Artificial rearing with docosahexaenoic acid and n-6 docosapentaenoic acid alters rat tissue fatty acid composition[S]

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Abstract Docosahexaenoic acid (DHA; 22:6n-3) and n-6 docosapentaenoic acid (DPAn-6; 22:5n-6) are components of enriched animal feed and oil derived from *Schizochytrium* species microalgae. A one generation, artificial rearing model from day 2 after birth onward (AR) and a dam-reared control group (DAM) were used to examine DPAn-6 feeding on the fatty acid composition of various rat tissues at 15 weeks of age. Four AR diets were based on an n-3 fatty acid-deficient, 18:2n-6-based artificial milk with 22:6n-3 and/or 22:5n-6 added: AR-LA, AR-DHA, AR-DPAn-6, and AR-DHA+DPAn-6. The 22:6n-3 levels for the DAM, AR-DHA, and AR-DHA+DPAn-6 groups tended to be similar and higher than in the AR-LA and AR-DPAn-6 groups. The levels of 22:5n-6 tended to be higher only in the absence of dietary 22:6n-3. Adipose levels of 22:5n-6 was the only exception, as 22:5n-6 was significantly higher in AR-DHA+DPAn-6 than was observed in either the DAM or the AR-DHA group. There were no differences in 20:4n-6 levels within the tissues examined. In conclusion, 22:5n-6 replaces 22:6n-3 in the absence of 22:6n-3 only and does not appear to compete with 22:6n-3 in the presence of dietary 22:6n-3, suggesting that oils containing 22:5n-6 and 22:6n-3 may be a good dietary source of 22:6n-3.—Stark, K. D., S-Y. Lim, and N. Salem, Jr. **Artificial rearing with docosahexaenoic acid and n-6 docosapentaenoic acid alters rat tissue fatty acid composition. *J. Lipid Res.* 2007.** 48: **2471–2477.**

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Increased dietary docosahexaenoic acid (DHA; 22:6n-3) is associated with several health benefits by its effects on various physiological systems through incorporation into cellular membranes and affecting cell signaling (1–3). The major source of 22:6n-3 in the food supply is marine fish and fish oil, but population-based strategies to increase 22:6n-3 intake by fish and fish oil intake alone are complicated by concerns about environmental contaminants (4) and the sustainability of fishing stocks (5). Commercial-scale cultivation of 22:6n-3-enriched microalgae such as *Crypthecodinium cohnii* and *Schizochytrium* species can serve as alternative sources of 22:6n-3. The *Schizochytrium* species have a relatively high 22:6n-3 biomass and oil yield for a low cost (6, 7), but they also contain considerable amounts of n-6 docosapentaenoic acid (DPAn-6; 22:5n-6) (7).

22:6n-3 and 22:5n-6 differ by a single, additional carbon-carbon double bond at the Δ19 position of 22:6n-3. 22:6n-3 and 22:5n-6 are largely incorporated into the *sn*-2 position of phospholipids along with and in competition with other highly unsaturated fatty acids (HUFAs; ⩾20 carbons, ⩾3 double bonds) such as arachidonic acid (20:4n-6) and eicosapentaenoic acid (20:5n-3) (8). In n-3 fatty acid deficiency studies using linoleic acid (LA; 18:2n-6)-based diets, decreases in 22:6n-3 can be compensated for to some degree by 22:5n-6, but it is not complete (9, 10). The rate of biosynthesis of 22:5n-6 from 18:2n-6 is low, as 20:4n-6 is the preferred biosynthetic end product (11). 18:2n-6 is also incorporated directly into complex lipids such as phospholipids and triacylglycerols as well as being β-oxidized (12). Therefore, n-3 fatty acid deficiency studies using 18:2n-6-based control diets have the potential to increase the content of 20:4n-6, which has significant bioactive properties. Given that the reciprocal replacement of 22:6n-3 with 22:5n-6 is incomplete in the cerebral cortex of developing rats (10), supplementing the deficient diets with preformed 22:5n-6 was necessary to attribute deficiencies in spatial task performances to low levels of 22:6n-3 rather

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Abbreviations: AR, artificially reared; DAM, dam-reared; DHA, docosahexaenoic acid; DPAn-6, n-6 docosapentaenoic acid; HUFA, highly unsaturated fatty acid; LA, linoleic acid.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of 9 tables.

TABLE 1. Fatty acid composition of artificial rat milks

Composition	AR-LA	AR-DPA	AR-DHA+DPA	AR-DHA
Fat source (g/100 ml milk)				
Medium-chain triacylglycerol oil	1.56	1.56	1.56	1.56
Hydrogenated coconut oil	3.24	3.24	3.24	3.24
18:1n-9 EE	5.40	5.28	5.24	5.28
18:2n-6 EE	1.80	1.80	1.80	1.80
22:5n-6 EE	—	0.12	—	—
22:6n-3 EE	—	—	—	0.12
22:6n-3/22:5n-6 EEs (2:1)			0.16	
Fatty acid composition (wt% of total fatty acids)				
Total saturates	33.0	33.6	33.0	35.6
Total monounsaturates	46.9	47.5	46.8	45.5
18:2 n-6	18.1	16.0	17.7	16.1
18:3 n-3	0.01	0.01	0.01	0.01
22:5 n-6	—	1.01	0.42	—
22:6 n-3	—	—	0.96	1.16

AR-LA, 18:2n-6 artificially reared diet; AR-DPA, 22:5n-6 artificially reared diet; AR-DHA+DPA, 22:6n-3 plus 22:5n-6 artificially reared diet; AR-DHA, 22:6n-3 artificially reared diet; EE, ethyl ester.

than low levels of 22:6n-3 plus 22:5n-6 (13). Reports of the effect of feeding 22:5n-6 on the fatty acid composition of tissues are limited (13), with no previous studies comparing diets supplemented with 22:5n-6 (free of 22:6n-3), 22:6n-3 (free of 22:5n-6), or a combination of 22:6n-3 plus 22:5n-6.

The purpose of the present study was to determine to what extent dietary 22:5n-6 is incorporated into lipids in various tissues. A second goal is to determine whether dietary 22:5n-6 is antagonistic to the incorporation of other HUFAs, particularly 22:6n-3, but also 20:4n-6. A unique, artificially reared (AR) suckling rat model was used to control the type of fatty acids fed and generate n-3 fatty acid-deficient animals in a single generation. AR groups included an 18:2n-6-based diet (AR-LA) and 18:2n-6 diets with 1% 22:6n-3 (AR-DHA), 1% 22:5n-6 (AR-DPAn-6), or 1% 22:6n-3 plus 0.4% 22:5n-6 (AR-DHA+DPAn-6). AR groups were also compared with a dam-reared control group (DAM). The fatty acid compositions of plasma, erythrocytes, brain, heart, liver, adipose (epididymal fat pads), kidney, skeletal muscle (soleus), and testes were determined.

METHODS

Animals and study design

The study design and experimental procedures were approved by the Animal Care and Use Committee of the National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health. The study design and experimental diets have been described in detail previously (13). In brief, pregnant, Long-Evans dams at day 3 of gestation were purchased from Charles River (Portage, MI) and maintained with ad libitum water at a controlled temperature (23 ± 1°C) and a 12 h light/dark cycle. Dams were immediately placed on a 22:6n-3-free diet with 3.1% of total fatty acids as α-linolenic acid and 18:3n-3 for n-3 fatty acid adequacy. Male pups were collected from each dam litter at 2 days after birth and randomized to five experimental groups: a DAM group and four AR groups on experimental milks. The experimental milks varied in specific fatty acid content but were based on fat from hydrogenated coconut oil (Dyets, Bethlehem, PA), medium-chain triglycerides (Mead Johnson Nutritionals, Evansville, IN), and oleic and linoleic ethyl esters with or without DPAn-6 and/or docosahexaenoic ethyl esters added (Nu-Chek Prep, Elysian, MN). Details of the diet preparations are presented elsewhere (13). The four AR groups were fed an 18:2n-6-based diet (AR-LA), an 18:2n-6-based diet with 1% of total fatty acids as 22:5n-6 (AR-DPAn-6), an 18:2n-6-based diet with 1% of total fatty acids as 22:6n-3 (AR-DHA), and an 18:2n-6-based diet with 1% of total fatty acids as 22:6n-3 and 0.4% of total fatty acids as 22:5n-6 diet (AR-DHA+DPAn-6) **(Table 1)**. Rat pups were hand-reared until capable of feeding from bottle nipples ad libitum after eye opening (postnatal day 14–15). Pups were eventually weaned to a pelleted diet with a similar fatty acid composition to their respective artificial milks; the DAM group was transferred to the maternal diet **(Table 2)**.

The rats were euthanized by decapitation at 15 weeks of age after completing various behavioral assessments (13). Trunk blood was collected and separated into plasma and erythrocytes, with the erythrocytes washed with phosphate-buffered saline according to Reed et al. (14). Tissues were excised, washed with phosphate-buffered saline, frozen immediately on dry ice, and

TABLE 2. Fatty acid composition of pelleted rat diets

Composition	DAM	AR-LA	AR-DPA	AR-DHA+DPA	AR-DHA
Fat source (g/100 g diet)					
Hydrogenated coconut oil	7.75	2.7	2.7	2.7	2.7
Safflower oil	1.77	—	—	—	—
Flaxseed oil	0.48	—	—	—	—
Medium-chain triacylglycerol oil	—	1.3	1.3	1.3	1.3
18:1n-9 EE	—	4.4	4.4	4.37	4.4
18:2n-6 EE	—	1.5	1.5	1.5	1.5
22:5n-6 EE	—	—	0.10		—
22:6n-3 EE	—	—	—		0.10
22:6n-3/22:5n-6 EEs (2:1)	—	—	—	0.13	—
Fatty acid composition (wt% of total fatty acids)	DAM	AR-LA	AR-DPA	AR-DHA+DPA	AR-DHA
Total saturates	77.2	27.0	27.3	26.5	22.0
Total monounsaturates	4.3	46.2	44.5	44.7	47.1
18:2 n-6	15.3	15.4	15.1	15.3	16.3
18:3 n-3	3.1	0.04	0.04	0.04	0.05
22:5 n-6	—	—	1.04	0.49	—
22:6 n-3	—	—	—	0.98	1.10

DAM, dam-reared; EE, ethyl ester.

stored at −80°C. Epididymal fat pads were collected to represent adipose tissue, and skeletal muscle is represented by soleus muscle. The fatty acid compositions of red and white gastrocnemius muscles are presented elsewhere (15).

Analysis of tissue fatty acid composition

Total lipids of tissues were prepared by a modified Folch extraction procedure (16) in the presence of docoasatrienoic (22:3n-3) methyl ester (Nu-Chek Prep) included as an internal standard. Lipid extracts were then transmethylated with 14% BF_3 in methanol (17) with a modification to include hexane (18). Fatty acid methyl esters were analyzed by gas chromatography as described previously (18) using a 28 component quantitative standard mixture (Prep 462; Nu-Chek Prep) to identify individual fatty acids. Fatty acid compositions were determined both qualitatively (weight percentage of total fatty acids) and quantitatively (concentration of fatty acids in tissue).

Statistical analyses

Statistical analyses were completed with SPSS for Windows statistical software (release 11.5.1; SPPS, Inc., Chicago, IL) with data expressed as means ± SD. The effects of the dietary groups on body and tissue weights and fatty acid composition data from tissues were compared by the general linear model procedure with individual means compared using the Tukey's honestly significant difference post hoc test if a significant F value was determined. Statistical significance was inferred at $P < 0.01$ rather than $P < 0.05$ because of the number of comparisons completed.

RESULTS

Tissue and organ weights

The body weights of the rats in the present study have been presented in detail previously (13). Briefly, the AR groups showed lower weight gain than the DAM group until the time of weaning, but the differences were no longer present by 8 weeks of age. At the time of euthanasia (15 weeks of age), there were no differences in body and excised tissue weights between the AR and DAM groups **(Table 3)**.

Fatty acid composition of tissues

The percentage of HUFAs varied according to each tissue, but the pattern of changes in the percentage of HUFAs in response to dietary treatments was relatively similar **(Fig. 1)**. Dietary groups with 22:6n-3 included (DAM, AR-DHA, and AR-DHA+DPAn-6) had significantly higher 22:6n-3 in all tissues compared with groups that did not have preformed dietary 22:6n-3 (AR-LA and AR-DPAn-6). The levels of 22:6n-3 were similar for the DAM, AR-DHA, and AR-DHA+DPAn-6 groups in plasma, brain, heart, liver, adipose, kidney, and muscle. DAM rats had higher 22:6n-3 in testes compared with AR-DHA and AR-DHA+DPAn-6 rats. In erythrocytes, the AR-DHA group had 22:6n-3 levels that were slightly higher than the AR-DHA+DPAn-6 group.

The AR-LA and AR-DPAn-6 groups tended to have significantly higher levels of 22:5n-6 in tissues except for the testes. The level of 22:5n-6 was very high in testes (13–14% of total fatty acids) in all groups, and dietary manipulation had no significant effect on these levels. In adipose, the 22:5n-6 levels did not differ between the AR-LA group and the AR-DHA+DPAn-6 group, whereas AR-DPAn-6 was significantly higher. Levels of 22:5n-6 were also significantly higher in AR-DPAn-6 compared with AR-LA in erythrocytes and heart.

The levels of 22:6n-3 were significantly lower and the levels of 22:5n-6 were significantly higher in all tissues from the AR-DPAn-6 rats compared with the AR-DHA rats, with no differences in 22:5n-6 levels of the testes as an exception. When 22:5n-6 was fed with 22:6n-3, most of these differences disappeared, with levels of 22:6n-3 and 22:5n-6 in the AR-DHA+DPAn-6 rats resembling the levels in AR-DHA rats. Exceptions included slightly higher levels of 22:5n-6 in adipose tissue and slightly lower 22:6n-3 levels in erythrocytes of AR-DHA+DPAn-6 rats.

The impact of diet manipulation and DHA and DPAn-6 supplementation was examined further by comparing the sum of DHA and DPAn-6 and the percentage of this sum in total HUFAs **(Table 4)**. Overall, the AR-LA group tended to have lower sums for 22:6n-3 plus 22:5n-6 in tissues, with the exception of the testes. Total HUFA levels did not differ between dietary groups for all tissues (Fig. 1) and it appears that in the AR-LA group there were slight but not significant increases of 20:4n-6. Levels of 22:4n-6 tended to be significantly higher in tissues (except liver, kidney, and testes) of AR-LA rats compared with tissues from rats on diets containing 22:6n-3 (see supplementary Tables I–IX). The AR-DPA rats had significantly higher 22:4n-6 compared with the AR-DHA+DPAn-6 rats in erythrocytes and brain. Levels of 22:4n-6 in the AR-DHA+DPAn-6 and AR-DHA groups did not differ in any tissue. Levels of 22:5n-3 were significantly lower in all AR groups compared with DAM rats, with the only exception being the 22:5n-3 in testes of AR-DHA+DPAn-6 rats being

TABLE 3. Body and organ weights of DAM and artificially reared rats

Tissue	DAM (n = 8)	AR-LA (n = 7)	AR-DPA (n = 7)	AR-DHA+DPA (n = 8)	AR-DHA (n = 7)
Body (15 weeks)	630 ± 69	669 ± 68	639 ± 55	652 ± 92	589 ± 54
Brain	2.12 ± 0.14	1.99 ± 0.08	1.95 ± 0.17	2.00 ± 0.12	2.01 ± 0.09
Heart	1.68 ± 0.17	1.76 ± 0.09	1.69 ± 0.26	1.59 ± 0.21	1.52 ± 0.16
Liver	23.8 ± 3.9	27.8 ± 5.2	28.1 ± 5.4	24.9 ± 2.0	25.8 ± 3.6
Epididymal fat pads	16.1 ± 4.4	13.7 ± 4.4	14.3 ± 2.1	15.8 ± 5.2	13.6 ± 3.0
Kidney	1.70 ± 0.20	1.82 ± 0.18	1.77 ± 0.22	1.76 ± 0.30	1.64 ± 0.18
Testes	3.52 ± 0.26	3.28 ± 0.22	3.40 ± 0.46	3.27 ± 0.30	3.17 ± 0.22

Values shown are g of tissue and are means ± SD. No differences were detected by one-way ANOVA.

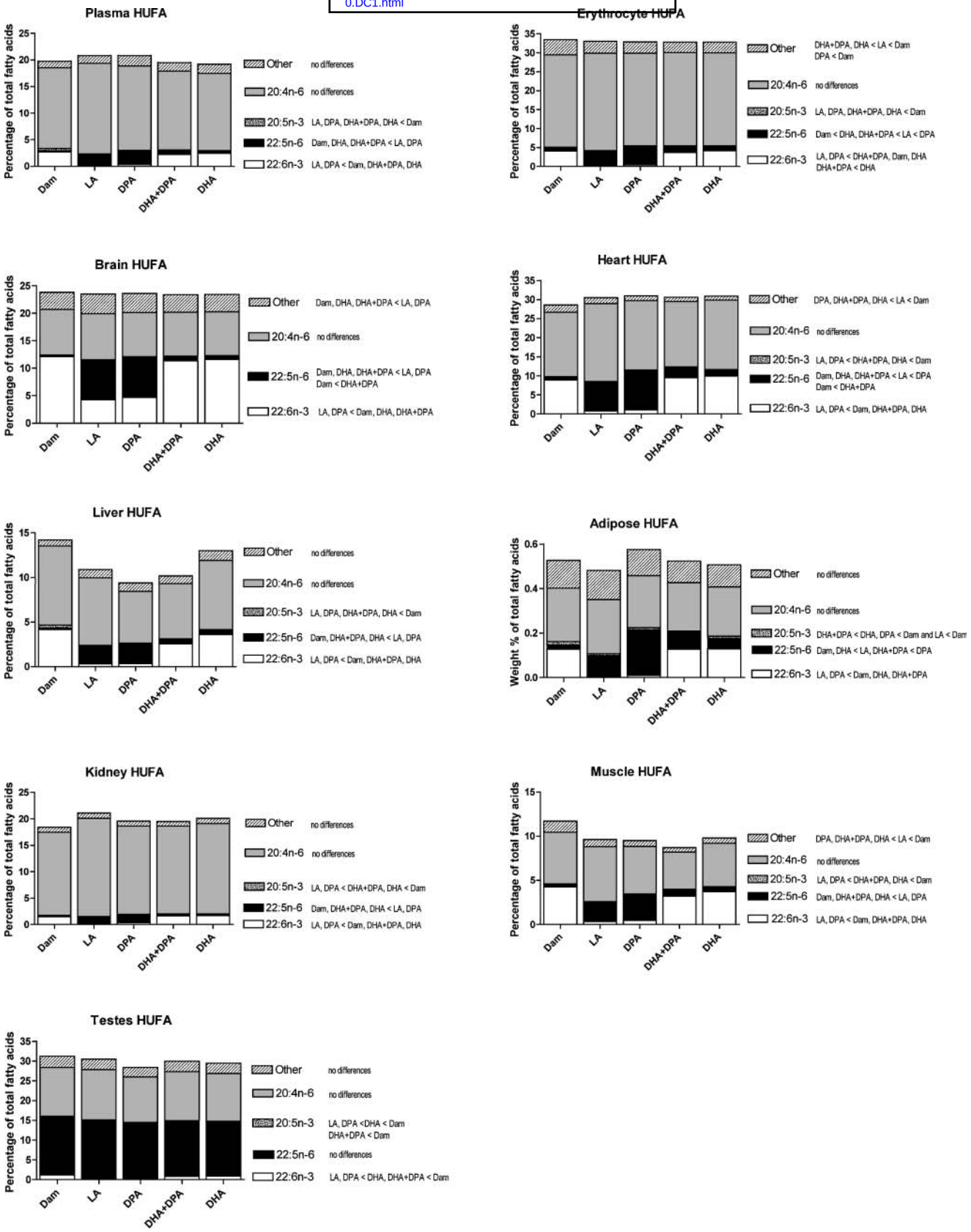

Fig. 1. Highly unsaturated fatty acids (HUFAs; ≥20 carbons and ≥3 double bonds) of tissues from dam-reared and artificially reared rats. Values are means and are derived from five to eight animals (see supplementary tables for details). Statistical differences were determined by Tukey's post hoc test after significance was detected by the general linear model procedure ($P < 0.05$). LA, 18:2n-6 artificially reared diet; DPA, 22:5n-6 artificially reared diet; DHA+DPA, 22:6n-3 + 22:5n-6 artificially reared diet; DHA, 22:6n-3 artificially reared diet.

TABLE 4. Impact of dietary manipulations on DHA and DPAn-6 tissue levels

Tissue	DAM	AR-LA	AR-DPA	AR-DHA+DPA	AR-DHA
			% by weight of total fatty acid		
Sum of DHA and DPA					
Plasma	2.91 ± 0.37	2.28 ± 0.79	2.96 ± 0.15	3.03 ± 0.37	2.93 ± 0.20
Erythrocyte	4.76 ± 0.22 a	4.16 ± 0.51 a	5.45 ± 0.38 b	5.42 ± 0.31 b	5.41 ± 0.32 b
Brain	12.4 ± 0.7	11.6 ± 0.6	12.1 ± 0.9	12.2 ± 0.3	12.3 ± 0.5
Heart	9.7 ± 2.4 a,b	8.5 ± 1.5 a	11.5 ± 1.7 a,b	12.3 ± 1.5 b	11.6 ± 1.2 b
Liver	4.34 ± 1.97	2.40 ± 1.43	2.64 ± 1.03	3.11 ± 1.26	4.14 ± 1.93
Adipose	0.15 ± 0.03 a,b	0.10 ± 0.0.3 a	0.21 ± 0.03 c	0.20 ± 0.04 c	0.18 ± 0.03 b,c
Kidney	1.59 ± 0.37	1.54 ± 0.28	1.92 ± 0.37	1.95 ± 0.42	1.94 ± 0.34
Muscle	4.52 ± 1.78	2.59 ± 0.85	3.47 ± 1.17	3.96 ± 2.22	4.27 ± 2.34
Testes	16.0 ± 1.5	15.1 ± 3.9	14.5 ± 2.0	14.9 ± 1.7	14.8 ± 1.5
Percentage of DHA plus DPA in total HUFA					
Plasma	15.0 ± 2.4 b	10.9 ± 1.8 a	14.4 ± 1.9 a,b	15.6 ± 1.6 b	15.7 ± 2.9 b
Erythrocyte	14.4 ± 0.7 b	12.6 ± 1.4 a	16.6 ± 1.2 c	16.6 ± 1.3 c	16.5 ± 0.7 c
Brain	52.2 ± 1.2 b	49.1 ± 1.3 a	51.2 ± 1.2 b	52.2 ± 1.0 b	52.6 ± 1.0 b
Heart	33.9 ± 2.0 b	27.9 ± 2.4 a	37.0 ± 1.6 b,c	40.1 ± 1.5 c	37.6 ± 2.3 b,c
Liver	30.3 ± 2.6 b	21.7 ± 2.9 a	28.0 ± 2.1 b	30.9 ± 2.5 b	31.7 ± 3.1 b
Adipose	27.6 ± 1.7 b	20.7 ± 2.1 a	37.4 ± 3.8 c	38.8 ± 2.6 c	34.5 ± 1.9 c
Kidney	8.6 ± 0.7 a,b	7.4 ± 1.0 a	9.8 ± 0.6 b	10.0 ± 1.1 b	9.8 ± 1.5 b
Muscle	37.9 ± 6.3 b	26.7 ± 2.2 a	36.4 ± 3.5 b	43.4 ± 6.4 b	42.8 ± 3.0 b
Testes	51.0 ± 1.5	49.3 ± 1.7	51.0 ± 1.0	49.7 ± 1.2	50.1 ± 1.4

Values are means ± SD. Means with different letters are significantly different across diet groups by Tukey's post hoc test after significance was detected by the general linear model procedure ($P < 0.05$). HUFA, highly unsaturated fatty acids (≥20 carbons and ≥3 double bonds).

similar to that in the DAM rats. In the AR groups, the levels of 22:5n-3 tended to be similar except in brain and kidney, where 22:5n-3 was higher in rats fed DHA (AR-DHA and AR-DHA+DPAn-6).

Detailed fatty acid compositions, including total fatty acid concentrations of each dietary group for each tissue, are included in supplementary Tables I–IX. Other differences in fatty acids were largely the result of artificial rearing versus dam rearing. Saturated fatty acids tended to be significantly higher in tissues of DAM rats, whereas monounsaturated fatty acids, particularly 18:1n-9, tended to be higher in AR rats. The higher intakes and tissue levels of 18:1n-9 in the AR rats may be partly responsible for the significantly higher levels of 18:2n-6 in all tissues of DAM rats despite similar 18:2n-6 dietary intakes in all dietary groups. The concentrations of total fatty acids were similar for all diet groups for all tissues except adipose tissue, in which the DAM group contained higher levels than the AR-LA, AR-DPAn-6, and AR-DHA groups (see supplementary Tables I–IX).

DISCUSSION

The present study demonstrates that 22:5n-6 is incorporated into a variety of body tissues and does not appear to compete with 22:6n-3 incorporation into tissues when 22:6n-3 is present in the diet. In the absence of 22:6n-3 in the diet, the inclusion of 22:5n-6 in the diet compensates completely for 22:6n-3 in the tissues examined, as demonstrated by comparisons between the AR-DPAn-6 and AR-DHA groups. Surprisingly, we saw no significant differences in tissue levels of 20:4n-6 with the dietary manipulations. However, there were small increases in 20:4n-6 levels in several tissues in the AR-LA group, with levels in the heart approaching significance ($F_{4,31} = 2.27$, $P = 0.08$).

The addition of 22:5n-6 to control diets compared with 18:2n-6-only control diets may be particularly useful in assessing the physiological effects of 22:6n-3. In the present study, fatty acid compositional differences between AR-LA and AR-DPA were limited, but they may be physiologically relevant. Levels of 22:5n-6 were higher in erythrocyte, adipose, and heart tissues in the AR-DPAn-6 group compared with the AR-LA group, whereas 22:4n-6 levels were higher in erythrocyte and heart of the AR-LA group. More importantly, the proportion of 22:5n-6 plus 22:6n-3 was significantly less in all tissues except plasma ($P = 0.038$) and testes ($P = 0.10$) from the AR-LA rats compared with the AR-DPAn-6 rats. The sum of 22:5n-6 plus 22:6n-3 did not differ between the AR-DPAn-6, AR-DHA, and AR-DHA+DPAn-6 groups. Maintaining levels of 22:5n-6 plus 22:6n-3 is important to be able to attribute functional observations to 22:6n-3 specifically.

The present study clearly demonstrates a preferential incorporation of 22:6n-3 over 22:5n-6 into all tissues except rat testes. The tissue fatty acid compositions of AR-DHA+DPAn-6 rats were largely similar to that of the AR-DHA rats. In the AR-DHA+DPAn-6 group, 22:5n-6 levels were significantly lower than in the AR-DPAn-6 rat tissues (except testes), but 22:5n-6 levels in AR-DHA+DPAn-6 rats were slightly higher than in AR-DHA rats in plasma and adipose (0.76 vs. 0.49 wt% and 0.08 vs. 0.05 wt%, respectively).

Dietary 22:6n-3 has been associated with decreased 20:4n-6 in various tissues (19–26), and dietary 22:6n-3 is considered to compete directly with 20:4n-6. It has been suggested that feeding 22:5n-6 along with 22:6n-3 may maintain 20:4n-6 levels in tissues via 22:5n-6 retroconversion (19). The results of the present study do not support a direct competition between 22:6n-3 and 20:4n-6, and as such there was no evidence of 22:5n-6 retroconversion to 20:4n-6. Conclusions regarding elongation, desaturation,

and retroconversion based on static determinations of fatty acid composition are speculative and require confirmation with stable isotope methodologies (11). Ward et al. (27) have demonstrated a lack of an effect of DHA feeding on 20:4n-6 levels in brain tissue.

The present study used a novel, single generation n-3-deficient artificial rearing method with dietary fat feeding persisting through adulthood until the time of euthanasia at 15 weeks of age. The pelleted diet fed to the dams and eventually to the weaned pups of the DAM group was an n-3 fatty acid-adequate diet based on the AIN-93 formulation used previously in this laboratory (28). Although levels of 18:2n-6 were matched between DAM rats and AR rats in this study, the DAM diets contained higher levels of saturates, lower levels of monounsaturates, and higher amounts of 18:3n-3. The fatty acid composition of milk from dams consuming the DAM diet has been presented previously (28). Briefly, the DAM milk has been shown to contain 74% saturates, 14% monounsaturates (10.5% 18:1n-9), 7.6% n-6 polyunsaturates (6.4% 18:2n-6 and 0.03% 22:5n-6), and 1.4% n-3 polyunsaturates (0.95% 18:3n-3 and 0.12% DHA). The buffering capacity of the dam also results in metabolic intermediate fatty acids such as 22:5n-3 (0.14%) being provided to the pups in the milk (28). The increased 22:5n-3 in DAM rat tissues is likely a reflection of the intake of preformed 22:5n-3 in the dam's milk during suckling. Therefore, the DAM group received a relatively uncontrolled diet, as energy and essential fatty acid distribution were not matched to the AR rats. The AR method used here allows for precise control of energy and nutrient balance and provides the unique ability to introduce a single fatty acid variable during postnatal development.

The DAM group did demonstrate greater initial growth; however, the growth advantage did not persist. The growth rate observed in the DAM group may be artificially high as all litter sizes (DAM and AR) were initially culled to 10 pups, thus limiting competition between pups for maternal feeding. Although it is possible that tissue fatty acid differences between the AR and DAM groups may reflect different developmental rates, tissue fatty acid comparisons were made at 15 weeks of age, when developmental differences were greatly diminished, and the DAM fatty acid compositions tend to reflect the DAM group diets.

Methodologies in previous studies vary in several parameters, including the timing and length of intervention and the fatty acid composition of the intervention diets. The total amount of fat in the diet provided can vary across studies, but within studies the total amount across different diets is usually held constant. The specific fatty acid composition of the dietary fat in many studies reflects commercially available oils (e.g., soybean oil, corn oil, cod liver oil, etc.), and control of individual fatty acids is often compromised. Although such an approach is relevant for and may better reflect dietary food choices in the human population, this approach does not allow for a true determination of the metabolic relationship between individual fatty acids. Mohrhauer and Holman (29) demonstrated that 20:4n-6 incorporation into tissues is very much influenced by levels of 18:2n-6 and 18:3n-3, with 18:3n-3 having a particularly inhibitory effect on 20:4n-6 incorporation.

Previous 22:5n-6 rat feeding trials include a 4 week diet intervention starting at 5 weeks of age (19) and a 12 month intervention study starting at 3 months of age (30). Feeding 22:5n-6 with 22:6n-3 resulted in higher levels of 20:4n-6 in tissues, including brain, liver, testes, and erythrocytes, compared with diets containing 18:2n-6 with 22:6n-3. Diets were composed of commercial oils mixed with a commercial diet, and there were significant differences in individual fatty acids, with 18:3n-3 and 18:2n-6 fed at higher levels in control diets and considerable variation in feeding levels across the experimental diets. In the present study, 18:2n-6 levels were matched across all diets and 18:3n-3 levels were matched in the artificial rearing diets. We provided DHA and DPAn-6 at a ratio of 2.3:1 to reflect the commercial oil derived from *Schizochytrium* species available in North America, but we also provided a 22:5n-6 diet free of DHA, which has not been accomplished previously.

Differences in 20:4n-6 may become more apparent if the fatty acid composition of individual phospholipids is analyzed rather than the fatty acid composition of total lipid extracts. In this initial study, given the lack of 22:5n-6 feeding studies, we decided to examine the potential incorporation into all lipid classes, despite the fact that HUFAs such as 22:5n-6 and 22:6n-3 are preferentially incorporated into phospholipids rather than triacylglycerols. The lack of differences in the total HUFA levels in all tissues and the fact that the sums of 22:5n-6 and 22:6n-3 levels in the AR-DPAn-6 group and the AR-DHA group were similar within brain (predominantly phospholipid) and adipose (predominantly triacylglycerols) indicate that 22:5n-6 is incorporated into lipid classes, similar to the 22:6n-3 with 22:6n-3 incorporation preferred in most tissues except testes.

A high level of 22:5n-6 in rat testes has been observed previously (19, 31) and has been attributed to biosynthesis in the testes (32) associated with maturation of the spermatids (31). In primates and humans, 22:5n-6 levels are much lower, with 22:6n-3 being the dominant HUFA in mature males (33). It is believed these HUFAs provide the necessary tail membrane fluidity to support optimal sperm motility (33).

Although numerous tissues were examined in the present study, the characterization of 22:5n-6 incorporation into tissue remains incomplete. In particular, we were unable to complete fatty acid composition analyses of retina, as samples were used for G-protein-coupled signaling assessments (to be presented elsewhere). In addition to potential differences in individual lipid classes that the present study is unable to discern, there may be regional and subcellular differences in the incorporation of 22:5n-6 into individual tissues and organs.

Overall, the present study suggests that dietary 22:5n-6 in the presence of dietary 22:6n-3 does not compete with 22:6n-3. It has been demonstrated previously that the AR-DHA+DPAn-6 group performed similarly to the DAM and AR-DHA groups in spatial task performance testing, although this effect was significantly different in AR-DHA+DPAn-6 rats compared with AR-LA and AR-DPA

rats for memory retention but not for escape latency (13). Administration of a 22:5n-6 plus 22:6n-3 oil to growing swine resulted in no adverse effects (34), and humans consuming a 22:5n-6 plus 22:6n-3 oil supplement had no adverse effect on cardiovascular disease risk (35). In an Alzheimer disease rat model, 22:5n-6 plus 22:6n-3 may decrease the efficacy of DHA treatment in reducing amyloid-β and -τ accumulation, but 22:6n-3 plus 22:5n-6 may have a greater effect than DHA alone on reducing early-stage phosphor-τ epitope levels (30). The mixture of 22:5n-6 and 22:6n-3 had little impact on the fatty acid composition of the examined tissues compared with 22:6n-3 alone. Although feeding 22:6n-3 plus 22:5n-6 has not been associated with significant adverse health effects to date, the number of studies is limited and further research is required to assess the effect of *Schizochytrium* species-based oils on various physiological processes relevant to human health and disease.

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