

# Effects of Diets Enriched in Omega-3 and Omega-6 Polyunsaturated Fatty Acids on Offspring Sex-Ratio and Maternal Behavior in Mice<sup>1</sup>

Emily D. Fountain,<sup>3</sup> Jiude Mao,<sup>3</sup> Jeffrey J. Whyte,<sup>3</sup> Kelly E. Mueller,<sup>3</sup> Mark R. Ellersieck,<sup>4</sup> Matthew J. Will,<sup>5</sup> R. Michael Roberts,<sup>6,7</sup> Ruth MacDonald,<sup>8</sup> and Cheryl S. Rosenfeld<sup>2,3</sup>

Departments of Biomedical Sciences,<sup>3</sup> Food Systems and Bioengineering, Agriculture Experimental Station-Statistics,<sup>4</sup> Psychological Sciences,<sup>5</sup> Animal Sciences,<sup>6</sup> and Biochemistry,<sup>7</sup> University of Missouri-Columbia, Columbia, Missouri 65211  
Department of Food Science and Human Nutrition,<sup>8</sup> Iowa State University, Ames, Iowa 50011

## ABSTRACT

There have been many trials describing the effects of polyunsaturated fatty acids (PUFA) on fecundity, neonatal development, and maternal behavior in humans, but few controlled studies in rodents. We examined the effects of a maternal diet high in omega 3 (N-3) or omega 6 (N-6) PUFA on NIH Swiss mice. Female mice were ad libitum fed one of three complete and balanced diets (N-3, enriched in menhaden oil; N-6, enriched in corn oil; C, control diet, Purina 5015) from age 4 wk until the end of the study. Mice were bred at ~19 wk and 27 wk of age, providing a total of 838 pups from 129 litters in two experiments. After weaning their pups from parity 1, behavior of dams was assessed on elevated-plus and open-field mazes. Although the fraction of male pups from the N-3 and C groups was not different from 0.5, dams on the N-6 diet birthed more daughters than sons (213 vs. 133;  $P < 0.001$ ). Although maternal stress has been reported to favor birth of daughters, the behavior of N-6 dams was not different from controls. By contrast, the N-3 dams displayed greater anxiety, spending less time in the open arms and more time in the closed arms of the elevated maze and traveling less distance and exhibiting less exploratory behavior in the open field ( $P < 0.05$ ). N-3 dams tended to produce smaller litters than C dams, and N-3-suckled pups gained less weight ( $P < 0.05$ ). In conclusion, the N-3 diet had negative effects on murine fecundity and maternal behavior, whereas the N-6 diet favored birth of daughters.

*anxiety, behavior, elevated-plus maze, mouse, nutrition, open-field maze, parturition, pregnancy, sex allocation, sex ratio*

## INTRODUCTION

Diets enriched in polyunsaturated fatty acids (PUFA), particularly diets containing supplements of omega 3 (N-3) components, which are fatty acids that are found in abundance in fish oils, have been widely publicized as promoting human health. Not surprisingly, there have been many clinical trials examining their effects, including several to determine whether supplementing maternal diet during pregnancy leads to improved maternal and fetal outcomes [1–7]. In general these studies have not been conclusive, but have indicated few, if any, negative side effects of supplementation. On the other

hand, there have been few controlled studies on the reproductive effects of N-3 supplementation in rodents [8–13].

Our laboratory is interested in the influence of maternal diet, particularly its fat composition, on offspring sex ratio [14–16]. We noted, for example, that two defined, ad libitum fed, nutritionally complete diets that differed primarily in the content of energy and amount of saturated fat had contrasting effects on the sex of offspring born to NIH Swiss and CF1 dams [14]. Diet 1 was low in saturated fat (LF), with the majority of calories provided as sugars and complex carbohydrate, while the second diet was very high in fat (VHF), with 54% of its energy provided as lard, which is 40% palmitic and stearic acids, 40% oleic acid, and approximately 10% linoleic acid, an omega 6 (N-6) fatty acid. Although there was little effect of these diets on sex ratio distortion in young mice bred at 10 wk of age, sex ratio skewing became obvious at later parities and in virgin mice bred when they were older than 20 wk. Specifically, the VHF diet provided a significantly higher number of male-biased litters and a sex ratio that favored sons over daughters by almost 2:1. The LF diet, by contrast, strongly favored the birth of female offspring. When consumption of the VHF diet was reduced by providing it in restricted amounts, however, the sex-ratio shifted to favor daughters [17], suggesting that it was the amount of calories consumed by the dams, rather than the fat content of the diet per se, that influenced the relative numbers of sons and daughters born. Whether these observations on sex ratio skewing have adaptive significance is unclear, but they are generally consistent with the sex allocation hypothesis of Trivers and Willard [18].

Previous studies in opossums and humans suggest that females on a diet supplemented with PUFA give birth to more sons than daughters [19, 20]. In the case of the opossums, the females were provisioned with sardines to provide extra N-3 fatty acids [19]. Based on these reports, we hypothesized that in our previous studies [14, 16, 17] on mice fed a diet rich in lard, the large amount of saturated fat consumed was sparing the PUFA from oxidative metabolism and was causing the skewing of the offspring sex ratio toward males. Accordingly, we speculated that PUFA might be governing the alteration in the offspring sex ratio. To test this hypothesis, we have herein repeated the study design above, but with diets enriched with either N-3 or N-6 fatty acids. In our experiments, the N-3 fatty acids were derived primarily from menhaden oil (Table 1), a particularly rich source of (20:5) eicosapentaenoic acid (EPA) and (22:6) docosahexaenoic acid (DHA), rather than from plant oils. The N-6-enriched diet was supplemented with corn oil to provide extra linoleic acid. Flaxseed and menhaden oil are routinely used to bolster the N-3 dietary content. We chose to use the latter because many previous rodent studies have employed this ingredient as the N-3 fatty acid source [9, 21–24]. Additionally, these studies were designed to replicate as close as possible the previous opossum study where the

<sup>1</sup>Supported by NIH grant HD 044042 to R.M.R. and C.S.R.

<sup>2</sup>Correspondence: Cheryl S. Rosenfeld, 440F Christopher S. Bond Life Sciences Center, Columbia, MO 65211-7310. FAX: 573 884 9676; e-mail: rosenfeldc@missouri.edu

Received: 16 August 2007.  
First decision: 6 September 2007.  
Accepted: 3 October 2007.

© 2008 by the Society for the Study of Reproduction, Inc.  
ISSN: 0006-3363. <http://www.biolreprod.org>

TABLE 1. Diet composition of the control, N-3, and N-6 diets.

Diet	PUFA source (%)			Proportion of PUFA (%)		Proportion of calories (%)				Energy density (kcal/g)		
	Safflower oil	Corn oil	Menhaden oil	Arachidonic acid	Linoleic acid	N-3 in diet (%)	N-6 in diet (%)	Ratio of N-3:N-6	Fat		Carbohydrate	Protein
Control	0	0	0	0.03	2.45	0.18	2.48	-	26	56	18	3.73
N-3	2	0	5	0.02	50.00	2.10	2.50	1.00	16	64	20	3.948
N-6	2	5	0	0.00	93.00	0.03	4.50	0.00	16	64	20	3.948

animals were provisioned with sardines [19, 20]. Moreover, fish oil has been touted to exert beneficial effects for in utero-exposed conceptuses, including promoting visual and neural development and preventing later adult-onset diseases, such as diabetes and mammary tumors [2, 4, 6, 8, 9].

During the course of our studies, we noted that the females on the two contrasting PUFA diets showed differences in behavior. In particular, the mice on the N-3 diet appeared to display aberrant “nesting” activities and poorer maternal behavior than those on the N-6 and control diets. Therefore, in addition to examining whether the diets enriched in PUFA influenced the sex ratio of pups born, the dams were subjected to behavioral tests designed to assess maternal anxiety.

## MATERIALS AND METHODS

### Diets

Diets containing specific fatty acid composition (N-3 and N-6 diets) were prepared from commercial ingredients following the standards defined by the American Society for Nutritional Sciences (Table 1). Both diets contained safflower oil to provide essential fatty acids. The N-6 diet contained corn oil as a source of N-6 fatty acids and the N-3 diet contained menhaden oil as a source of N-3 fatty acids. Neither the N-3 nor N-6 diets had lard added in the diet. The N-3 diet contained 16.45 g/kg of saturated fatty acids, which comprised 25.43% of the total fatty acids in the diet. The saturated fatty acids in the N-6 were 8.81% of the fatty acid profile, and the diet contained 5.89 g/kg of saturated fatty acids. The control diet had 0.30 g/kg of saturated fatty acids. Diets were isocaloric, isonitrogenous, and equivalent in nutrient composition other than the fatty acid profile. The female mice were fed ad libitum the N-3, N-6, or LabDiets Purina 5015 control diet (PMI Nutrition International, LLC, Brentwood, MO; Table 1)

### Animals

Three-week-old NIH Swiss mice (Harlan, Madison, WI) were maintained and bred at the Animal Sciences Research Center, University of Missouri-Columbia. Experiments were carried out in accordance with NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Missouri Animal Care and Use Committee.

### Breeding and Offspring Gender Determination

Four-week-old females were fed ad libitum the N-3 (n=26), N-6 (n=30), and Purina 5015 (PMI Nutrition International; n=26) control diet for 15 wk before the initial breeding, and they were maintained on the diets throughout the experiment. NIH Swiss males (n=42) were age-matched with the females and first employed as breeders when they were 19 wk old. They were maintained on a 5001 chow diet from LabDiets Purina Mills (PMI Nutrition International), except when paired with females, during which time they consumed the test diets. As previously demonstrated [17], there is no effect on either sex ratio of offspring or X/Y sperm counts when males are exposed to diets that have major effects on the dam.

The experiment consisted of two replicate trials. At 19 wk of age, dams were bred and permitted to carry the pregnancy to term. Offspring gender was determined by two independent individuals who measured ano-genital distance of the pups at 2 and 21 days of age. Both male and female pups were weighed at 2, 14, and 21 days of age; in trial 2, the female pup weight was further recorded at 35 and 40 days of age. At 21 days of age, the pups were weaned from their mother. For trial 1, 2 wk after weaning the pups, the dams were rebred to the same male used for the first parity. The same weighing and sexing procedure was followed for the second parity. For both trials, observed dead pups were removed from the cage and, if intact, sexed as above. Partially

cannibalized pups were sexed by using fluorescent in situ hybridization with probes specific to X and Y chromosomes [25]. For trial 2, 1 wk after weaning the pups from parity 1, the dams were tested in the behavioral mazes (detailed below) over the course of 2 wk. One week after the last maze test, the dams were rebred to the same male used in parity 1. The same weighing and sexing procedure was used for parity 2.

### Maternal Behavioral Observations

Maternal behavior was observed and documented at parturition and throughout lactation. Instances of cannibalism and barbering and general pup condition were digitally recorded with an Olympus Stylus 600 digital camera (Olympus, Center Valley, PA). Additionally, the size (length, width, depth, and volume) and overall quality of each nest was also assessed during lactation.

### Elevated-Plus and Open-Field Mazes

Two rodent behavioral assessment methods, an elevated-plus maze and an open-field maze, were used to determine the anxiety of the dams [26–28]. One week after weaning the pups from parity 1 or 2, dams were randomly selected to test in the elevated-plus or open-field maze. After recuperating for 1 wk, the same dams were assessed in the alternate maze. All surviving dams that had carried litters to term were tested in the mazes.

The elevated-plus maze measures anxiety behavior [27] and was constructed of black plexiglass with two opposite open arms (30 cm), a middle platform (5 cm × 5 cm), and two opposite closed arms (30 cm). It was supported 77.5 cm from the floor and lit by a Philips TL70 F32T8 fluorescent bulb (Philips Electronics North America Corporation, New York, NY) placed 140 cm above the center of the maze. Each mouse was placed on the center platform and allowed to explore the maze for 5 min. After each test, the apparatus was cleaned thoroughly with 10% bleach solution before reuse. Each trial was recorded via a Logitech Quickcam Orbit MP webcam (Logitech, Fremont, CA) connected to an HP Pavilion dv6000 laptop (HP, Palo Alto, CA) and later analyzed for time spent in the open and closed arms, and in the center of the maze. An entry into the arms was defined as both front paws and shoulders placed into the area. Anxious mice are more likely to spend time in the closed arms, where they feel less threatened. In contrast, normal mice spend approximately equal time in the open and closed arms, correlating to equal exploration of the entire maze [27].

The open-field maze measures exploratory and motor behavior by analyzing the distance the mouse travels during the course of 5 min and the number of times the mouse displays behaviors associated with normal exploration [29, 30]. The open field was made of a clear plexiglass circular floor (40 cm diameter) surrounded by a white plastic wall (40 cm high). A black square (5 cm × 5 cm) was drawn in the middle of the floor to mark the center. The maze was illuminated by a fluorescent bulb as above. Again, the mouse was placed in the center of the maze and permitted to explore for 5 min, after which the platform was cleaned as above. Each trial was recorded and later analyzed for time spent in the center and total distance traveled. The following behaviors were also documented: rearing (as demarcated by standing up on the hind legs), smelling, and grooming. When the mice were at rest, the number of times they began to groom or smell the surrounding area was recorded. The greater distance traveled by the mouse corresponds to higher ambulatory movement and less anxiety [28]. The more time spent in the center of the open-field maze correlates to the time spent in the open arms for the elevated-plus maze. This pattern of behavior is typical following administration of anxiolytics or relaxation drugs, such as benzodiazepines, e.g., valium [28].

### Statistical Analysis

Female body weights before the initiation of the diet (Week 0), at 15 wk on the diets, at conception, before and after parturition, and at weaning in both parities 1 and 2 were analyzed by using the general linear model (GLM) procedure of Statistical Analysis Systems (SAS) [31]. The ANOVA model contained the effects of trial and diet. Each female served as the experimental

TABLE 2. Dam body weight during different phases of the experiment.

Diet	Before breeding		Parity 1			Parity 2				
	Week 0	Week 15	At conception	Before delivery	After delivery	At weaning	At conception	Before delivery	After delivery	At weaning
Control	23.3 ± 0.4	33.1 ± 0.8 <sup>a</sup>	32.5 ± 0.9 <sup>a</sup>	49.5 ± 1.2 <sup>a</sup>	37.2 ± 0.9 <sup>a</sup>	33.3 ± 0.7	37.7 ± 0.9 <sup>a</sup>	55.1 ± 1.3 <sup>a</sup>	42.9 ± 1.0 <sup>a</sup>	41.0 ± 0.9 <sup>a</sup>
N-3	23.7 ± 0.4	29.6 ± 0.9 <sup>b</sup>	29.4 ± 1.0 <sup>b</sup>	39.9 ± 1.4 <sup>b</sup>	31.8 ± 0.9 <sup>b</sup>	31.6 ± 0.7	33.9 ± 1.0 <sup>b</sup>	42.4 ± 1.5 <sup>b</sup>	35.9 ± 1.4 <sup>b</sup>	35.9 ± 1.2 <sup>b</sup>
N-6	23.5 ± 0.4	29.9 ± 0.8 <sup>b</sup>	30.6 ± 0.9 <sup>a,b</sup>	45.9 ± 1.2 <sup>c</sup>	34.6 ± 0.8 <sup>c</sup>	33.0 ± 0.7	33.8 ± 0.8 <sup>b</sup>	51.0 ± 1.2 <sup>c</sup>	38.5 ± 0.9 <sup>c</sup>	37.2 ± 0.8 <sup>b</sup>

<sup>a,b</sup> Group means within columns that have different superscripts are significantly different ( $P < 0.05$ ).

unit. Differences in body weights between females on the diets were determined by using the Fisher Least Significant Difference.

The pup sex ratio at 2 and 21 days of age was analyzed by using a Generalized Linear Model (GENMOD) of SAS [31]. The data were distributed as a binomial and transformed by means of a logit-link function. The model was a “repeated measures design” since each dam had repeated parities. The Generalized Estimating Equation contained the effects of weight (weight of dam at the time she was bred), diet, parity number, and the interaction of diet and parity (diet\*parity). The parameter of maternal weight was used as a covariate to determine whether the body mass of the dams influenced the sex ratio of pups born. Chi-square methodology was employed to test deviation from a 1:1 ratio, i.e., a value of 0.5 for fraction of males born, as well as differences in the sex ratio between groups. The antilog of the logit and the antilog of the differences between logit estimates produce the odds and odds ratio, respectively. Pup weight was analyzed by a split plot in time.

The dependent variables for time spent in the open and closed arms, and in the center of the maze, the numbers of entries into the open and closed arms, total distance traveled in the behavioral studies, number of rearings, grooming, smelling and inactive periods, gestation length, litter size at birth, and pup body weights at 2, 14, and 21 days of age were analyzed by using the GLM procedure of SAS with trial, diet, parity, and the interaction between diet and parity as the primary effects. The nest size (length, width, depth, and volume) and proportion of nesting material used were measured at weeks 1, 2, and 3 of lactation. The average of these three observations was used for final data analysis with trial, diet, parity, and diet by parity interaction as main effects. Mean differences were determined by using the Fisher Least Significant Difference. All data are expressed as least squares means ± SEM.

**RESULTS**

*Dam Weights*

During the 15 wk on the diet prior to breeding, females on the N-3 and N-6 diets showed similar average body weight gains (Table 2), but by 15 wk, the females on the N-3 and N-6 diets weighed less than the mice on the control diet ( $P < 0.05$ ). Mice on all three diets appeared healthy. Before the delivery of their first litters, however, the N-3 dams weighed significantly less than both the control and the N-6 mice (Table 2), possibly in part because they carried slightly smaller litters (Table 3), although this variation in litter size may not account for the entire body weight difference. The body weight variance between dietary groups seen during the first pregnancy was lost by the time the dams had weaned their first litter of pups, but

reemerged during the second pregnancy and persisted until the end of the experiment ( $P < 0.05$ ).

*Offspring Sex Ratio*

Pup gender was determined on 2 and 21 days of age. Although there was some pup mortality during the experiment, particularly instances of partial cannibalism in the first week for parity 2 in the N-3 group ( $P = 0.01$ , data not shown), the mortality rate for male and female pups did not differ (data not shown). Surprisingly, the N-6 dams produced more daughters (~0.65) than sons at both parities compared to the N-3 and control dams ( $P < 0.001$ ; Table 3). The N-6 dams had a preponderance of female-biased litters (31 female vs. 15 male) compared to the N-3 dams (14 female vs. 13 male;  $P < 0.05$ ). No sex-ratio skewing was evident in the controls (Table 3).

Because dam weights varied among the three groups (Table 2), we considered whether dam weight might influence offspring sex ratio. Maternal body weight did not influence the likelihood of producing more daughters than sons in any of the dietary groups. For both parities, no significant differences existed in gestation length or total litter size for the three diet groups.

*Offspring Weights*

To determine whether the maternal diet influenced offspring weight, the pups were weighed at 2, 14, and 21 days of age. There were no differences between the mean weights of male and female pups born to the different dietary groups. Pups born to the N-3 dams on average weighed less than the pups from the control dams at 14 and 21 days of age, but not at birth (Fig. 1). The pups born to the dams on the N-6 diet weighed less than the control pups by 21 days of age. Thus, in general the pups from the N-3 and N-6 dams were lighter than those from the control dam when they were weaned.

*Behavioral Observations*

As the experiments proceeded, it became apparent that the N-3 dams showed more instances of abnormal maternal

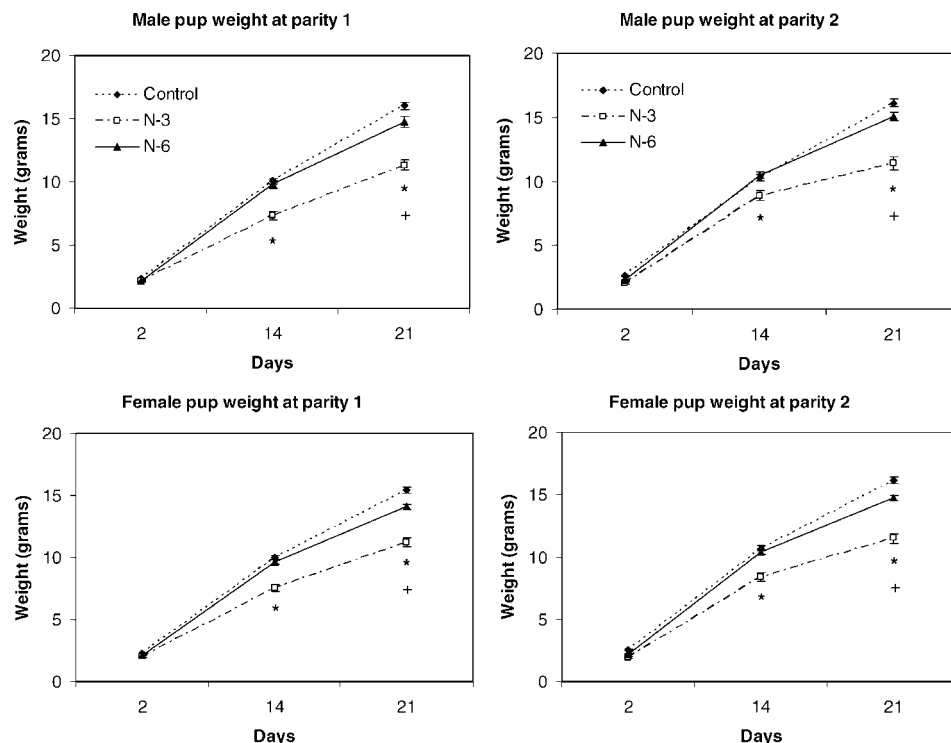
TABLE 3. Gestation length, litter size, total pups born, and sex ratio at Day 2 after parturition.

Diet	No. of litters	Gestation length (days)	Litter size	No. of pups			Percentage of males and females		No. of sex-biased litters		
				Total	Male	Female	Male	Female	Male	Eq*	Female
<b>Parity 1</b>											
Control	26	19.5 ± 0.3	6.9 ± 0.6	182	97	85	53.3	46.7 <sup>a</sup>	12	3	11
N-3	20	19.5 ± 0.4	5.5 ± 0.7	109	59	50	54.1	45.9 <sup>a</sup>	8	5	7
N-6	27	18.6 ± 0.3	6.9 ± 0.6	187	74	113	39.6	60.4 <sup>b</sup>	8	2	17
<b>Parity 2</b>											
Control	19	18.9 ± 0.4	6.1 ± 0.7	118	57	61	48.3	51.7 <sup>a</sup>	5	4	10
N-3	15	19.5 ± 0.5	5.6 ± 0.8	83	37	46	44.6	55.4 <sup>a</sup>	5	3	7
N-6	22	18.9 ± 0.4	7.3 ± 0.7	159	59	100	37.1	62.9 <sup>b</sup>	7	1	14

\* Eq = Number of litters with equivalent number of female and male offspring.

<sup>a,b</sup> Percentage of female pups with different superscript are significantly different among diet groups within parity ( $P < 0.001$ ).

FIG. 1. The male and female pup weights at 2, 14, and 21 days of age. Within a time, if the differences between any two means were greater than the least significant difference of 1.135, then they are considered different at  $P < 0.05$ . \* indicates where the N-3 pups differed from the 5015 control group and + indicates where the N-6 pups differed from the 5015 control group.



behavior relative to the N-6 and control dietary groups. For example, instances of cannibalism in parity 2 ( $P = 0.01$ , data not shown) and barbering of pups appeared to be more common in the N-3 group than the N-6 and C groups.

There were also measurable aberrations in nesting activity among the N-3 females. A large proportion of the N-3 dams (0.67), for example, failed to build any nests at all compared to the N-6 (0.18) and control (0.07) dams. The nests that were built by N-3 dams were more often constructed out of the aspen bedding than the provided 2"-square neslet material (Ancare Corp., Bellmore, NY) used by the majority the N-6 and control dams. By parity 2, these differences in aspen versus neslet construction material reached statistical significance ( $P < 0.05$ ; Table 4). The nests built by the N-3 dams were shorter (for parities 1 and 2) and narrower (for parity 2) compared to those built by the other two groups, although these differences might reflect the nature of the construction materials used.

As a result of observing such anomalous behavior, we next determined whether the N-3 and N-6 showed more anxiety than control dams in behavioral tests, because anxious mothers might be expected to demonstrate less-than-optimal maternal activities such as nest building and barbering pups. In addition, because maternal stress has been correlated with low sex ratios

[15, 32, 33] and the N-6 group of dams produced significantly more daughters than sons, we sought to determine whether N-6 dams were more anxious, i.e., possibly more stressed, than either the N-3 or control groups.

Both the elevated-plus and open-field maze tests indicated signs of anxiety in the N-3 dams, but not in either of the other two groups. The N-3 dams, for example, spent less time in the open arms ( $24.48 \pm 12.9$  sec) than the control ( $56.6 \pm 9.7$  sec) and N-6 ( $77.2 \pm 9.47$  sec) dams ( $P < 0.05$ ; Table 5). Instead, the N-3 dams exhausted more of their allotted 5 min in the closed arms ( $222.83 \pm 13.8$  sec) of the maze, as opposed to the control and N-6 dams, who only spent  $196.3 \pm 10.4$  and  $181.5 \pm 10.1$  sec, respectively ( $P < 0.05$ ; Table 5). The amount of time spent in the middle of the maze and the number of entries into the open and closed arms did not differ among the three dietary groups. In the open-field maze, the N-3 dams traveled only about one-third the distance ( $517.7 \pm 222.5$  cm) of the N-6 ( $1617.7 \pm 163.2$  cm) and 5015 control ( $1629.8 \pm 159.9$  cm) diet groups ( $P < 0.05$ ; Table 6).

## DISCUSSION

The main goal of this work was to test the hypothesis that in mice a maternal diet enriched with either N-3 or N-6 fatty acids

TABLE 4. The length, width, depth, volume (length  $\times$  width  $\times$  depth) and proportion of nesting material used in the control, N-3, and N-6 groups.

Diet group (n)*	Length (cm)	Width (cm)	Depth (cm)	Volume (cm <sup>3</sup> )	Proportion of nesting material used (%)
Parity 1					
Control (15)	$8.1 \pm 0.4^a$	$7.1 \pm 0.4$	$2.6 \pm 0.2$	$138.8 \pm 12.7$	$52.8 \pm 11.2$
N-3 (9)	$5.8 \pm 0.6^b$	$6.8 \pm 0.5$	$2.8 \pm 0.3$	$113.7 \pm 17.3$	$35.6 \pm 15.2$
N-6 (18)	$8.4 \pm 0.4^a$	$7.6 \pm 0.3$	$1.7 \pm 0.2$	$106.3 \pm 11.8$	$40.6 \pm 10.3$
Parity 2					
Control (14)	$8.1 \pm 0.5^a$	$7.8 \pm 0.4^a$	$1.5 \pm 0.2$	$91.4 \pm 13.0$	$64.7 \pm 11.4^a$
N-3 (5)	$6.2 \pm 0.8^b$	$6.2 \pm 0.6^b$	$1.7 \pm 0.4$	$65.8 \pm 22.6$	$15.9 \pm 19.8^b$
N-6 (19)	$8.5 \pm 0.4^a$	$7.5 \pm 0.3^a$	$1.4 \pm 0.2$	$88.5 \pm 11.1$	$44.6 \pm 9.8^{ab}$

\* n = Number of dams that built a nest.

<sup>a,b</sup> Group means within columns that have different superscripts are significantly different ( $P < 0.05$ ).

TABLE 5. Results for the number of entries into maze arms and time spent in each arm of the elevated plus maze.

Diet*	Time in open arms (sec)	Time in closed arms (sec)	Time in the middle (sec)	No. of entries into open arms	No. of entries into closed arms
Control (n = 22)	56.6 ± 9.7 <sup>b</sup>	196.3 ± 10.4 <sup>a,b</sup>	45.3 ± 6.5	6.1 ± 1.2	9.2 ± 1.2
N-3 (n = 14)	24.48 ± 12.9 <sup>a</sup>	222.83 ± 13.8 <sup>a</sup>	54.1 ± 8.6	4.6 ± 1.6	6.5 ± 1.5
N-6 (n = 23)	77.2 ± 9.47 <sup>b</sup>	181.5 ± 10.1 <sup>b</sup>	41.1 ± 6.3	7.8 ± 1.2	9.6 ± 1.1

\* n = Number of dams.

<sup>a,b</sup> Group means within columns that have different superscripts are significantly different ( $P < 0.05$ ).

would shift offspring sex ratio to favor males [19, 20], as observations in humans and a field study with opossums had suggested [19, 20]. Unexpectedly, the corn oil-enriched N-6 diet we employed shifted offspring sex ratio in a highly significant manner ( $P < 0.001$ ) towards females in two separate trials that provided 346 pups from 49 litters. Female-biased litters exceeded those skewed to males by more than 2:1, and over 60% of the pups born were female. Equally surprising was the fact that the fish oil-enriched N-3 diet had no effect on sex ratio, with an almost equal number of male and female offspring born, and provided no observable shift in the relative numbers of male- and female-biased litters relative to control dams. This experiment not only suggests that the kind of PUFA consumed can influence the sex ratio of offspring born to mice, but argues against our earlier hypothesis that female mice on a very high-fat diet comprised largely of lard produced more male offspring because the high content of palmitic, stearic, and oleic acids in lard had a sparing action on the metabolism of the essential PUFA. The present experiments argue the opposite, namely, that excess N-6 PUFA favor females. Nor are the present data consistent with the view that the caloric density of the food consumed by dams has a major influence on sex ratio. The N-6 and N-3 diets were identical in total metabolizable energy and total fat content, and in both of these regards they exceeded the control diet, although this caloric advantage did not lead to an increased weight gain of the dams. Accordingly, it is difficult to formulate any general hypothesis that might explain the outcomes of this and earlier studies [19, 20]. The fat composition of diets, especially the fatty acids, could influence numerous events in the reproductive process, including oocyte maturation and the timing of ovulation [34], the production of chemoattractants by the oocyte [35], prostaglandin synthesis [24, 36, 37], and properties of the reproductive tract [38], which might, in turn, adjust the relative fertilization abilities of X and Y sperm or provide an advantage to conceptuses of one sex over the other during their development [38–41].

Our experiments do not support the notion that supplementing the diet of mothers (or at least of mouse mothers) with menhaden oil, which is enriched in EPA and DHA, is beneficial to pregnancy outcomes. The N-3 mice had slightly smaller litters, they gained weight more slowly than controls, and their pups failed to thrive as well as those in the other two groups. Importantly, the pups born to N-3 dams had similar body masses to those in the other two groups at birth, but failed to gain weight

at the same rate as the others during the postnatal period. One possibility is that dams on the omega-enriched diet produce either less milk or poorer-quality milk than control females. Mammary duct development is attenuated in female mice on a diet enriched with menhaden oil [23, 42, 43], and this defect likely compromises their ability to secrete normal amounts of milk. Another possibility is that the in utero exposure to EPA and DHA alters the pups' suckling responses. Several reports, for example, have indicated that in utero or perinatal exposure to omega-supplemented diets alters the behavioral responses, growth, and metabolic hormones of the offspring [44–47].

The N-3 dams, in addition to their altered reproductive features, exhibited abnormal behavior, presumably as a result of consuming the fish oil. Based on anecdotal observations, they cannibalized or barbered their pups more frequently than the other two groups, and a majority did not build a nest. The nests that were built were unlike the ones observed with the 5015 control and N-6 group. Together, these data suggest that the N-3 diet led to altered behavioral responses in the dams. When the dams were tested in the elevated-plus maze, their responses, including lack of investigatory and ambulatory behaviors, are consistent with high anxiety levels [26]. The interpretation of their responses in the open-field maze is more equivocal. The N-3 dams spent a similar time in the center of the maze compared to animals from the N-6 and control groups. Several of our N-3 dams, however, did not move from the initial starting point at the center of the open field for several minutes, thereby lengthening the mean center time for the N-3 diet group. The lack of any response or ambulatory movement when placed in the open-field maze may be a result of anxiety. We noted no obvious change in gait or movement of the N-3 dams that might suggest motor impairment; therefore, we conclude that these mice are more tentative and fearful than the mice on the other two diets. The observations are generally consistent with some previous reports that N-3 oversupplementation in rodents can alter neurological and behavioral responses [26, 30, 48].

A potential problem with any diet supplemented with N-3 is accumulating peroxide activity. We attempted to minimize the accumulation of these undesirable products by including the antioxidant *tert*-butylhydroquinone (TBHQ) in the diet to limit oxidative breakdown of the fatty acids, keeping the stored formulation in a freezer, and frequently replenishing the food provided. Peroxide content of the diets did increase over the course of the study (from the start of the diet at Week 0 to

TABLE 6. The total distance traveled, time in center, and number of behavioral displays in the open field maze.

Diet*	Total distance traveled (cm)	Time spent in center (sec)	No. of rears	No. of times grooming	No. of times smelling	No. of inactive periods
Control (n = 22)	1629.8 ± 159.9 <sup>b</sup>	34.2 ± 6.2	14.4 ± 2.9	0.9 ± 0.3	21.2 ± 3.1	6.9 ± 0.9
N-3 (n = 14)	517.7 ± 222.5 <sup>a</sup>	23.0 ± 8.6	6.6 ± 3.4	0.6 ± 0.4	15.3 ± 4.3	3.2 ± 1.2
N-6 (n = 23)	1617.7 ± 163.2 <sup>b</sup>	25.5 ± 6.3	17.2 ± 2.9	1.4 ± 0.3	25.8 ± 3.2	5.6 ± 0.9

\* n = Number of dams.

<sup>a,b</sup> Group means within columns that have different superscripts are significantly different ( $P < 0.05$ ).

Week 36). However, the mice on the diet appeared to show no aversion to their food and gave consistent outcomes in both trials. Unfortunately, our animal facility is not equipped with metabolic cages and not all food taken into the cages was eaten; thus, it was not possible to make an accurate measure of food consumption in any of the three dietary groups. However, except for their behavioral peculiarities, the N-3 mice appeared to consume similar amounts of food as the dams on the N-6 and 50I5 diets. Additionally, they remained healthy and did not exhibit significantly higher morbidity or mortality compared to the other two groups.

In summary, the dams on the N-6 supplemented diet had a shift in offspring sex ratio favoring daughters, without any measurable increase in maternal anxiety. In contrast, the N-3 dams displayed greater maternal anxiety, but with no accompanying changes in offspring sex ratio. Thus, maternal diet-induced alteration of offspring sex ratio is seemingly due to mechanisms other than stress. These experiments confirm that marked changes in offspring sex ratio in mice can be brought about by altering the fat content of the mother's diet and indicate that menhaden oil supplementation had negative effects on maternal behavior and pregnancy outcomes.

## ACKNOWLEDGMENTS

We thank Wayne Shoemaker, David Card, and Leon Toebben, Bond Life Sciences Center, University of Missouri-Columbia, for construction of the mazes. Assistance with maze analysis, animal husbandry, and manuscript preparation was provided by Ms. Jessica Berry, University of Missouri-Columbia, MO.

## REFERENCES

- Decsi T, Campoy C, Koletzko B. Effect of N-3 polyunsaturated fatty acid supplementation in pregnancy: the Nuheal trial. *Adv Exp Med Biol* 2005; 569:109–113.
- Eilander A, Hundscheid DC, Osendarp SJ, Transler C, Zock PL. Effects of n-3 long chain polyunsaturated fatty acid supplementation on visual and cognitive development throughout childhood: a review of human studies. *Prostaglandins Leukot Essent Fatty Acids* 2007; 76:189–203.
- Helland IB, Saugstad OD, Saarem K, Van Houwelingen AC, Nylander G, Drevon CA. Supplementation of n-3 fatty acids during pregnancy and lactation reduces maternal plasma lipid levels and provides DHA to the infants. *J Matern Fetal Neonatal Med* 2006; 19:397–406.
- Helland IB, Saugstad OD, Smith L, Saarem K, Solvoll K, Ganes T, Drevon CA. Similar effects on infants of n-3 and n-6 fatty acids supplementation to pregnant and lactating women. *Pediatrics* 2001; 108:E82.
- Hosli I, Zanetti-Daellenbach R, Holzgreve W, Lapaire O. Role of omega 3-fatty acids and multivitamins in gestation. *J Perinat Med* 2007; 35(suppl 1): S19–S24.
- Jensen CL. Effects of n-3 fatty acids during pregnancy and lactation. *Am J Clin Nutr* 2006; 83:1452S–1457S.
- Krauss-Etschmann S, Shadid R, Campoy C, Hoster E, Demmelmair H, Jimenez M, Gil A, Rivero M, Veszpremi B, Decsi T, Koletzko BV. Effects of fish-oil and folate supplementation of pregnant women on maternal and fetal plasma concentrations of docosahexaenoic acid and eicosapentaenoic acid: a European randomized multicenter trial. *Am J Clin Nutr* 2007; 85:1392–1400.
- Joshi S, Rao S, Golwilkar A, Patwardhan M, Bhonde R. Fish oil supplementation of rats during pregnancy reduces adult disease risks in their offspring. *J Nutr* 2003; 133:3170–3174.
- Olivo SE, Hilakivi-Clarke L. Opposing effects of prepubertal low- and high-fat n-3 polyunsaturated fatty acid diets on rat mammary tumorigenesis. *Carcinogenesis* 2005; 26:1563–1572.
- Wainwright PE, Huang YS, Bulman-Fleming B, Levesque S, McCutcheon D. The effects of dietary fatty acid composition combined with environmental enrichment on brain and behavior in mice. *Behav Brain Res* 1994; 60:125–136.
- Carrie I, Guesnet P, Bourre JM, Frances H. Diets containing long-chain n-3 polyunsaturated fatty acids affect behaviour differently during development than ageing in mice. *Br J Nutr* 2000; 83:439–447.
- Sosenko IR, Innis SM, Frank L. Menhaden fish oil, n-3 polyunsaturated fatty acids, and protection of newborn rats from oxygen toxicity. *Pediatr Res* 1989; 25:399–404.
- Yeh YY, Gehman MF, Yeh SM. Maternal dietary fish oil enriches docosahexaenoate levels in brain subcellular fractions of offspring. *J Neurosci Res* 1993; 35:218–226.
- Rosenfeld CS, Grimm KM, Livingston KA, Brokman AM, Lamberson WE, Roberts RM. Striking variation in the sex ratio of pups born to mice according to whether maternal diet is high in fat or carbohydrate. *Proc Natl Acad Sci U S A* 2003; 100:4628–4632.
- Rosenfeld CS, Roberts RM. Maternal diet and other factors affecting offspring sex ratio: a review. *Biol Reprod* 2004; 71:1063–1070.
- Whyte JJ, Alexenko AP, Davis AM, Ellersieck MR, Fountain ED, Rosenfeld CS. Maternal diet composition alters serum steroid and free fatty acid concentrations and vaginal pH in mice. *J Endocrinol* 2007; 192:75–81.
- Alexenko AP, Mao J, Ellersieck MR, Davis AM, Whyte JJ, Rosenfeld CS, Roberts RM. The contrasting effects of ad libitum and restricted feeding of a diet very high in saturated fats on sex ratio and metabolic hormones in mice. *Biol Reprod* 2007; 10:1095.
- Trivers RL, Willard DE. Natural selection of parental ability to vary the sex ratio of offspring. *Science* 1973; 179:90–92.
- Austad S, Sunquist M. Sex-ratio manipulation in the common opossum. *Nature* 1986; 324:58–60.
- Crawford MA, Doyle W, Meadows N. Gender differences at birth and differences in fetal growth. *Hum Reprod* 1987; 2:517–520.
- Hilakivi-Clarke L, Cho E, Cabanes A, DeAssis S, Olivo S, Helferich W, Lippman ME, Clarke R. Dietary modulation of pregnancy estrogen levels and breast cancer risk among female rat offspring. *Clin Cancer Res* 2002; 8:3601–3610.
- Clarke SD, Benjamin L, Bell L, Phinney SD. Fetal growth and fetal lung phospholipid content in rats fed safflower oil, menhaden oil, or hydrogenated coconut oil. *Am J Clin Nutr* 1988; 47:828–835.
- Abraham S, Faulkin LJ, Mitchell DJ. Attenuation of mammary duct development by menhaden oil in BALB/c mice. *Proc Soc Exp Biol Med* 1991; 196:222–229.
- Henderson CD, Black HS, Wolf JE Jr. Influence of omega-3 and omega-6 fatty acid sources on prostaglandin levels in mice. *Lipids* 1989; 24:502–505.
- Whyte JJ, Roberts RM, Rosenfeld CS. Fluorescent in situ hybridization for sex chromosome determination before and after fertilization in mice. *Theriogenology* 2007; 67:1022–1031.
- Fedorova I, Salem N Jr. Omega-3 fatty acids and rodent behavior. *Prostaglandins Leukot Essent Fatty Acids* 2006; 75:271–289.
- Pellow S, Chopin P, File SE, Briley M. Validation of open/closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods* 1985; 14:149–167.
- Crusio WE. Genetic dissection of mouse exploratory behaviour. *Behav Brain Res* 2001; 125:127–132.
- Raygada M, Cho E, Hilakivi-Clarke L. High maternal intake of polyunsaturated fatty acids during pregnancy in mice alters offspring's aggressive behavior, immobility in the swim test, locomotor activity and brain protein kinase C activity. *J Nutr* 1998; 128:2505–2511.
- Chalon S, Delion-Vancassel S, Belzung C, Guilloteau D, Leguisquet AM, Besnard JC, Durand G. Dietary fish oil affects monoaminergic neurotransmission and behavior in rats. *J Nutr* 1998; 128:2512–2519.
- Littell RC, Henry PR, Ammerman CB. Statistical analysis of repeated measures data using SAS procedures. *J Anim Sci* 1998; 76:1216–1231.
- Uller T, Meylan S, De Fraipont M, Clobert J. Is sexual dimorphism affected by the combined action of prenatal stress and sex ratio? *J Exp Zool A Comp Exp Biol* 2005; 303:1110–1114.
- Wells JC. Natural selection and sex differences in morbidity and mortality in early life. *J Theor Biol* 2000; 202:65–76.
- Bilby TR, Block J, do Amaral BC, Sa Filho O, Silvestre FT, Hansen PJ, Staples CR, Thatcher WW. Effects of dietary unsaturated fatty acids on oocyte quality and follicular development in lactating dairy cows in summer. *J Dairy Sci* 2006; 89:3891–3903.
- Kubagawa HM, Watts JL, Corrigan C, Edmonds JW, Sztul E, Browse J, Miller MA. Oocyte signals derived from polyunsaturated fatty acids control sperm recruitment in vivo. *Nat Cell Biol* 2006; 8:1143–1148.
- Thatcher WW, Guzeloglu A, Mattos R, Binelli M, Hansen TR, Pru JK. Uterine-conceptus interactions and reproductive failure in cattle. *Theriogenology* 2001; 56:1435–1450.
- Wamsley NE, Burns PD, Engle TE, Enns RM. Fish meal supplementation alters uterine prostaglandin F2alpha synthesis in beef heifers with low luteal-phase progesterone. *J Anim Sci* 2005; 83:1832–1838.
- Pratt NC, Huck UW, Lisk RD. Offspring sex ratio in hamsters is correlated with vaginal pH at certain times of mating. *Behav Neural Biol* 1987; 48: 310–316.
- Dominko T, First NL. Timing of meiotic progression in bovine oocytes

- and its effect on early embryo development. *Mol Reprod Dev* 1997; 47: 456–467.
40. Gutierrez-Adan A, Perez G, Granados J, Garde JJ, Perez-Guzman M, Pintado B, De La Fuente J. Relationship between sex ratio and time of insemination according to both time of ovulation and maturational state of oocyte. *Zygote* 1999; 7:37–43.
  41. Martin JF. Length of the follicular phase, time of insemination, coital rate and the sex of offspring. *Hum Reprod* 1997; 12:611–616.
  42. Miyamoto-Tiaven MJ, Hillyard LA, Abraham S. Influence of dietary fat on the growth of mammary ducts in BALB/c mice. *J Natl Cancer Inst* 1981; 67:179–188.
  43. Welsch CW, O'Connor DH. Influence of the type of dietary fat on developmental growth of the mammary gland in immature and mature female BALB/c mice. *Cancer Res* 1989; 49:5999–6007.
  44. Korotkova M, Gabriellson BG, Holmang A, Larsson BM, Hanson LA, Strandvik B. Gender-related long-term effects in adult rats by perinatal dietary ratio of n-6/n-3 fatty acids. *Am J Physiol Regul Integr Comp Physiol* 2005; 288:R575–R579.
  45. Song C, Leonard BE, Horrobin DF. Dietary ethyl-eicosapentaenoic acid but not soybean oil reverses central interleukin-1-induced changes in behavior, corticosterone and immune response in rats. *Stress* 2004; 7:43–54.
  46. Oken E, Kleinman KP, Olsen SF, Rich-Edwards JW, Gillman MW. Associations of seafood and elongated n-3 fatty acid intake with fetal growth and length of gestation: results from a US pregnancy cohort. *Am J Epidemiol* 2004; 160:774–783.
  47. Allen KGD, Harris MA. The role of n-3 fatty acids in gestation and parturition. *Exp Biol Med* 2001; 226:498–506.
  48. Levant B, Radel JD, Carlson SE. Decreased brain docosahexaenoic acid during development alters dopamine-related behaviors in adult rats that are differentially affected by dietary remediation. *Behav Brain Res* 2004; 152: 49–57.