Normally, motor activity is gradually reduced over 5 days of testing as the animals become habituated to the cage environment (Bidzinski et al., 1998; Thiel et al., 1998). However, an increase in motor activity in the F3 generation n-3 Def rats was observed on days 2–5. This result suggests that the n-3-deficient diet may affect habituation and spontaneous motor activity. Several other investigators have noted an increase in motor activity in n-3 Def animals (Umezawaet al., 1995; Wainwright et al., 1994, 1997); however, there is one report of decreased activity (Enslen et al., 1991).

On the first day of testing in the elevated plus-maze test, a naive rat will explore the open arms of the maze. The ratio of day 2/day 1 in open arm entries and visiting time usually decreases as the rats become aware of the danger of falling (File et al., 1993; File and Gonzalez, 1996). These ratios in the n-3 Def group were slightly

TABLE 5. *Effects of n-3 fatty acid deficiency on fatty acyl composition of rat pup stomach contents (g/100 g of total fatty acids)*

	F2 generation		F3 generation	
Fatty acid	n-3 Def (n = 4)	n-3 Adq (n = 4)	n-3 Def (n = 3)	n-3 Adq (n = 4)
10:0	7.16 ± 0.18	8.60 ± 0.61	6.46 ± 2.90	5.06 ± 1.52
12:0	19.08 ± 1.03	20.44 ± 0.43	15.38 ± 5.36	11.18 ± 3.28[a]
14:0	14.56 ± 1.02	16.01 ± 0.25	12.46 ± 3.50	8.93 ± 2.02[a]
16:0	22.77 ± 0.66	23.94 ± 0.62	23.73 ± 1.24	22.69 ± 0.91
18:0	5.38 ± 0.24	4.82 ± 0.25	5.25 ± 0.34	4.43 ± 0.32
20:0	0.09 ± 0.003	0.08 ± 0.003	0.09 ± 0.003	0.07 ± 0.011
22:0	0.03 ± 0.001	0.03 ± 0.001	0.04 ± 0.005	0.03 ± 0.003
24:0	0.04 ± 0.004	0.04 ± 0.004	0.06 ± 0.016	0.03 ± 0.005
Total saturated	69.30 ± 2.80	74.14 ± 0.65	63.67 ± 10.35	52.62 ± 6.02[a]
12:1	0.03 ± 0.003[b]	0.02 ± 0.001	0.03 ± 0.010	0.04 ± 0.007[a]
14:1	0.20 ± 0.02[b]	0.14 ± 0.01	0.15 ± 0.04	0.22 ± 0.04
16:1n-7	2.48 ± 0.41	1.85 ± 0.07	1.43 ± 0.92	4.52 ± 0.71[c]
18:1n-9	14.14 ± 1.59	10.52 ± 0.50	16.91 ± 5.99	21.70 ± 3.81[a]
18:1n-7	1.61 ± 0.30	1.03 ± 0.07	2.54 ± 1.02	3.30 ± 0.53[a]
20:1n-9	0.23 ± 0.05	0.14 ± 0.02	0.53 ± 0.30	0.33 ± 0.08
Total monounsaturated	18.73 ± 2.35	13.75 ± 0.49	22.67 ± 8.30	30.17 ± 5.11[a]
18:2n-6	7.01 ± 0.06[d]	6.41 ± 0.12	7.45 ± 0.84	9.72 ± 1.00[a]
18:3n-6	0.17 ± 0.02	0.13 ± 0.01	0.11 ± 0.03	0.14 ± 0.03
20:2n-6	0.30 ± 0.05	0.21 ± 0.03	0.56 ± 0.23	0.39 ± 0.07
20:3n-6	0.31 ± 0.04	0.24 ± 0.01	0.25 ± 0.04	0.26 ± 0.04
20:4n-6	0.68 ± 0.05[b]	0.49 ± 0.04	1.25 ± 0.33	1.06 ± 0.13[c]
22:4n-6	0.21 ± 0.03[b]	0.10 ± 0.01	0.39 ± 0.15	0.26 ± 0.04[c]
22:5n-6	0.12 ± 0.01[e]	0.03 ± 0.002	0.30 ± 0.10	0.08 ± 0.02
Total n-6	8.81 ± 0.21[d]	7.63 ± 0.15	10.32 ± 1.65	11.92 ± 0.14[c]
18:3n-3	0.023 ± 0.001[e]	0.95 ± 0.03	0.023 ± 0.002[d]	0.99 ± 0.13
20:3n-3	ND[e]	0.039 ± 0.004	ND[e]	0.043 ± 0.002
20:5n-3	0.004 ± 0.002[e]	0.15 ± 0.01	0.002 ± 0.002[d]	0.15 ± 0.02
22:5n-3	0.006 ± 0.002[e]	0.14 ± 0.01	0.006 ± 0.003[e]	0.25 ± 0.03[a]
22:6n-3	0.020 ± 0.002[e]	0.12 ± 0.01	0.018 ± 0.006[b]	0.52 ± 0.11[a]
Total n-3	0.054 ± 0.002[e]	1.40 ± 0.03	0.049 ± 0.006[e]	1.95 ± 0.13[c]
22:5n-6/22:6n-3	6.0 ± 0.8[e]	0.20 ± 0.01	16.8 ± 1.2[e,f]	0.16 ± 0.02
22:5n-6 + 22:6n-3	0.14 ± 0.01	0.15 ± 0.01	0.32 ± 0.11	0.60 ± 0.14[a]
n-6 + n-3	8.9 ± 0.2	9.5 ± 0.2	10.4 ± 1.7	13.9 ± 1.2[c]
Total fatty acids (μg/mg of milk)	257 ± 20	265 ± 20	209 ± 31	278 ± 29

Stomach contents were collected in 15-day-old animals. Data are mean ± SEM values for three or four litters. ND, not detected.

[a] $p < 0.05$, [c] $p < 0.01$, [f] $p < 0.001$ versus same diet group in F2 generation; [b] $p < 0.05$, [d] $p < 0.01$, [e] $p < 0.001$ versus n-3 Adq group by Student's *t* test.

higher than that of the n-3 Adq group in both generations. The difference in the number of entries/visiting time between the F2 and F3 sets of experiments has no obvious explanation but may relate to seasonal variations in activity.

The spatial learning test does not involve aversive stimuli and is based on more natural behavior than passive and conditioned avoidance tests. Escape latency in the water maze test is generally believed to be a parameter reflecting spatial learning and cognitive capacity (Morris et al., 1982; Morris, 1984; McNamara and Skelton, 1993). The n-3 Def group showed significantly longer escape latency and a delay in the acquisition of this task. These results may indicate a poorer learning ability for the n-3 Def group and are consistent with the results obtained with other learning tasks (Coscina et al., 1985; Nakashima et al., 1993; Frances et al., 1996; Jensen et al., 1996). Furthermore, in the fourth session of the learning trial, both diet groups in the F2 generation showed a similar escape latency, whereas in the F3 generation, the n-3 Def group escape latency was longer compared with its control group. Although in both the F2 and F3 generation experiments the differences due to diet were statistically significant, the magnitude of the effect and the level of significance were greater in the F3 generation. It therefore appeared that the learning ability of the F3 generation was somewhat poorer than that of the F2 generation. To analyze the escape latency in detail, this parameter was separated into swimming time and resting time. Depressant actions have been shown to be associated with an increase in the immobilized time during forced swimming (Porsolt et al., 1977; Hascoet et al., 1994). The resting times were unchanged, indicating that this was not an important contributor to the increased escape latencies in the n-3 Def animals. However, the swimming times were increased in all groups where the escape latencies were increased. This combination of results suggests that the escape latency was primarily a measure of spatial learning and memory.

In the memory retention trial, the number of crossings of the platform position (region A) is a parameter used for the evaluation of spatial memory. In the F3 generation, after four sessions the number of crossings of region A in the n-3 Adq group was significantly greater than those of other regions; however, in the n-3 Def group, the rats swam randomly. This result indicates that the n-3 Adq rats swam selectively around the former location of the platform. It was clear that in the F3 generation, the working memory of the n-3 Def group was inferior to that of the n-3 Adq group. On the other hand, in the F2 generation, the number of crossings of region A was significantly greater in both diet groups. This may have been a consequence of overtraining as the F2 generation rats received a greater number of learning sessions (six) relative to the F3 generation (four) before the memory retention trial.

The deficiency of n-3 fatty acids is known to impact adversely visual system measures in nonhuman primates (Neuringer et al., 1986) as well as human infants (Uauy et al., 1990; Carlson et al., 1993; Makrides et al., 1995; Carlson and Werkman, 1996; for review, see Birch et al., 1998; Hamosh and Salem, 1998). Therefore, the question may arise as to whether the poorer performance of the n-3 Def group in the spatial tasks may have been due to a visual sensory deficit. First, it is noted that there were obvious visual cues, e.g., a sheet of dazzling, crinkled metallic paper and a dark, contrasting, large door were used as cues in the water maze test. Therefore, subtle visual discriminations did not appear to be necessary to perform well on the spatial tasks. In addition, the magnitude of the loss in visual function expected in an n-3-deficient animal is unlikely to be sufficient to affect performance in a spatial task. This contention is supported, for example, by the demonstration that blind (enucleated) rats were not profoundly impaired on the Morris water maze task (Lindner et al., 1997). This may in part reflect the fact that rats are macrosmatic (Slotnick, 1990) and have relatively poor vision (Artal et al., 1998). Thus, rats may use olfactory cues to perform spatial tasks such as the Morris water maze (van Rijzingen et al., 1995; Lavenex and Schenk, 1996). Moreover, Oakley and colleagues suggested that decortication did not impair instrumental performance in a simple visual discrimination (horizontal/vertical stripe problem) and did not introduce any limiting sensory or motor deficit (Oakley, 1981; Goldstein and Oakley, 1987). Indeed, in our experiment, there were no significant differences between the two groups in the number of successful animals in the visible trial. Furthermore, differences in performance were also observed in n-3-deficient animals in other nonvisual cued tasks, including two-odor olfactory discriminations (Greiner et al., 1999) and shock avoidance (Bourre et al., 1989; Umezawa et al., 1995). Therefore, differences in sensation between the n-3 Def and n-3 Adq groups in the water maze are minimal under these conditions with regard to their effects on performance.

Another explanation for the difference in behavior between dietary groups may be through an influence on the motivational state. Although this hypothesis cannot be ruled out, there is some evidence that would mitigate against this interpretation. In the various measures of general activity, including ambulatory time and distance as well as entries and time spent in the open arms of the plus-maze, there were few differences between dietary groups. The swimming speed was another measure of activity/motivation in the Morris water maze. In this regard, there was a significantly greater swimming speed in the n-3 Def group of the F2 generation (the F3 was also increased but not significantly); this result is opposite to that predicted by the motivation hypothesis. It is also noteworthy that the swimming speed of the n-3 Def group increased over time during the water maze tests. This may indicate a reduction of cognitive capacity in these deficient animals rather than greater motivational activity as reported in other trials (Wainwright et al., 1994, 1997).

The levels of total n-3 fatty acids in the frontal cortex of the n-3 Def group were reduced by 83% in the F2 generation and by 87% in the F3 generation compared with that of the n-3 Adq groups; the decline was compensated for by an increase in levels of n-6 fatty acids. The levels of total n-6 fatty acids in the n-3 Def groups were more than twofold greater than that of the n-3 Adq group for both generations. This reduction in levels of the n-3 fatty acids is comparable to levels achieved in artificially reared rats (Ward et al., 1996). These results suggest that the poorer performance in spatial tasks in the n-3 Def group was due to the change in the balance between the n-3 and the n-6 fatty acids. Moreover, the ratio of 22:5n-6/22:6n-3 in both the frontal cortex and the milk (stomach contents) significantly increased in the n-3 Def group in the F3 generation compared with that of the F2 generation. Because the n-3 Def animals of both the F2 and F3 generations received the same diet after weaning, any difference in their brain composition would most directly be ascribed to differences in their fatty acid intake during the period of lactation. Although the milk DHA content was only slightly lower in the F3 generation dam milk, the 22:5n-6 content was higher; thus, the 22:5n-6/22:6n-3 ratio was significantly greater in the F3 milk in comparison with the F2 milk. The reports by Yonekubo et al. (1993) and Francois et al. (1998) that n-3 fat supplements in the maternal diet led to increases in the n-3 fatty acid composition of mother's milk are consistent with our study of milk alterations caused by n-3 deficiency.

In a closely related experimental paradigm, Wainwright et al. (1994, 1997) examined n-3-deficient diet and water maze tasks but found no differences in performance. There are several differences between that work and the data presented here that may explain this apparent discrepancy. In our experiment, there was a greater decrease in the percentage of brain n-3 fats. In our study, the whole brain showed an 83% decrease in brain DHA content in F2 animals, whereas Wainwright et al. (1994) reported an ~40% decrease in DHA content in brain phosphatidylethanolamine and subsequently a 51% de-

crease in forebrain phospholipid DHA content (Wainwright et al., 1998). Second, there were differences in experimental procedures and conditions in administering the Morris water maze task. For example, differences in such variables as the number of trials allowed per day, the pool size in relation to the animal size, the height of the wall from the water, the intervals used, and the resting time on the hidden platform can alter the difficulty of the task. Our studies indicate that differences in behavior related to the rather subtle experimental manipulation of brain biochemistry made in n-3 deficiency studies can be optimally observed when the degree of difficulty of the task is increased.

Losses in neural function in studies of dietary n-3 deficiency have usually been ascribed to the loss in nervous system DHA (Tinoco, 1982; Salem, 1989; Okuyama et al., 1997). Alternatively, the behavioral effects may be linked to the increase in neuronal phospholipid 22:5n-6 content. Another valid hypothesis is that there is an inadequate amount of total 22-carbon polyunsaturate during a critical stage in early nervous system development and would predict that 22:5n-6 supplementation would prevent losses in behavioral performance associated with n-3 deficiency. The present study demonstrates that as the brain 22:5n-6/22:6n-3 ratio increases (for example, when the F2 and F3 generation are compared), this is associated with poorer water maze performance; still, it does not distinguish between these alternate hypotheses. In the present study, we conclude that a dietary n-3 fatty acid deficiency adversely impedes learning and cognitive-related behaviors in rats via the change in the fatty acid composition of the CNS. This change was influenced not only by the weanling diet, but also by the milk received from the mother during the lactation period.

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