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Vitamin E Deficiency Decreases Long-Chain PUFA in Zebrafish (*Danio rerio*)[1–3]

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Abstract

α-Tocopherol is a required, lipid-soluble antioxidant that protects PUFA. We hypothesized that α-tocopherol deficiency in zebrafish compromises PUFA status. Zebrafish were fed for 1 y either an α-tocopherol-sufficient (E^+; 500 mg α-tocopherol/kg) or -deficient (E^-; 1.1 mg α-tocopherol/kg) diet containing α-linolenic (ALA) and linoleic (LA) acids but without arachidonic acid (ARA), EPA, or DHA. Vitamin E deficiency in zebrafish decreased by ~20% (n-6) ($P < 0.05$) and (n-3) ($P < 0.05$) PUFA and increased the (n-6):(n-3) PUFA ratio ($P < 0.05$). In E^- compared to E^+ females, long chain-PUFA status was impaired, as assessed by a ~60% lower DHA:ALA ratio ($P < 0.05$) and a ~50% lower ARA:LA ratio ($P < 0.05$). *fads2* ($P < 0.05$) and e*lovl2* ($P < 0.05$) mRNA expression was doubled in E^- compared to E^+ fish. Thus, inadequate vitamin E status led to a depletion of PUFA that may be a result of either or both increased lipid peroxidation and an impaired ability to synthesize sufficient PUFA, especially (n-3) PUFA. J. Nutr. 141: 2113–2118, 2011.

Introduction

Zebrafish are an emerging model organism for lipid metabolism studies. Analysis of gene expression in zebrafish has revealed the presence of many, but not all, of the enzymes involved in LC-PUFA[9] synthesis in mammals. Specifically, Elovl2 and Elovl5 with specificity to C20–22 and C18–20 PUFA, respectively, are expressed by zebrafish (1,2). In contrast to mammals, zebrafish do not express FADS1 but express a dual function Δ5/Δ6 FADS2 desaturase (3). The characterization and presence of these enzymes suggest that zebrafish synthesize LC-PUFA through a similar pathway to the one used by humans (4,5).

PUFA, notably ARA [20:4(n-6)], EPA [20:5(n-3)], and DHA [22:6(n-3)], are implicated in a wide variety of cellular functions, including gene regulation, membrane fluidity, and function, and as precursors for several classes of signaling molecules (4). However, due to the high degree of unsaturation in the aliphatic tail, PUFA are particularly sensitive to lipid peroxidation. α-Tocopherol specifically scavenges peroxyl radicals, preventing further radical propagation and radical-mediated degradation of lipids (6). Thus, it is likely that inadequate α-tocopherol status will lead to marked lipid peroxidation and loss of PUFA.

We developed a vitamin E-deficient zebrafish model by feeding the fish a defined α-tocopherol–deficient diet and observed that both the α-tocopherol–deficient adults and embryos developed abnormalities, especially behavioral abnormalities (7). We hypothesized that the observed abnormalities might be a result of increased lipid peroxidation and decreased PUFA concentrations. Therefore, in the present study, we examined the impact of α-tocopherol (E) deficiency on PUFA status and gene expression of enzymes responsible for PUFA synthesis in zebrafish fed defined diets containing ALA and LA, but without ARA, EPA, and DHA.

Materials and Methods

Fish husbandry. Tropical 5D strain zebrafish (*Danio rerio*) were housed in the Sinnhuber Aquatic Research Laboratory at Oregon State University and studied in accordance with protocols approved by the Institutional Animal Care and Use Committee.

Adult zebrafish were kept at standard laboratory conditions of 28°C on a 14-h-light/10-h-dark photoperiod in fish water, consisting of reverse osmosis water supplemented with a commercial salt solution (0.6% Instant Ocean, Spectrum Brands). Zebrafish were fed either a defined experimental diet (described below) or a conventional zebrafish diet comprising of artemia (Inve Aquaculture) and a combination of com-

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[3] Supplemental Tables 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.

[9] Abbreviations used: acox, acyl-coA oxidase; ARA, arachidonic acid; ALA, α-linolenic acid; E^-, vitamin E-deficient experimental diet; E^+, vitamin E-sufficient experimental diet; FADS, fatty acid desaturase; Elovl, fatty acid elongase; L, conventional zebrafish diet; LA, linoleic acid; LC-PUFA, long-chain PUFA; sdc1, stearoyl CoA desaturase; SREBP, sterol regulatory element binding protein.

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mercial flake foods including, Aquatox Flake (77% by weight, Zeigler Brothers), Cyclopeez (6% by weight, Argent Laboratories), Golden Pearls (8.5% by weight, Artemia International), and Hikari Micropellets (8.5% by weight, Hikari), which was previously described (7) and hereafter referred to as L diet. Thus, the L diet is a mix of commercially available foods containing undefined ingredients with large amounts of fish oil and fishmeal.

The composition of the defined experimental diets was previously described (7), with the exception of the lecithin source. The lecithin (Lipoid PC 18:0/18:0, Lipoid) used for the present study is a structured phospholipid synthesized with 2 stearic acids as the only fatty acyls. Defined diets were prepared in 300-g batches with (E^+, 500 mg *RRR*-α-tocopherol/kg diet) or without (E^-) added vitamin E and stored at −20°C until fed to the zebrafish. Zebrafish were fed twice daily with an amount of food sufficient for the fish to consume in ~5 min. Zebrafish were fed the E^-, E^+, or L diet for 1 y prior to sampling for analytical measurements.

Measurement of tocopherols and α-tocopherol depletion kinetics. Fish were deprived of food for 12 h prior to sampling for whole body vitamin E, killed by an overdose of tricaine, and stored at −80°C until analyzed. Fish sampled for visceral α-tocopherol were deprived of food for 12 h, killed by an overdose of tricaine, viscera removed, and all eggs (if female) cleaned from viscera, and stored at −80°C until analyzed. Whole fish, viscera, as well as diet and α- and γ-tocopherol concentrations were determined by HPLC with electrochemical detection, as previously described (8).

α-Tocopherol depletion kinetics were calculated using GraphPad Prism (GraphPad Software) by fitting a linear regression line to the logarithmic-transformed α-tocopherol concentrations up to ~80 d postinitiation of diet. We previously determined that from 80 to 300 d, α-tocopherol concentrations remain at a minimal level without further detectable decreases (7). The data reported herein are from fish fed a diet that contained the synthetic lecithin, not the diet with soybean lecithin previously reported (7).

Lipid extraction and fatty acid analysis. Adult zebrafish were deprived of food for 36 h prior to sampling for fatty acid analysis. This time period was chosen because adult rainbow trout take up to 36 h to evacuate 95% of ingested food from their gut (9), whereas zebrafish fry, after 24 h of food deprivation, may still have food remaining in the gut (10). Zebrafish were killed by an overdose of tricaine. Whole viscera (includes the heart, liver, kidney, stomach, intestines, spleen, gall bladder, pancreas, and ovaries/testis) were collected, frozen in liquid nitrogen, and stored at −80°C until analysis. For female zebrafish, eggs were removed from the viscera prior to collection. Zebrafish embryos were obtained as a result of the natural spawning of adult zebrafish according to methods in (11). Embryos were collected immediately postspawning (0 h postfertilization), pooled into 50 embryos/sample, frozen in liquid nitrogen, and stored at −80°C until analyzed.

Total lipids were extracted as previously described (12). Fatty acids were converted to FAME and analyzed using GC with flame ionization detection (Agilent) as previously described (12). Peaks were integrated using Agilent OpenLab CDC software (Agilent). The pmol injected were calculated using external FAME standards (Nu-Chek Prep) and the total of all the fatty acids was summed; each of the fatty acids was then calculated as a mol percentage of the total. Visceral fatty acid distribution is reported for completeness rather than for statistical comparisons, as discussed in the "Statistics" section.

The experimental diets lacked ARA, EPA, and DHA; therefore, the visceral fatty acid composition was measured to confirm that the fish fed the experimental diets were able to elongate and desaturate the precursors, LA and ALA, to their respective C20–22 fatty acids. The percentage of (n-6) fatty acids was calculated by summing the mol percentages of the following fatty acids: 19:2(n-6), 20:2(n-6), 20:3(n-6), 20:4(n-6), 22:2(n-6), 22:4(n-6), 22:5(n-6), and 22:6(n-6). [18:2(n-6) and 18:3(n-6) were excluded, because these latter fatty acids are available in the diet.] For the fish fed the defined diets, this percentage equaled all (n-6) fatty acids synthesized from LA; for fish fed the L diet, this percentage equaled both fed and synthesized (n-6) fatty acids. The percentage of (n-3) fatty acids was calculated by summing the mol percentages of the following fatty acids: 20:5 (n-3), 20:3 (n-3), 22:3 (n-3), 22:5 (n-3), and 22:6 (n-3) (ALA was excluded, because this fatty acid is available in the diet). For the fish fed the defined diets, this percentage equaled all (n-3) fatty acids synthesized from ALA; for fish fed the L diet, this percentage equaled both fed and synthesized (n-3) fatty acids. The ratio of (n-6):(n-3) fatty acids was calculated as the percentage (n-6) divided by the percentage (n-3) fatty acids.

Quantitative real-time PCR. Fish were deprived of food for 36 h and killed by tricaine overdose and the viscera removed and homogenized immediately in TRIzol Reagent, followed by total RNA extraction per the manufacturer's instructions (Invitrogen). RNA concentrations and purity were determined by UV absorption (NanoDrop ND-1000 UV-Vis Spectrophotometer, Thermo Scientific). cDNA was synthesized following the manufacturer's directions using Superscript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). Specific primers were designed for each target gene (**Supplemental Table 1**) using Primer3 (13). PCR products were generated using PlatinumTaq DNA polymerase (Invitrogen) and sequenced to verify the correct sequence of the product (Center for Genome Research and Biocomputing core facility, Oregon State University). Plasmid clones were generated for each primer set according to the manufacturer's protocols (TOPO TA cloning kit, Invitrogen) and used to generate an absolute copy number standard curve. Samples were analyzed using AB Power SYBR Green on an Applied Biosystems 7900HT Fast Real-Time PCR (Applied Biosystems). Gene expression was normalized using *GAPDH* expression; data are reported as fold of the normalized values obtained for male fish fed the L diet (set at 1.0). There were no significant interactions between diet and gender for any of the genes examined; however, there were main effects of each diet and gender. The males and females were combined to show the between-diet differences. To show gender differences, female fish fed the 3 diets were combined and compared with males fed the 3 diets.

Statistics. Statistical analysis was performed using GraphPad Prism (GraphPad Software) and JMP (SAS Institute). When unequal variances were observed between groups, the data were logarithmically transformed and then statistics performed on the normalized data. All comparisons between diets and genders were evaluated using a 2-way ANOVA. If the 2-way interaction (diet × gender) was not significant, the significance of the main effect (either diet or gender) is reported; paired comparisons were used for post hoc analysis (Tukey's honestly significant differences). Comparisons between diets for embryo fatty acids were evaluated using an unpaired t test. Correlations of gene expression were calculated using multivariate correlation analysis (JMP). Differences were considered significant at $P < 0.05$. Values shown are mean ± SEM.

Results

Diet α-tocopherol concentrations and fatty acid distributions. The E^- diet contained 1.1 mg α-tocopherol/kg, which was substantially less than the E^+ diet (~400–500 mg/kg) and was similar to the diets we previously reported for α-tocopherol in our defined zebrafish diets (7). The defined diets (E^+ and E^-) for the present study lacked PUFA with carbon chains longer than LA or ALA (**Supplemental Table 2**).

Vitamin E depletion in adult zebrafish and visceral α-tocopherol concentrations. By d 21 of consuming the diets, whole body α-tocopherol concentrations were lower ($P < 0.05$) in the E^- fish compared with either the E^+ or L fish and remained significantly lower for every subsequent time point (**Fig. 1***A*). E^- fish α-tocopherol concentrations decreased at an exponential rate of 0.013 ± 0.001 nmol/d. Whole body α-tocopherol concentrations did not differ between male and female fish after feeding the experimental diet for 90 d; examples shown are from Figure 1*B*.

After 1 y, viscera from fish fed either the E^+ ($n = 3$) or L ($n = 3$) diet contained α-tocopherol concentrations that were 400–600

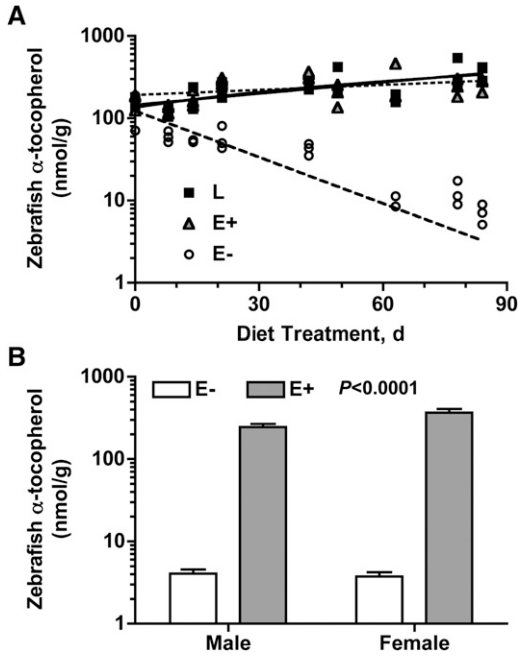

FIGURE 1 α-Tocopherol depletion kinetics (*A*) and total body concentrations at 1 y (*B*) in zebrafish fed a L, E^+, or E^- diet beginning at 6 wk of age. In E^- fish, α-tocopherol concentrations decreased at a rate of 0.013 ± 0.001 nmol/d and were lower than in E^+ or L fish by d 21 and remained lower for the remainder of the study ($P < 0.05$). Values in *B* are mean + SE, n = 12 (E^- female), n = 11 (E^+ female), n = 15 (E^- male), n = 13 (E^+ male) zebrafish fed the respective E^- and E^+ diets for 1 y. The P value refers to the effect of the diet. E^-, vitamin E-deficient experimental diet; E^+, vitamin E-sufficient experimental diet; L, conventional zebrafish diet.

times greater (1280 ± 479 and 857 ± 583 nmol/g, respectively) than viscera from the E^- fish (4 ± 1 nmol/g; $n = 3$; $P < 0.0001$).

Adult viscera fatty acid contents. Most fatty acids of interest were present in detectable amounts in the viscera, including oleic acid, LA, ALA, ARA, EPA, and DHA. Additional fatty acids detected included 16:0, 20:0, 20:2(n-6), 20:3(n-6), 22:4(n-6), 22:5(n-6), and 22:5(n-3). The following highly unsaturated fatty acids, 20:3(n-3), 22:3(n-3), and 22:6(n-6), were not present in detectable amounts in the viscera of fish fed the experimental diets.

Percentages (n-6) and (n-3) fatty acids. In zebrafish fed the L diet, the percentage of (n-6) fatty acids in female fish (3.3 ± 0.2%; $n = 5$) was similar to that in males (3.7 ± 0.3%; $n = 6$). In fish fed the E^+ or E^- diet, the percentage of (n-6) PUFA varied with the vitamin E content of the diet or with gender, but there was no significant interaction of diet vitamin E level with gender. E^- fish (7.7 ± 0.8%; $n = 11$) contained a lower percentage of (n-6) PUFA than did E^+ fish (9.5 ± 1.5%; $n = 8$; $P = 0.03$). Irrespective of diet, female fish (11.7 ± 0.9%; $n = 8$) contained nearly double the percentage of (n-6) fatty acids compared with the male fish (6.1 ± 0.4%; $n = 11$; $P < 0.0001$).

In zebrafish fed the L diet, the percentage of (n-3) PUFA in female fish (17.3 ± 1.0%; $n = 5$) was greater than in male fish (10.2 ± 0.4%; $n = 6$; $P = 0.003$). In fish fed the E^+ or E^- diet, the percentage (n-3) fatty acids varied with diet or gender, but there was no significant interaction. E^- fish (2.3 ± 0.3; $n = 11$) contained a lower percentage of (n-3) fatty acids than did the E^+ fish (3.4 ± 0.6%; $n = 8$; $P = 0.02$). Irrespective of diet, female fish (3.8 ± 0.5%; $n = 8$) contained nearly double the percentage of (n-3) fatty acids compared with the male fish (2.0 ± 0.2; $n = 11$; $P = 0.001$).

In eggs collected immediately upon zebrafish spawning, the percentage (n-6) or (n-3) fatty acids did not vary with dietary vitamin E. The percentage of (n-6) fatty acids in zebrafish eggs collected from adult fish fed the E^+ (16.6 ± 2.3%; $n = 6$) or E− (15.9 ± 2.3%; $n = 6$) diet was ~4 times greater than the percentage of (n-3) fatty acids (E^+ were 4.4 ± 0.8%, $n = 6$ and E^- were 4.7 ± 0.8%, $n = 6$).

The ratio of (n-6):(n-3) fatty acids was 20% higher in the E^- fish (3.5 ± 0.2) compared with E^+ fish (2.9 ± 0.1; $P = 0.05$).

ARA:LA and DHA:ALA ratios. To assess the ability of zebrafish fed the defined diets to generate ARA from LA, the ARA:LA ratios were calculated. Both gender and diet affected this ratio (*P*-interaction = 0.01) (**Fig. 2***A*). In E^+ female fish, the ARA:LA ratios were double those of E^- females, whereas males had lower ARA:LA ratios than any of the females. The ARA:LA ratio was not different between E^+ and E^- males.

To assess the ability of zebrafish to generate DHA from ALA, the DHA:ALA ratio was calculated. Both gender and diet affected this ratio (*P*-interaction = 0.007) (Fig. 2*B*). In E^+ female fish, the DHA:ALA ratios were triple those of E^- females, whereas males had lower DHA:ALA ratios. The DHA:ALA ratio was not different between E^+ and E^- males.

Viscera mRNA abundance of genes encoding enzymes for fatty acid synthesis. Given the differences observed in fatty acid percentages and ratios between the E^- and E^+ fish, the transcription of several genes encoding enzymes necessary for fatty acid synthesis was assessed to determine if vitamin E modulation of fatty acid status occurs at the transcriptional level (**Tables 1** and **2**).

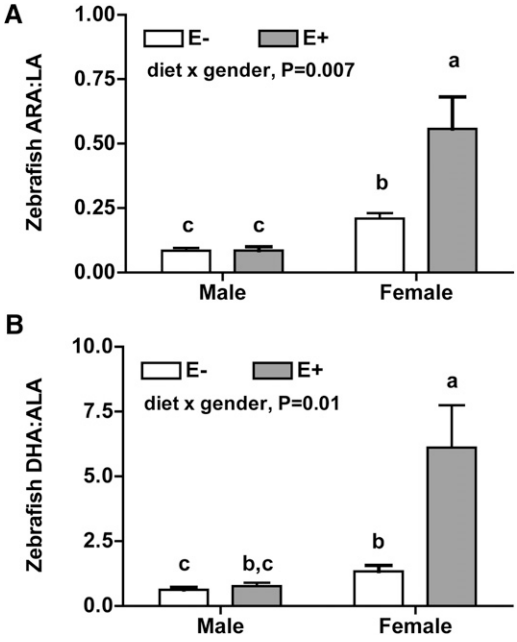

FIGURE 2 The ratios of ARA:LA (*A*) and of DHA:ALA (*B*) in E^- female ($n = 5$), E^+ female ($n = 3$), E^- male ($n = 6$), and E^+ male ($n = 5$) zebrafish fed the respective E^- and E^+ diets for 1 y. Means without a common letter differ, $P < 0.05$. ARA, arachidonic acid; ALA, α-linolenic acid; E^-, vitamin E-deficient experimental diet; E^+, vitamin E-sufficient experimental diet; LA, linoleic acid.

There was a doubling of *fads2* mRNA expression in viscera from E^- fish compared with E^+ or L diet fish ($P < 0.05$), with no significant differences between those fed the E^+ and L diets. Female fish from all diets had a nearly 4-fold higher *fads2* expression than did male fish ($P < 0.0001$).

There was a doubling of *elovl2* mRNA expression in E^- fish compared with E^+ or L diet fish ($P < 0.05$), with no significant differences between E^+ and L. Expression of *elovl2* was not significantly different in females compared with males and *elovl5* mRNA expression was not different between E^+ and E^- fish; however, male fish had double the expression compared with females ($P < 0.0001$).

Two splice variants of *acox1* are present in zebrafish, *acox1–3I* and *acox1–3II*, which are equally expressed in the intestine and liver of zebrafish (14). Expression of *acox1* for both splice variants doubled in fish fed the E^- compared with the L diet ($P < 0.05$); however, the E^+ and E^- fish did not differ. Gender did not affect *acox1* expression for either splice variant.

SREBP2 regulates the genes involved in cholesterol metabolism (15). Female compared with male fish had higher *SREBP2* expression ($P = 0.04$); however, *SREBP2* expression was unaffected by diet. *SREBP1*, which regulates de novo lipogenesis (15), was unaffected by either diet or gender.

Although SFA and MUFA concentrations are not discussed in this paper, the enzymes responsible for their synthesis and elongation deserve mention, because little information is available on their expression in zebrafish. It is important to note that these enzymes have not yet been functionally characterized in zebrafish. Expression of *elovl1a* in females was double that of males, *elovl6I* expression was nearly 100 times greater, and *elovl7b* expression was 60 times greater; all were unaffected by diet. Finally, *scd1* and *acox3* expression were unaffected by either gender or diet.

Correlations between all the genes with the corresponding correlation coefficients and *P* values are summarized in **Table 3**. Notably, the genes for enzymes critical for DHA synthesis were all strongly and positively correlated with one another (*fads2*, *elovl2*, *acox1–3I*, and *acox1-3II*).

Discussion

The aim of our study was to determine the impact of vitamin E deficiency on fatty acid status in zebrafish. Vitamin E deficiency caused marked decreases in the percentage of (n-6) and (n-3) fatty acids regardless of gender as well as reductions in the DHA:ALA and ARA:LA ratios in E^- females (Fig. 2), suggesting that inadequate tissue α-tocopherol altered the ability of the zebrafish to maintain PUFA status. Various mechanisms to explain the low PUFA in the E^- fish are possible, including decreased synthesis, inadequate availability of essential fatty acids as substrates, or increased lipid peroxidation. We discuss these possibilities below.

We observed that the E^- compared with E^+ zebrafish had a higher expression of the genes responsible for PUFA synthesis (Table 1). Although the genes are not necessarily indicative of protein concentrations or enzyme activities, the direction of the change is opposite to that which would cause decreased PUFA status. In support of our observations, vitamin E deficiency in salmon resulted in increased recovery of the elongation and desaturation products of ALA and EPA from isolated salmon hepatocytes (16), suggesting that vitamin E deficiency in salmon promoted an increase in PUFA synthesis. However, Tu et al. (17) reported that PUFA synthesis is regulated by substrate availability for the elongase and desaturase enzymes. Thus, the amount of essential fatty acids provided in the zebrafish experimental diets may have been insufficient for the fish fed the E^- diet to synthesize sufficient amounts of LC-PUFA. It should be noted, however, that diet ALA and LA concentrations were measured and did not differ in the E^- and the E^+ diets (Supplemental data). Moreover, the percentage fatty acid distribution (Supplemental data) shows similar distributions for the male fish fed the defined diets, whereas the relative percentages of LA and ALA were both higher in the E^- compared with E^+ female fish. Thus, inadequate substrate does not seem a likely explanation for the lower ARA:LA and DHA:ALA ratios documented in the E^- female fish given that the E^- females had higher relative concentrations of the necessary substrates. Although we show that gene expression is upregulated by α-tocopherol deficiency in zebrafish, it is unclear from our studies if elongase and desaturase enzyme expression or activities are upregulated or if the E^- fish are unable to sufficiently upregulate PUFA synthesis to meet needs.

Our data suggest that PUFA were depleted in the E^- fish faster than they could be replaced; i.e. compensatory mechanisms in the E^- zebrafish were insufficient to normalize PUFA concentrations. Although we did not study antioxidant mechanisms or measures of lipid peroxidation, salmon or trout fed vitamin E^-deficient diets had increased antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, as well as increased RBC lipid hydroperoxides (18) and development of severe lipoid liver degeneration (16). These latter findings support the hypothesis that the E^- zebrafish have increased lipid peroxidation. Of note, an interaction between diet and gender was not observed for the percentage (n-6) and (n-3) fatty acids; thus, regardless of gender, vitamin E deficiency resulted in PUFA depletion.

High levels of (n-6) fatty acids relative to low levels of (n-3) fatty acids [i.e., high (n-6):(n-3) fatty acid ratios] have been positively associated with markers of inflammation (19,20). Vitamin E deficiency in zebrafish also led to an increase in the (n-6):(n-3) fatty acid ratio, suggesting that the (n-6) LC-PUFA were

TABLE 1 Survey of the mRNA abundance of genes encoding enzymes for fatty acid synthesis or cholesterol synthesis in viscera from zebrafish fed E^-, E^+, or L diets for 1 y[1]

Diet	*n*	*fads2*	*elovl2*	*elovl5*	*acox1–3I*	*acox1–3II*	*acox3*	*scd1*	*elovl1a*	*elovl6I*	*elovl7b*	*SREBP1*	*SREBP2*
							fold of L males						
E^-	10	12 ± 2.2^a	4.8 ± 1.1^a	0.4 ± 0.1	2.6 ± 0.4^a	2.2 ± 0.3^a	1.1 ± 0.2	6.3 ± 2.5	3.6 ± 1.1	65 ± 37	50 ± 25	2.6 ± 0.5	1.4 ± 0.2
E^+	11	5.5 ± 1.1^b	2.2 ± 0.3^b	0.5 ± 0.1	2.0 ± 0.3^{ab}	1.7 ± 0.2^{ab}	1.1 ± 0.1	7.3 ± 2.6	2.2 ± 0.5	39 ± 16	26 ± 15	1.9 ± 0.2	1.5 ± 0.2
L	12	4.5 ± 1.1^b	1.6 ± 0.3^b	0.6 ± 0.1	1.2 ± 0.2^b	1.2 ± 0.2^b	1.1 ± 0.1	2.5 ± 1.2	1.9 ± 0.6	36 ± 22	29 ± 20	1.6 ± 0.1	1.3 ± 0.2
ANOVA *P*		0.0004	0.006	0.2	0.004	0.03	0.8	0.3	0.3	0.9	0.9	0.7	0.1

[1] Values are mean ± SEM expressed relative to *GAPDH*, with the mean of L males set to 1. Means in a column with superscripts without a common letter differ, $P < 0.05$. See Table 2 for gender differences. E^-, vitamin E-deficient experimental diet; E^+, vitamin E-sufficient experimental diet; L, conventional zebrafish diet.

TABLE 2 Survey of the effect of gender on mRNA abundance of genes encoding enzymes for fatty acid synthesis or cholesterol synthesis in viscera from zebrafish fed E^-, E^+, or L diets for 1 y[1]

Gender	*n*	*fads2*	*elovl2*	*elovl5*	*acox1–3I*	*acox1–3II*	*acox3*	*scd1*	*elovl1a*	*elovl6l*	*elovl7b*	*SREBP1*	*SREBP2*
							fold of L males						
Female	17	11 ± 1.2	3.6 ± 0.7	0.3 ± 0.0	1.8 ± 0.2	1.8 ± 0.2	1.1 ± 0.1	4.9 ± 0.9	3.5 ± 0.7	88 ± 24	66 ± 19	2.3 ± 0.3	1.5 ± 0.1
Male	16	3.0 ± 3.3	2.0 ± 0.5	0.7 ± 0.3	1.9 ± 0.3	1.6 ± 0.3	1.1 ± 0.1	5.6 ± 2.4	1.4 ± 0.3	0.9 ± 0.2	1.0 ± 0.2	1.7 ± 0.3	1.3 ± 0.2
ANOVA *P*		<0.0001	0.08	<0.0001	0.7	0.7	0.98	0.7	0.02	0.003	0.006	0.2	0.04

[1] Values are mean ± SEM expressed relative to *GAPDH*, with the mean of L males set to 1. See Table 1 for diet differences. E^-, vitamin E-deficient experimental diet; E^+, vitamin E-sufficient experimental diet; L, conventional zebrafish diet.

spared relative to the (n-3) fatty acids. This finding is consistent with the higher relative susceptibility of (n-3) fatty acids to lipid peroxidation (21). Additionally, because DHA inhibits AA synthesis [by suppressing SREBP1 nuclear abundance (22)], the postulated DHA losses associated with α-tocopherol deficiency could have potentiated an increase in (n-6) fatty acid synthesis in the E^- fish. Regardless of the mechanism by which the vitamin E deficiency led to an increase in the (n-6):(n-3) fatty acid ratio, the E^- fish were also susceptible to a greater inflammatory stress due to a potential increase in (n-6) fatty acid-derived eicosanoids, which are generally considered inflammatory, and a reduction in (n-3) fatty acid-derived eicosanoids, which are generally considered antiinflammatory (4,5).

Our study also demonstrates that fatty acid composition varies in a gender-specific manner in adult zebrafish. The females compared with males contained a greater percentage of both (n-6) and (n-3) fatty acids as well as increased expression of *fads2* (Table 1). These results are consistent with studies in rodents and humans that have previously shown a greater PUFA synthetic capacity in females and increased expression of the necessary desaturase enzyme (23,24), the rate-limiting step in LC-PUFA synthesis. Interestingly, α-tocopherol deficiency resulted in reduced DHA:ALA and ARA:LA ratios only in the female zebrafish. Adult female fish producing eggs show specific changes in hepatic metabolism, especially in association with vitellogenesis (25). During vitellogenesis, vitamin E and lipids are mobilized from the liver and muscle to the ovary to supply the developing oocyte (26). As the ovarian follicles mature, the expression of elongase and desaturase enzymes as well as the fatty acid profile change (27). Surprisingly, the eggs from the E^+ and E^- female zebrafish contained similar percentages of (n-3) and (n-6) fatty acids and were similar to those in the E^+ female zebrafish. Thus, the E^- female zebrafish must either decrease egg production or increase synthesis to maintain the fatty acid composition of the eggs. We did not observe a decrease in the egg production, suggesting that the observation that α-tocopherol deficiency resulted in lower DHA:ALA and ARA:LA ratios only in female, not male, zebrafish could be due to an increased synthetic requirement to meet the PUFA requirements of both the adult female zebrafish and the developing oocytes. It is thus possible that the higher expression of specific genes in females is associated with the need for more lipids and PUFA that are mobilized to the developing oocytes. Although we did not measure the total lipid content, in general, females have a higher total body fat that also must be maintained and may add to the increased gene expression. Additional studies are needed to clarify the regulation of PUFA synthesis during vitellogenesis.

We also present herein a defined zebrafish diet containing the essential fatty acids, ALA and LA, but not longer PUFA, compared with a typical feeding regime of commercial zebrafish food. The defined diets contained a greater percentage total (n-6) fatty acids and a reduction in the percentage of total (n-3) fatty acids relative to the L diet. The defined diets are similar to an average Western diet, with high levels of saturated fat and a high ratio of dietary (n-6):(n-3) fatty acids (~8:1 LA:ALA). A ratio of 0.6 mg *RRR*-α-tocopherol/g LA is recommended for humans, with increasing α-tocopherol needed as the degree of unsaturation of the fatty acid increases (28). The α-tocopherol–sufficient diet contains 500 mg *RRR*-α-tocopherol/kg diet, equivalent to 11.1 mg α-tocopherol/g LA, far surpassing this recommendation; therefore, the E^+ diet contains sufficient α-tocopherol to prevent alterations in PUFA metabolism due to lipid peroxidation.

TABLE 3 Correlations of individual mRNA abundance of genes from zebrafish fed E^-, E^+, or L diets for 1 y[1]

Gene	*fads2*	*elovl2*	*elovl5*	*acox1–3I*	*acox1–3II*	*acox3*	*scd1*	*elovl1a*	*elovl6l*	*elovl7b*	*SREBP1*	*SREBP2*
						r						
fads2		0.76*	−0.59**	0.36#	0.40#							0.40#
elovl2	0.76*			0.55**	0.59**	0.39#						0.59**
elovl5	−0.59**							−0.38#	−0.45#	-0.41#		
acox1–3I	0.36#	0.55**			0.95*	0.62*		0.38#			0.52**	0.64
acox1–3II	0.40#	0.59**		0.95*		0.69*		0.36#			0.54**	0.73*
acox3		0.39#		0.62*	0.69*						0.54**	0.66*
scd1											0.52**	0.38#
elovl1a			−0.38#	0.38#	0.36#				0.91*	0.88*		
elovl6L			−0.45#					0.91*		0.86*		
elovl7b			−0.41#					0.88*	0.86*			
SREBP1				0.52**	0.54**	0.54**	0.52**					0.74*
SREBP2	0.40#	0.59**		0.64*	0.73*	0.66*	0.38#				0.74*	

[1] *P* values shown are $*P \leq 0.0001$; $**P \leq 0.005$; $\#P \leq 0.05$. Blank cells indicate that the correlation was not significant, $P > 0.05$. E^-, vitamin E-deficient experimental diet; E^+, vitamin E-sufficient experimental diet; L, conventional zebrafish diet.

Based on α-tocopherol's role as a lipid-soluble antioxidant (6), the depletion of PUFA in E^- fish is most likely due to lipid peroxidation. We hypothesize that, to compensate for PUFA depletion, zebrafish have induced PUFA synthesis based on the increased expression of *elovl2* and *fads2*. However, it is also possible that α-tocopherol deficiency stimulates PUFA β-oxidation or causes diminished PUFA synthesis in females. Future studies are required to resolve the mechanisms accounting for the changes observed in visceral PUFA. In summary, α-tocopherol deficiency alters PUFA metabolism in zebrafish, ultimately resulting in decreased concentrations of both long chain (n-3) and (n-6) fatty acids.

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Literature Cited

1. Monroig O, Rotllant J, Sanchez E, Cerda-Reverter JM, Tocher DR. Expression of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis genes during zebrafish Danio rerio early embryogenesis. Biochim Biophys Acta. 2009;1791:1093–101.
2. Agaba M, Tocher DR, Dickson CA, Dick JR, Teale AJ. Zebrafish cDNA encoding multifunctional Fatty Acid elongase involved in production of eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids. Mar Biotechnol (NY). 2004;6:251–61.
3. Hastings N, Agaba M, Tocher DR, Leaver MJ, Dick JR, Sargent JR, Teale AJ. A vertebrate fatty acid desaturase with Delta 5 and Delta 6 activities. Proc Natl Acad Sci USA. 2001;98:14304–9.
4. Jump DB. The biochemistry of n-3 polyunsaturated fatty acids. J Biol Chem. 2002;277:8755–8.
5. Schmitz G, Ecker J. The opposing effects of n-3 and n-6 fatty acids. Prog Lipid Res. 2008;47:147–55.
6. Traber MG, Atkinson J. Vitamin E, antioxidant and nothing more. Free Radic Biol Med. 2007;43:4–15.
7. Miller GW, Labut EM, Lebold KM, Floeter A, Tanguay RL, Traber MG. Zebrafish (Danio rerio) fed vitamin E deficient diets produce embryos with increased morphologic abnormalities and mortality. J Nutr Biochem. Epub 2011 Jun 16.
8. Podda M, Weber C, Traber MG, Packer L. Simultaneous determination of tissue tocopherols, tocotrienols, ubiquinols and ubiquinones. J Lipid Res. 1996;37:893–901.
9. Nichols JW, Fitzsimmons PN, Whiteman FW, Kuehl DW, Butterworth BC, Jenson CT. Dietary uptake kinetics of 2,2′,5,5′-tetrachlorobiphenyl in rainbow trout. Drug Metab Dispos. 2001;29:1013–22.
10. Field HA, Kelley KA, Martell L, Goldstein AM, Serluca FC. Analysis of gastrointestinal physiology using a novel intestinal transit assay in zebrafish. Neurogastroenterol Motil. 2009;21:304–12.
11. Westerfield M. The zebrafish book: a guide for the laboratory use of zebrafish (Danio rerio). 5th ed. Eugene (OR): University of Oregon Press; 2007.
12. Tripathy S, Torres-Gonzalez M, Jump DB. Elevated hepatic fatty acid elongase-5 activity corrects dietary fat-induced hyperglycemia in obese C57BL/6J mice. J Lipid Res. 2010;51:2642–54.
13. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol. 2000;132:365–86.
14. Morais S, Knoll-Gellida A, Andre M, Barthe C, Babin PJ. Conserved expression of alternative splicing variants of peroxisomal acyl-CoA oxidase 1 in vertebrates and developmental and nutritional regulation in fish. Physiol Genomics. 2007;28:239–52.
15. Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell. 1997;89:331–40.
16. Bell JG, McEvoy J, Tocher DR, Sargent JR. Depletion of alpha-tocopherol and astaxanthin in Atlantic salmon (Salmo salar) affects autoxidative defense and fatty acid metabolism. J Nutr. 2000;130:1800–8.
17. Tu WC, Cook-Johnson RJ, James MJ, Muhlhausler BS, Gibson RA. Omega-3 long chain fatty acid synthesis is regulated more by substrate levels than gene expression. Prostaglandins Leukot Essent Fatty Acids. 2010;83:61–8.
18. Puangkaew J, Kiron V, Satoh S, Watanabe T. Antioxidant defense of rainbow trout (Oncorhynchus mykiss) in relation to dietary n-3 highly unsaturated fatty acids and vitamin E contents. Comp Biochem Physiol C Toxicol Pharmacol. 2005;140:187–96.
19. Kalogeropoulos N, Panagiotakos DB, Pitsavos C, Chrysohoou C, Rousinou G, Toutouza M, Stefanadis C. Unsaturated fatty acids are inversely associated and n-6/n-3 ratios are positively related to inflammation and coagulation markers in plasma of apparently healthy adults. Clin Chim Acta. 2010;411:584–91.
20. Weaver KL, Ivester P, Seeds M, Case LD, Arm JP, Chilton FH. Effect of dietary fatty acids on inflammatory gene expression in healthy humans. J Biol Chem. 2009;284:15400–7.
21. Halliwell B, Gutteridge J. Free radicals in biology and medicine. 3rd ed. New York: Oxford University Press; 1999.
22. Jump DB, Botolin D, Wang Y, Xu J, Demeure O, Christian B. Docosahexaenoic acid (DHA) and hepatic gene transcription. Chem Phys Lipids. 2008;153:3–13.
23. Extier A, Langelier B, Perruchot MH, Guesnet P, Van Veldhoven PP, Lavialle M, Alessandri JM. Gender affects liver desaturase expression in a rat model of n-3 fatty acid repletion. J Nutr Biochem. 2010;21:180–7.
24. Giltay EJ, Gooren LJ, Toorians AW, Katan MB, Zock PL. Docosahexaenoic acid concentrations are higher in women than in men because of estrogenic effects. Am J Clin Nutr. 2004;80:1167–74.
25. Arukwe A, Goksoyr A. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. Comp Hepatol. 2003;2:4.
26. Palace V, Werner J. Vitamins A and E in the maternal diet influence egg quality and early life stage development in fish: a review. Sci Mar. 2006;70:41–57.
27. Ishak SD, Tan SH, Khong HK, Jaya-Ram A, Enyu YL, Kuah MK, Shu-Chien AC. Upregulated mRNA expression of desaturase and elongase, two enzymes involved in highly unsaturated fatty acids biosynthesis pathways during follicle maturation in zebrafish. Reprod Biol Endocrinol. 2008;6:56.
28. Valk EE, Hornstra G. Relationship between vitamin E requirement and polyunsaturated fatty acid intake in man: a review. Int J Vitam Nutr Res. 2000;70:31–42.