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Zebrafish (*Danio rerio*) fed vitamin E deficient diets produce embryos with increased morphologic abnormalities and mortality

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Abstract

Vitamin E (α-tocopherol) is required to prevent fetal resorption in rodents. To study α–tocopherol's role in fetal development, a non-placental model is required. Therefore, the zebrafish, an established developmental model organism, was studied by feeding the fish a defined diet with or without added α–tocopherol. Zebrafish (age: 4–6 w) were fed the deficient (E-), sufficient (E+), or lab diet up to 1 y. All groups showed similar growth rates. The exponential rate of α–tocopherol depletion up to ~80 day in E- zebrafish was 0.029 ± 0.006 nmol/g, equivalent to a depletion half-life of 25 ± 5 days. From age ~80 d, the E- fish (5 ± 3 nmol/g) contained ~50 times less α–tocopherol than the E+ or lab diet fish (369 ± 131 or 362 ± 107, respectively, $P<0.05$). E-depleted adults demonstrated decreased startle response suggesting neurologic deficits. Expression of selected oxidative stress and apoptosis genes from livers isolated from the zebrafish fed the three diets were evaluated by quantitative polymerase chain reaction and were not found to vary with vitamin E status. When E-depleted adults were spawned, they produced viable embryos with depleted α–tocopherol concentrations. The E- embryos exhibited a higher mortality ($P<0.05$) at 24 h post fertilization (hpf) and a higher combination of malformations and mortality ($P<0.05$) at 120 hpf than embryos from parents fed E+ or lab diets. This study documents for the first time that vitamin E is essential for normal zebrafish embryonic development.

Keywords

alpha tocopherol; gamma tocopherol; embryonic development; liver; ascorbic acid; morphology

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Introduction

Vitamin E (α-tocopherol) was discovered in 1922 by Evans and Bishop, who demonstrated that rats fed diets containing rancid fat were unable to carry offspring to term [1]. Both low fetal and maternal α-tocopherol concentrations may be important factors during fetal resorption. Retention of uterine α-tocopherol by the mother appears essential to maintain pregnancy because the α-tocopherol transfer protein (TTP) is increased at the site of implantation [2,3] and is also expressed in the placenta [3–6]. Both TTP and biomarkers of lipid peroxidation (malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE)) have been detected in the first trimester placenta [6]. Moreover, early failure of pregnancy is associated with lipid peroxidation with resultant damage to the placental syncytiotrophoblast [7]. Thus, it is likely that α-tocopherol is needed by the mother to protect her from the oxidative stress of the rapidly growing fetus.

TTP is a liver protein in adult rodents and humans. However, Jauniaux et al. [8] showed that not only does the human placenta and uterus express TTP, the human yolk sac expresses TTP. They further suggest that during very early human fetal development, the human embryo obtains α-tocopherol from the yolk sac. Our hypotheses are dependent upon the observation that TTP is expressed during the first 48 h of zebrafish embryonic development [9].

The zebrafish is an established vertebrate model organism frequently used in developmental studies [10–13]. Embryos undergo maturation from a single cell to an autonomous juvenile fish in 120 hours (5 days) [14]. During development, embryos are optically clear, allowing for noninvasive observation of the entire embryo. TTP mRNA is expressed in embryonic zebrafish as early as 24 hours post fertilization (hpf) [9]. Because the zebrafish reproduces by spawning and does not have a placenta, the vitamin E requirements of the embryo can be separated from those of the mother. However, available commercial diets contain many ingredients making the study of a specific nutrient difficult [15]. Many “fish foods” also contain ambiguous ingredients, such as “fish oil” and/or “fish meal”, the sources of which are not immediately apparent. The feeding of live food sources (artemia, paramecium, etc) to zebrafish present similar difficulties [16]. This problem has been addressed using zebrafish and other teleost fish in the past with varying levels of success by partially defining laboratory diets [17–20].

To address these challenges, we hypothesized that development of a defined diet, which is sufficient to sustain zebrafish health, growth, and reproduction, will allow us to study the action of vitamin E during embryogenesis. We further hypothesize based on studies in rodent models of vitamin E deficiency that 1) we can deplete adult zebrafish of α-tocopherol, 2) these adults will remain reproductively active, 3) the embryos that are produced will also be deficient in α-tocopherol, and 4) α-tocopherol deficient embryos will show phenotypic abnormalities as a result of their deficiency.

Methods

Fish Husbandry

Tropical 5D strain zebrafish (Danio rerio) were housed in the Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University. The zebrafish were 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 (FW) consisting of reverse osmosis water supplemented with a commercially available salt solution (0.6% Instant Ocean®, Spectrum Brands, Inc.,

Atlanta, GA). Zebrafish were fed experimental diets (described below) or a conventional lab diet comprising of artemia (Inve Aquaculture Inc. Ogden UT) and a combination of commercial flake foods including: Aquatox Flake (77% by weight, Zeigler Brothers Inc., Gardners, PA), Cyclopeez (6% by weight, Argent Laboratories, Redmond, WA), Golden Pearls (8.5% by weight, Artemia International LLC, Fairview, TX), and Hikari Micropellets (8.5% by weight, Hikari, Hayward, CA); hereafter called "lab" diet. Zebrafish were fed twice daily (AM and PM) with an amount of food sufficient for the fish to consume in ~5 minutes. Embryos used for our studies were not fed, but instead received all their nutrition from their yolk sacs. At 120 hpf, embryos were euthanized by an overdose of tricaine. Note that zebrafish younger than 1 month cannot consume standard food because their mouths are too small and instead consume paramecium, artemia and specially created diets (EZ Larval Diet, Zeigler Brothers Inc., Gardners, PA). Thus, we are currently unable to generate adult zebrafish that were spawned from deficient parents and have only consumed the vitamin E deficient diet.

Diet Preparation

Ingredients for the defined diets (Table 1) were obtained from Dyets Inc. (Bethlehem, PA) with exceptions, as indicated: wheat gluten (#402100), casein (#400627), egg whites (#401600), cellulose (#401850), vitamin mix (#310064), mineral mix (#210087), tocopherol-stripped soybean oil (#404365), Stay C (Vitamin C-3, Argent Chemical Laboratories Inc., Redmond, WA), modified food starch (National Starch Food Innovation, Bridgewater, NJ), lecithin (Ultralec without added tocopherol), and Vitamin E (*RRR*-α-tocopherol). Both the lecithin and the vitamin E were generous gifts from Archer Daniels Midland (Decatur, IL).

Diets were prepared in 100 g batches without added vitamin E (E-) or with (E+, ~500 mg *RRR*-α-tocopherol/kg diet). Each diet was prepared by mixing ingredients until homogenous, spreading the batter on a large baking sheet and then oven-drying at 212°F for one hour. The diet was then cooled, crushed into a powder, and stored frozen at −20°C until fed to the zebrafish. Diets were used within four months of preparation.

α- and γ-Tocopherol Measurements

Tocopherol concentrations of the diets, fish, and embryos were determined by high-pressure liquid chromatography using electro-chemical detection (HPLC-ECD, Shimadzu, Columbia, MD and LC-4C, Bioanalytical Systems, Inc. West Lafayette, IN, respectively), as described previously [21]. In brief, the samples were weighed (diet samples ~50 mg, whole fish 5–900 mg, and 5–30 embryos (embryo estimated wet-weight was 1 mg), saponified in alcoholic KOH with 1% ascorbic acid at 70°C for 30 minutes (young fish, embryos and diets) or up to 1 hour (adult fish). The samples were cooled, extracted with hexane, dried under nitrogen gas, resuspended in 50:50 ethanol:methanol, and an appropriate aliquot injected into the HPLC-ECD. Tocopherols were quantified by comparison to calibration curves generated from authentic α- and γ-tocopherol standards.

Ascorbic Acid Measurements

Sample collection methods were based on Moreau et al [22] and Matamoros et al [23]. Briefly, fish were euthanized by an overdose of tricaine (MS 222, ethyl 3-aminobenzoate methanesulfonate salt, Sigma-Aldrich, St Louis, MO), weighed, and the entire fish homogenized with buffer (5% trichloroacetic acid [TCA, Sigma-Aldrich, St Louis, MO], 0.08% diethylenetriaminepenta-acetic acid [DTPA, Acros Organics, Morris Plains, NJ], 250 mM perchloric acid [PCA, Fisher Scientific, Fair Lawn, NJ], and 0.4 mM dithioerythritol [DTE, Sigma-Aldrich, St Louis, MO]). Samples were then centrifuged, the supernatants

frozen and stored at −80°C until analysis. Samples were analyzed by HPLC-ECD, as described previously [24].

Zebrafish α-Tocopherol Depletion Kinetics

At 4–6 weeks of age, zebrafish were randomly separated into three dietary groups: E-, E+, or lab diets. Zebrafish (n = 3/dietary group) were euthanized by overdose of tricaine at each of the indicated time points. Zebrafish were weighed and kept frozen until analysis for vitamin concentrations, as described above. This experiment was repeated using three separate generations of fish. α-Tocopherol depletion kinetics were calculated using Microsoft Excel (Microsoft Corporation, Santa Rosa, CA) by fitting a linear regression analysis to the logarithmic-transformed α-tocopherol concentrations measured during the first ~80 days after initiation of the E- diet.

Zebrafish Sensory Testing

To evaluate sensory responses, computer-assisted video monitoring of swimming behavior was assess using modification of the method of Eddins et al [25]. Adult zebrafish (n=6) from each diet (E+, E- and Lab; 221 days on diet) were placed in individual 1.75 L tanks containing ~1.5 L FW. Tanks were set in-line on shelves with the broadside facing the camera, separated by dividers to isolate individual fish. Tanks were backed with blank white paper, evenly backlit. Room temperature was controlled at 28° C. Fish were fasted for the duration of the behavior trials and were given 24 h to acclimatize prior to beginning the trials.

Trials were recorded using a Sony HD camcorder (Sony Handycam HDR-SR11) coupled with the Noldus Etho-Vision XT V 7.0 analysis software (Leesburg, VA). The swimming velocity was recorded for 16 minute sessions (separated into 2 minute intervals), and the first 6 minutes were averaged to obtain the average velocity for the trial, the next 10 minutes were not used for assessment (rest time). Two trials with no stimulus were used to generate “background” swimming information and results from each fish were averaged to generate its own baseline information. Two trials were done using a “single tap” generated by an electro-magnetic solenoid to tap the tank at two minutes; results from each fish were averaged to generate its own single tap information. A final “multiple tap” trial was generated using the solenoids to strike the tanks once every 5 seconds starting at 2 minutes for 90 seconds (18 taps total). Rest time was given between each stimulus trial to allow a return to baseline behavior.

Zebrafish Embryo Handling and Scoring

Fertilized eggs were obtained from natural spawning of adult zebrafish according to methods in *The Zebrafish Book* [15]. Embryos were collected and staged, as described by Kimmel et al [14]. Embryos were treated with a dilute bleach solution (0.0033% = 0.55 ml household bleach diluted to 1 L FW) to clean their chorions, and were then rinsed twice with FW before being placed into 10 cm petri dishes containing methylene blue (0.0002%) to inhibit fungal growth.

To assess morphology of embryos over time, embryos were placed individually in wells of 96-well plates in 150 μl methylene blue (0.0002%) and observed daily using stereomicroscopy, up to 120 hours post-fertilization (hpf). Scoring was assessed on visible phenotypes (mortality at 24 hpf; mortality at 120 hpf, delayed development, lack of motility, abnormal touch response, spastic movement, and malformations of heart, brain, yolk sac, notochord, body axis, trunk, circulatory system, eye, jaw, somites, snout, otic, fin, pigmentation, or swim bladder).

Quantitative real-time polymerase chain reaction (qPCR)

Fish were euthanized by tricaine overdose, livers removed and placed in RNALater (Qiagen, Valencia, CA) and stored at −20° C until RNA extraction. Total RNA was extracted using an RNeasy kit with DNase I treatment per manufacture's directions (Qiagen). RNA concentrations and purity were determined by UV absorption (NanoDrop ND-1000 UV-Vis Spectrophotometer, Thermo Scientific, Wilmington, DE). cDNA was synthesized following manufacture's directions using Superscript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA). Primers were designed for each target gene using the Primer-BLAST program (Primer3 combined with BLAST, NCBI website) (Table 2). Plasmids were cloned from each primer product (TOPO TA cloning kit, Initrogen), sequenced to verify correct product (ABI Prism 3730 Genetic Analyzer, ABI Prism 3730 Data Collection Software v. 3.0, ABI Prism DNA Sequencing Analysis Software v. 5.2, with BigDye Terminator v. 3.1 Cycle Sequencing Kit, Center for Genome Research and Biocomputing core facility, Oregon State University), and concentrations measured by spectrophotometer. These plasmids were used to generate an absolute copy number standard curve for real-time PCR quantification. Samples were analyzed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) using a DNA Engine Opticon 2 System (Bio Rad, Hercules, CA) with the Opticon Monitor Version 3.0 software for real-time PCR detection. Results were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, or β-2-microglobulin (β2M) expression. Data are reported as fold-changes relative to values from lab control fish livers. There were no statistically significant differences in the housekeeping genes between the diet groups. The β2M gene was chosen because its abundance was similar to our genes of interest.

Statistics

GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) and JMP (SAS Institute, Cary NC) were used for statistical analyses. The data was logarithmically transformed, when unequal variances were observed between groups. α-Tocopherol depletion kinetics were calculated using Microsoft Excel (Microsoft Corporation, Santa Rosa, CA) by fitting a linear regression analysis on the log-transformed data to the first ~80 days after initiation of diet feeding. Sensory evaluation was estimated using average velocity of the fish generated by the Noldus EthoVision software. Comparisons between lab, E+, and E- diets were calculated using one-way ANOVA, comparisons between E+ and E- by Tukey's honestly significant differences, HSD. Relationships between the three diet groups over time were analyzed using two-way ANOVA. Post-hoc tests were carried out using paired comparisons (Tukey's honestly significant differences, HSD). Data are reported as means ± SEM; differences were considered significant at $P<0.05$.

Results

Diet Vitamin E Concentrations

The E- and E+ defined diets were formulated identically with the exception that the E- diet had no added α-tocopheryl acetate. The E- diet contained 400-times less α-tocopherol than the E+ diet ($P<0.0001$ Figure 1), which contained ~500 mg/kg. γ-Tocopherol concentrations were also less in the E- than the E+ diets (6.2 ± 1.1 and 10.4 ± 1.0 mg/kg, respectively, $P<0.0001$). The lab diet contained both α- and γ-tocopherols. The flake food components contained the following α- and γ-tocopherol concentrations, respectively: Aquatox Flake, 440 ± 18 and 16 ± 1 mg/kg; Golden Pearls 305 ± 8 and 10 ± 1; and Hikari Micropellets 283 ± 4 and 5.9 ± 0.1 (n=3 measurements).

Zebrafish Growth on Defined Diets and Tocopherol Concentrations

Zebrafish at ~4–6 weeks of age were divided into three groups: E-, E+ and lab diets; then were fed exclusively the indicated diets and the whole body vitamin E concentrations measured at the indicated time on diet (up to 310 days, Figure 2). Although the zebrafish increased weight during the first 3–4 months on diet (time effect $P<0.0001$), the various dietary treatments (E-, E+ and lab) had no significant effects on body weights over the course of the study (Figure 2A–C). The rate of weight increase was 2.3 ± 0.1, 2.1 ± 0.1, and 2.0 ±0.1 g/d for the E-, E+ and lab fish.

Prior to initiating the experimental diets, zebrafish α-tocopherol concentrations were 33.5 ± 2.5 nmol/g. When fed the E+ or lab diets, the α-tocopherol concentrations of zebrafish initially increased over time (Figure 2E or 2F). In contrast, when zebrafish consumed the E- diet, the α-tocopherol concentrations decreased exponentially for the first ~80 days at a rate of −0.029 ± 0.006 nmol/g (n=3 experiments), which yields a half-life of 25 ± 5 days (Figure 2D). After 3 half-lives, hypothetically only 12.5% of the starting vitamin E concentration should remain. Consistent with this prediction, between 80 and 300 days of diet consumption, the zebrafish fed the E- diets had α-tocopherol concentrations that were approximately 10% of their starting concentrations and more than ~50-times lower than zebrafish consuming either the E+ or lab diets (Figure 2E or F, Table 3, diet effect, $p=0.0007$, paired comparisons, $P<0.05$). After this observed initial decrease, the fish maintained consistently low α-tocopherol concentrations (Figure 2D, Table 3).

After 80 days, γ-tocopherol concentrations in zebrafish consuming the E- and E+ diets were lower than those in the fish consuming the lab diet; those of the zebrafish consuming the E- diet were less than half of those consuming the other diets (Figure 2G, H, I, Table 3, diet effect, $P<0.0001$, paired comparisons, $P<0.05$).

Impaired Sensory Perception in Zebrafish Consuming E- Diets

Sensory neuropathy is one of the first symptoms of vitamin E deficiency in humans [26]. To assess sensory neuropathy in zebrafish we devised a “startle response” measurement using a percussive “tap” with responses monitored by video recording. In the baseline trial, no significant differences were observed in the average swimming velocity between the diet groups. Application of a single synchronized solenoid tap showed a characteristic startle response in the E+ and Lab diet zebrafish [25]. When startled by a single tap the E+ and Lab zebrafish both swam faster, while E- fish had an attenuated response (Figure 3, diet effect $P<0.003$, paired comparisons, $P<0.05$). These differences disappeared when the fish were exposed to a higher level of stimulation in the “multiple tap” trial, suggesting that a greater stimulus was needed to elicit a response from the E- fish.

Embryo Tocopherol Concentrations

Fish from each of the diet groups were group-spawned starting at 3–4 months of age (~80 days on diets). Despite differences in α-tocopherol concentrations, all dietary groups of zebrafish routinely produced viable gametes. The embryos were observed up to 120 hours post-fertilization (hpf) without being fed. Normally, zebrafish embryos do not consume food during this period, but receive their nutrition from the yolk sac [13,14].

Embryo α-tocopherol concentrations reflected those of their parents. For example, adult zebrafish that had consumed the diets for >250 days were spawned and the α- and γ-tocopherol concentrations were measured in representative adult fish and in 48 hpf embryos (Figure 4). Adult zebrafish consuming the E+ and lab diets contained nearly 50-times higher α-tocopherol concentrations than did those consuming the E- diet; the embryos from the fish consuming the E+ and lab diets contained nearly 30-times higher α-tocopherol

concentrations than embryos in the E- group (diet × lifestage interaction, P=0.016; P<0.05 for comparisons, Figure 4A).

The adult zebrafish consuming the E+ and lab diets contained nearly 3-times higher γ-tocopherol concentrations than did the adults consuming the E- diet. However, in the the E- and lab embryos the γ-tocopherol concentrations were almost double those of the E+ embryos (diet × lifestage interaction, P<0.0001, E+ compared with lab P<0.05, Figure 4B). Thus, E- embryo γ-tocopherol concentrations were not significantly different from the E- adults' γ-tocopherol concentrations, while the γ-tocopherol concentrations of the embryos from the E+ and lab diet groups were lower than those of their parents (E+ or lab adults, P<0.05). These findings indicate that the adult female zebrafish transfer very little γ-tocopherol to the eggs, even during vitamin E deficiency.

Embryo vitamin E status did not vary significantly from 48 to 120 hpf (Data not shown).

Embryo Morphology and Mortality

Embryos from spawns of each diet group were observed up to 120 hpf for morphological changes. Low levels of abnormalities are inherent in zebrafish embryos and were observed in embryos from all the diet groups. In the E- embryos, there was increased mortality at 24 hpf and at 120 hpf compared with the other diet groups (Figure 5A, diet × time interaction, P<0.0001), but at 24 hpf, the differences between diet groups did not reach statistical significance for paired comparisons. At 120 hpf, the E- embryos displayed significantly higher levels of mortality compared with the E+ or lab diet embryos (diet effect p=0.005, P<0.05 paired comparisons).

At 120 hpf, nearly 70% of the E- embryos experienced either malformations or death. These levels were higher than those observed in the E+ or lab diet embryos; E+ had greater malformations than did lab diet embryos (Figure 5B, P<0.05). Malformations commonly observed included cranial-facial malformations, bent anterior-posterior axis, pericardial edema, swim bladder malformations, and yolk-sac edema (Figure 6).

Whole Fish Ascorbic Acid Concentrations

Ascorbic acid concentrations were measured in adult zebrafish after consuming E-, E+ or lab diets for various amounts of time (>181 days). Ascorbic acid concentrations of the fish on the defined diets were nearly double those of the fish consuming the lab diet (diet effect P<0.0035, P<0.05 paired comparisons, Figure 7).

Liver Gene Expression

To assess long-term effects of vitamin E deficiency on mRNA expression levels of liver genes of interest, we used using real-time quantitative polymerase chain reaction (qPCR) to measure transcripts of genes involved in oxidative stress response, lipid metabolism, vitamin E trafficking, and cell death [27–31] (genes are listed in Table 2). Expression levels were normalized to β2M, β-actin or GAPDH; all three housekeeping genes yielded similar results; there were no statistically significant differences between the diets for each of the housekeeping genes. The data shown are normalized to β2M and are shown as fold changes over the average of the lab diet control liver mRNA, set to 100. We observed no significant changes in mRNA levels due to chronic vitamin E deficiency in the genes assayed. However, the expression of four genes, which have previously been identified to be important in nerve protection [32], were found to be significantly reduced in the zebrafish fed E- or E+ diets relative to those in the control fish (Figure 8).

Discussion

Vitamin E deficiency caused developmental abnormalities and death in zebrafish embryos. This finding elucidates for the first time that the embryo, as well as the mother, requires vitamin E. The expression of TTP by the human embryo-yolk sac [8], as well as by the zebrafish embryo-yolk sac [9], suggested that the embryo itself might require vitamin E. Initially in 1922 vitamin E was discovered because rats fed rancid fat failed to carry their offspring to term [1]. This finding was the basis for the “fetal resorption test” that remains in use today as an assay of biologic activities of various vitamin E forms [33]. In order to prevent fetal resorption, vitamin E must be administered to the vitamin E deficient rat mother on post-fertilization days 5 to 9 [34,35]. Interestingly, this is the same critical period where the 12/15-lipoxygenase-dependent pathway appears to mediate implantation [36]. Similarly, glutathione peroxidase 4 (GPx4) expression increases at day 7.5 and is the same time at which GPx4-knockout mice embryos are resorbed [37]. Taken together these findings suggest that during days 5 to 9 of rodent embryogenesis, lipid peroxidation can be especially damaging. Importantly, vitamin E is a potent, lipid-soluble antioxidant and thus may be critical for the embryo to have sufficient amounts to protect against lipid peroxidation [38].

Both low fetal and maternal α-tocopherol concentrations may be important factors during fetal resorption. Retention of uterine α-tocopherol by the mother appears essential to maintain pregnancy because TTP is increased at the site of implantation [2,3] and is also expressed in the syncytiotrophoblast of the human placenta [3–6]. Early failure of pregnancy is associated with lipid peroxidation with resultant damage to the syncytiotrophoblast in the placenta [7]. Thus, it is likely that α-tocopherol is needed by the mother to protect her from the oxidative stress of the rapidly growing fetus. Herein, we demonstrate that the embryo independently requires vitamin E because the E- zebrafish embryos suffered increased developmental abnormalities, as well as increased mortality (Figures 5 & 6). These E- embryos contained significantly lower α-tocopherol concentrations than the embryos in the other diet groups (Figure 4),

Importantly, the E- diet depleted the adult zebrafish of α-tocopherol (Figure 2, Table 3). The exponential rate of depletion predicted that the zebrafish fed the E- diet would experience nearly 90% depletion of their initial α-tocopherol concentration by 80 days on diet. The α-tocopherol measurements beyond 100 days showed that further depletion did not occur, or at least was slowed to statistically imperceptible changes. Given that the E-diet had some α-tocopherol and that the zebrafish experienced deficiency symptoms, these data suggest that the dietary α-tocopherol concentration (approximately 2 mg/kg diet) is below the requirement for the zebrafish. Using the estimate that a fish weighing 500 mg eats about 10 mg food [39], the zebrafish is probably consuming about 50 pmol α-tocopherol, then after 80 days on the E- diet its whole body contains about 2500 pmol α-tocopherol.

Remarkably, like humans [40,41], zebrafish show a preference for α- over γ-tocopherol. This finding is most easily appreciated in the comparison of the vitamin E distribution within the E- diets compared with the E- zebrafish. The E- diets had approximately 5-times more γ- than α-tocopherol (Figure 1), while the E- zebrafish contained approximately 2.5-times more α- than γ-tocopherol (Table 3). Given that the zebrafish expresses TTP gene [9], it is not surprising that the zebrafish shows a preference for α-tocopherol. More remarkable is the apparent lack of γ-tocopherol transfer to the embryos (Figure 4). Although the E+ and lab adult zebrafish contained γ-tocopherol at 3-times the E- adult zebrafishes’ γ-tocopherol concentrations, the E- embryo γ-tocopherol concentrations were not significantly different from those of either the E+ and lab embryos. These data emphasize that the embryos are preferentially enriched in α-tocopherol and that the E- embryos were limited in γ-tocopherol

even when the zebrafish adults were severely limited in both forms of vitamin E. These findings are reminiscent of those in TTP null mice that were fed γ-tocopherol-enriched diets yet did not accumulate γ-tocopherol in tissues or plasma [42]. Thus, vitamin E deficiency does not drive the use of γ-tocopherol in place of α-tocopherol.

This study also demonstrates that the adult zebrafish require vitamin E because we detected abnormal swimming responses to a “tap” test (Figure 3). In general, testing of neurologic responses of adult zebrafish has been limited. Eddins et al [25] studied chlorpyrifos neurotoxicity that was induced during early development and then persisted into adulthood. They showed that the neurotoxicity caused an increase in swimming velocity in the affected adult zebrafish [25]. In contrast, we found that vitamin E deficiency in zebrafish *decreased* their responsiveness, as shown by decreased swimming velocity, suggesting diminished sensory perception. It should be noted that muscle degeneration is also associated with vitamin E deficiency, but in the current study the zebrafish were apparently able to swim faster in response to multiple taps, they just did not respond quickly to a single stimulus. This finding suggests that it took greater stimulus to elicit a response. Given that the vitamin E deficiency symptom in humans is a dying back of the sensory neurons [43], it seems likely that the E- zebrafish also had a sensory deficit, but further studies are needed to evaluate the extent and mechanism of the deficit.

To assess long term effects of vitamin E deficiency on mRNA expression levels of genes involved in oxidative stress response, lipid metabolism, vitamin E trafficking, and cell death [27–31]. Liver expression of these genes was not dependent upon vitamin E status since the fish from the E- and E+ groups displayed similar responses. However, the lab diet contains various ingredients, such as fish oil, that are not present in the defined diets. These “control” livers had higher expression of GPX4a, PLA2, PLA2gIV, and AIF, suggesting that the more oxidizable lipids had induced expression of these protective enzymes. However, we have not measured lipid oxidation products in these fish, so the mechanisms for the differential expression remains to be investigated. It is also apparent that the embryo is more sensitive than is the adult fish to insufficient vitamin E. We plan further studies to investigate vitamin E-dependent gene regulation in the embryo.

We formulated a diet containing sufficient nutrients for the zebrafish based on previous research in other fresh water teloest fish [17–20]. Our diet formulation (Table 1) was sufficient for zebrafish nutrition based on growth and body weights (Figure 2, Table 3). Animal health, behavior and mortality were observed daily during feeding and no overt visual changes were noted in fish consuming the defined diets. Previously, commercially available diets for zebrafish were insufficient to maintain growth and had to be supplemented with live food [39]. Our diets are, therefore, a major step forward in zebrafish husbandry given that 45-day old zebrafish fed these diets grow as well as those fed the commercial mixture of foods and live food.

In addition, we analyzed whole zebrafish ascorbic acid concentrations, as vitamin C status has been linked to vitamin E status [44], and teleost fish lack the L-gulonolactone oxidase gene and thus cannot synthesize ascorbic acid [45]. Zebrafish ascorbic acid concentrations were higher in the fish fed the defined diets than in the lab controls, showing that the defined diets are more than sufficient in ascorbic acid (Figure 7).

In conclusion, our findings document for the first time that vitamin E is required, not only for the mother, but also for the embryo during development. This zebrafish model has allowed the successful separation of maternal and embryonic nutrition, permitting the study of embryogenesis without the fetal resorption that occurs in mammalian models.

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Abbreviations used

AIF (pdcd8)	apoptosis-inducing factor (programmed cell death 8)
β2M	beta-2-microglobulin
BA	bent anterior-posterior axis
BAD	BCL2-antagonist of cell death
β-actin	beta-actin
CF	cranial facial malformations
E- diet	α-tocopherol deficient diet
E+ diet	α-tocopherol sufficient diet
FW	fish water
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCLc	glutamate-cysteine ligase, catalytic subunit
GPx4a	glutathione peroxidase 4a
GPx4b	glutathione peroxidase 4b
GSTp1	glutathione S-transferase pi
hfp	hours post-fertilization
HPLC-ECD	high-pressure liquid chromatography coupled with electro-chemical detection
lab diet	standard zebrafish diet (see methods)
LOX	arachidonate 12-lipoxygenase
NRF2	nuclear factor (erythroid-derived 2)-like 2
PE	pericardial edema
PLA2gIV	phospholipase A2, calcium dependent
PLA2gVI	phospholipase A2, calcium independent
SB	swim bladder malformation
TTP	α-tocopherol transfer protein
YSE	yolk-sac edema

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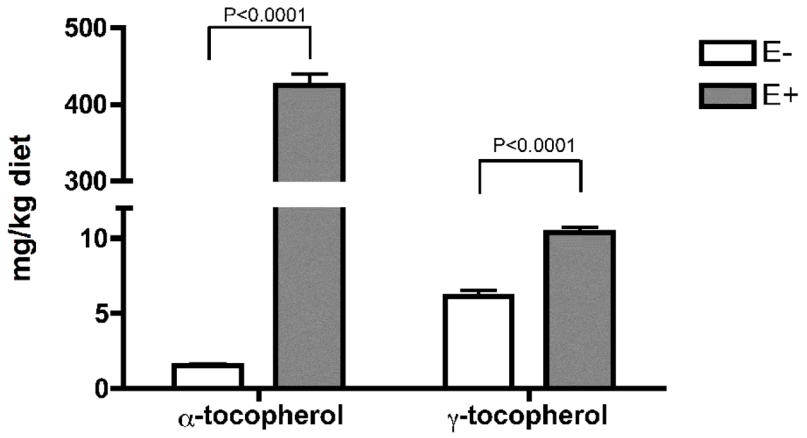

Figure 1. Diet Vitamin E Concentrations

Measured α- and γ-tocopherol concentrations (means ± SEM, n=3 separate diet batches) of experimental diets.

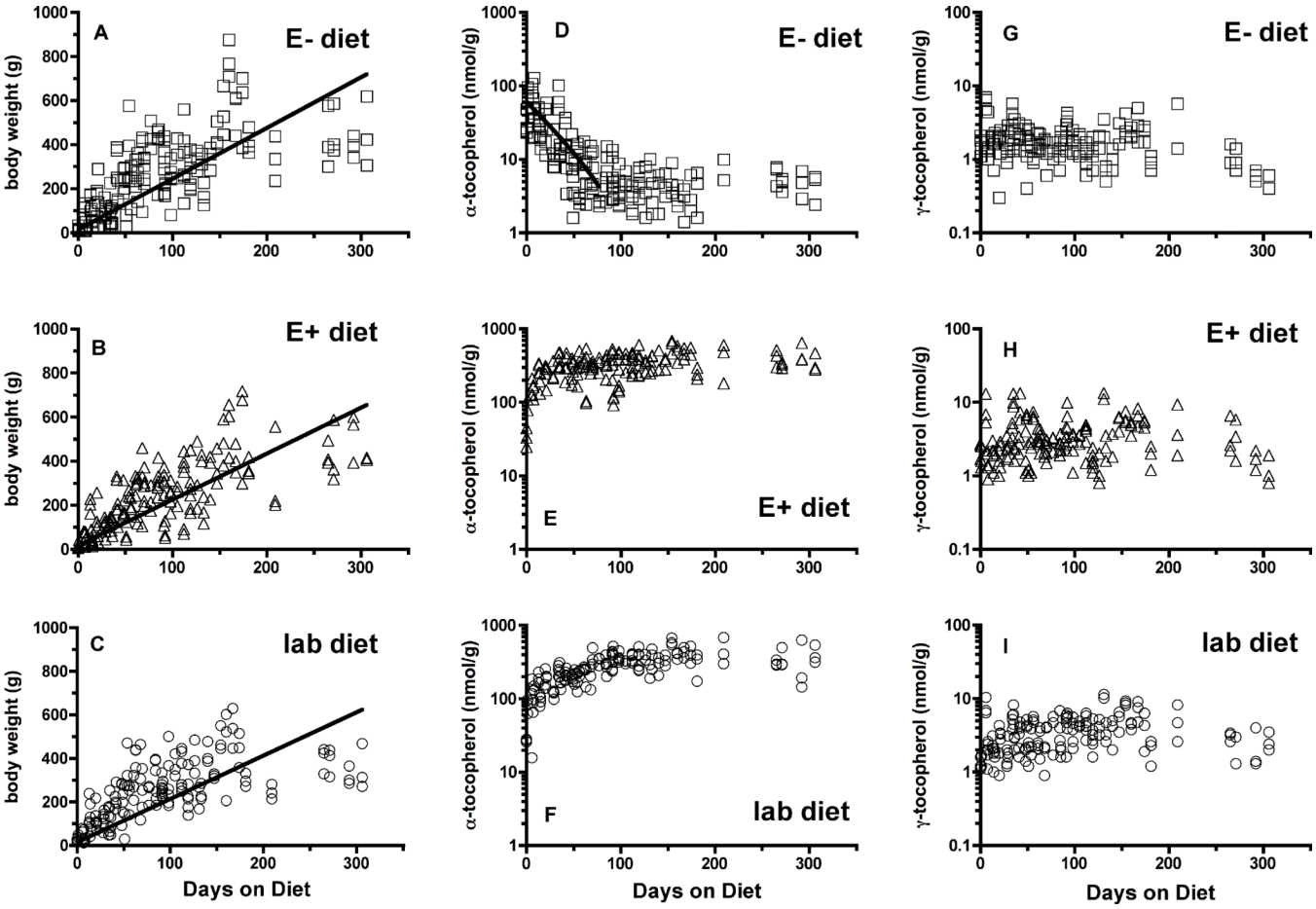

Figure 2. Vitamin E Depletion Kinetics

Time course of body weights (panels **A,B,C**) and whole body concentrations of a- (panels **D,E,F**) and γ-tocopherol (panels **G,H,I**) from individual zebrafish fed E- (squares, n=172, panels A,D,G), E+ (triangles, n=177, panels B,E,H) or lab (circles, n=168, panels C,F, I) diets. Results are from three separate generations of fish, samples taken at noted days after initiation of dietary treatments. Body weights (**A–C**) were not significantly different between the diet groups, but increased over time (time effect $P<0.0001$; month 1< month 2< month 3–5 < month 6–10, Tukey paired comparisons, each $P<0.05$). (**D,E,F**) By month 2 (>30 days) and thereafter, the α-tocopherol concentrations of the E- zebrafish were significantly less than those of the other groups [diet × time interaction, $p=0.0007$; E- for month 2, month 3–5, and month 6–10, different than E+ or lab diet groups (which were not different from each other at each time interval), Tukey paired comparisons, $P<0.05$]. The line indicates the exponential rate of depletion. (**G,H,I**) Within the first month (<30 days) and thereafter, the γ-tocopherol concentrations of the E- zebrafish were significantly less than those of the other groups [diet × time interaction, $P<0.0001$; E- for month 1, month 2, month 3–5, and month 6–10, different than E+ or lab (which were not different from each other at each time interval), Tukey HSD $P<0.05$].

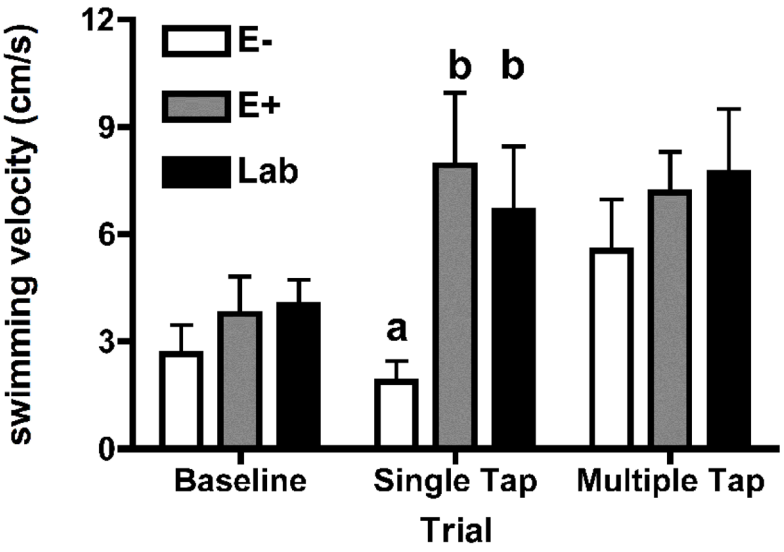

Figure 3. Vitamin E Deficiency Alters Startle Responses

Adult zebrafish (n=6) from each diet (E+, E- and Lab; 221 days on diet) were placed in individual 1.75 L tanks containing ~1.5 L of FW. In the baseline trials and multi-tap trials, no significant differences were observed in the average swimming velocity between the diet groups. When startled by a single tap the E+ and Lab zebrafish swam faster, while E- fish had an attenuated response (diet effect $P<0.003$, bars not sharing the same letter are significantly different, Tukey HSD $P<0.05$). When exposed to multiple taps, the fish did not show significant differences in swimming velocity.

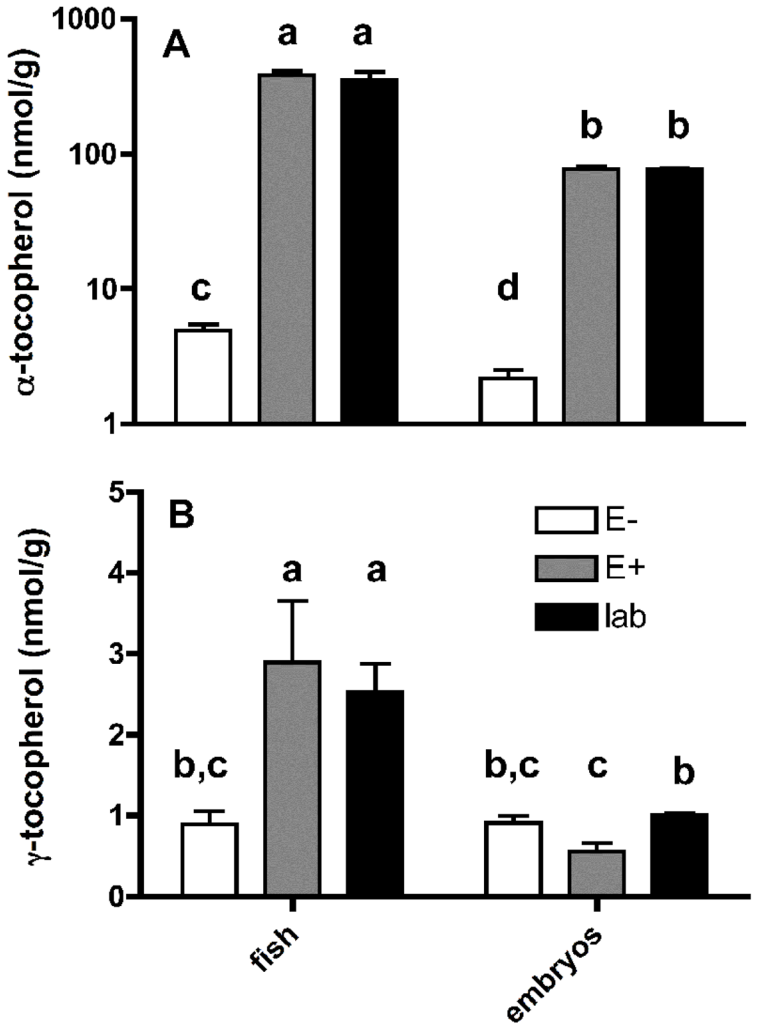

Figure 4. α- and γ-Tocopherol Concentrations of Embryos and Adults

Adult zebrafish (n= 12 per diet) were collected for vitamin E analysis between 250 and 300 days of consuming the diets. Zebrafish from this generation were spawned at 270, 278, and 284 days. Embryos were collected for vitamin E analysis at 48 hpf in groups of 15 embryos (E- n=16, E+ n=11, lab n=12 replicates). (**A**) All embryo α-tocopherol concentrations (mean ± SEM: logarithmic scale) were less than those of the adult zebrafish; E- embryo α-tocopherol concentrations were less than those of E- adults; E- embryos compared with E+ or lab embryos had the lowest α-tocopherol concentrations (diet × lifestage interaction, p=0.016; bars not sharing the same letter are significantly different, Tukey HSD P<0.05). (**B**) Adult E+ and lab zebrafish γ-tocopherol concentrations (mean ± SEM) were greater than the adult E- zebrafish and γ-tocopherol concentrations in all of the embryos; the lab embryo γ-tocopherol concentrations were greater than those of the E+ embryos; E- embryo γ-tocopherol concentrations were not significantly different from the E- adults γ-tocopherol concentrations, while E+ and lab embryos were less than those of the adults (diet × lifestage interaction, P<0.0001, bars not sharing the same letter are significantly different, Tukey HSD P<0.05).

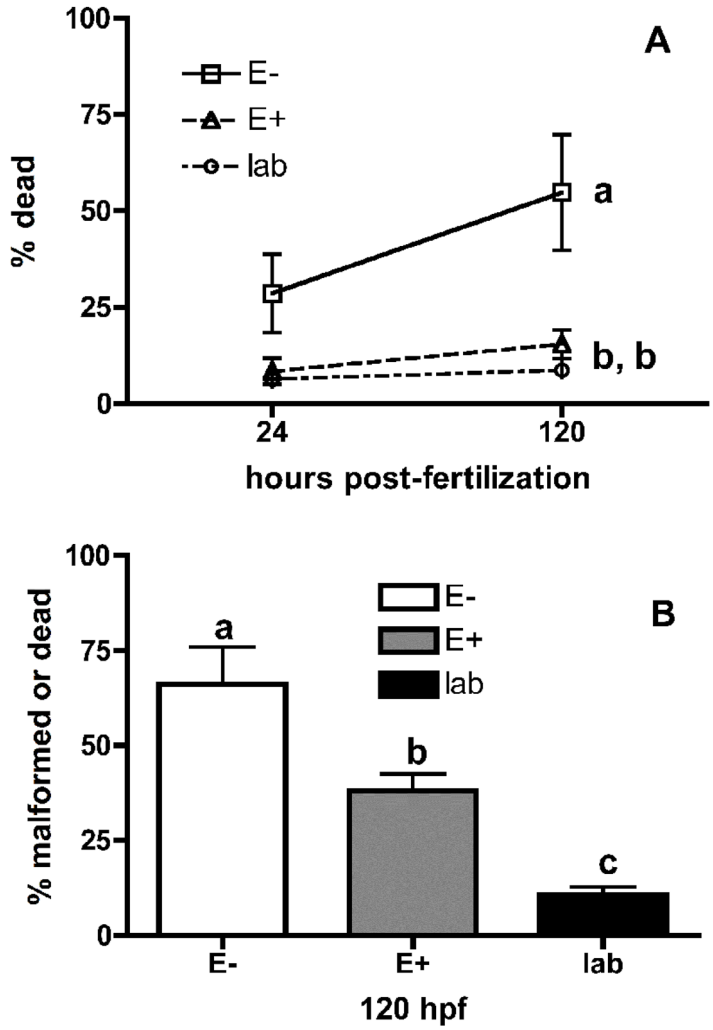

Figure 5. Malformations and Mortality of Zebrafish Embryos

(**A**) Increased mortality (mean ± SEM) was observed in the E- embryos at 24 hpf and at 120 hpf compared with the other diet groups (diet × time interaction, $P<0.0001$), but at 24 hpf the differences between diet groups did not reach statistical significance. Mortality increased from 24 to 120 hpf in the E- (squares, $P<0.01$) and the E+ embryos (triangles, $P<0.01$), but not in the lab diet embryos (circles). At 120 hpf, the E- embryos displayed significantly higher levels of mortality compared with the E+ and lab diet embryos (diet effect $p=0.005$; E- (a) > E+ (b) or lab (b), $P<0.05$ paired comparisons). (**B**) Higher levels of both malformations and mortality were observed at 120 hpf in the E- embryos compared with E+ ($P<0.05$, a) or lab diet embryos ($P<0.001$, b); E+ had greater malformations than did lab diet embryos ($P<0.05$, c). Embryos were analyzed in 96-well plates, one embryo per well with 48 to 120 embryos per group per spawn. Results are expressed as percentages affected per total number of embryos (n= 6 spawns per group).

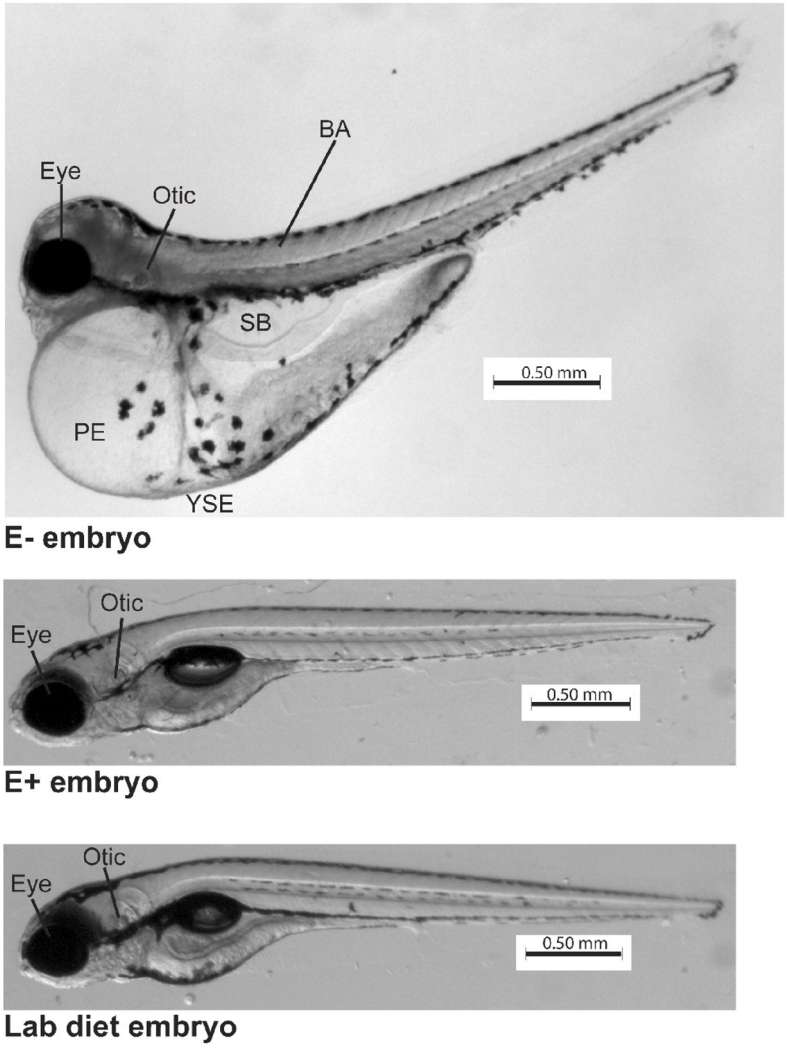

Figure 6. Typical Zebrafish Morphology at 120 hpf

Representative pictures from the three diet groups are shown after 5 days (120 h). The eye and otic vesicle are indicated on all fry; malformations are illustrated on the image of the deficient fish. CF=cranial-facial malformation, BA=bent anterior-posterior axis, PE=pericardial edema, SB=swim bladder malformation, and YSE=yolk-sac edema.

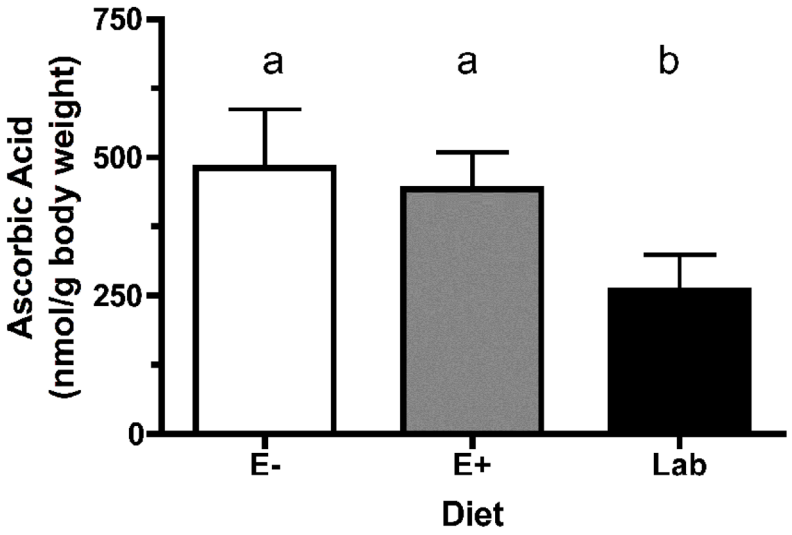

Figure 7. Ascorbic Acid Concentrations in Adult Fish

Ascorbic acid concentrations (mean ± SEM) were analyzed from two generations of zebrafish (n=7 per diet). (Diet effect $P<0.0035$, bars not sharing the same letter are significantly different $P<0.05$).

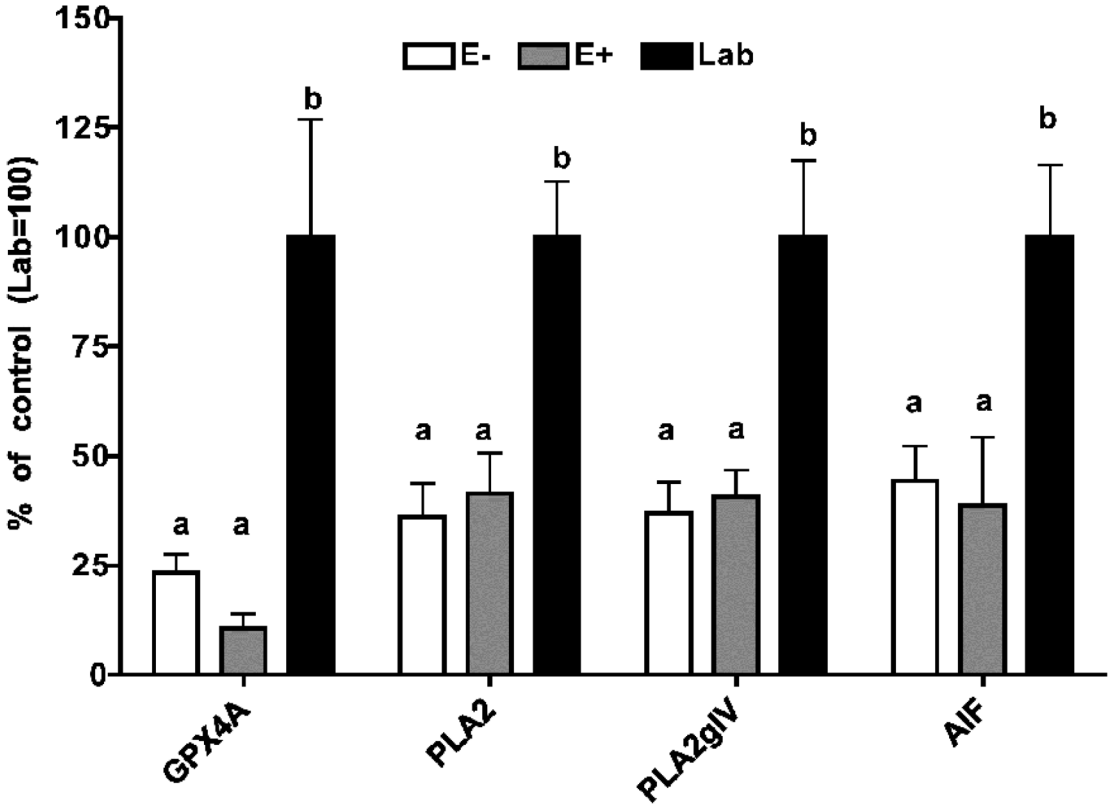

Figure 8. mRNA Expression in Adult Zebrafish Liver

mRNA expression (mean ± SEM) in adult zebrafish livers from fish from each diet group (n=4 per group, on diets >200 days) was analyzed by qPCR. Genes are defined and primers shown in Table 2. Expression levels were normalized to β2M and are shown as fold change over the average of the lab diet control liver mRNA, set to 100. (Diet effect for GPX4a P=0.0011, PLA2 P=0.0027, PLA2GIV P=0.0058, AIF P=0.0222; bars not sharing the same letter are significantly different P<0.05).

Table 1

Diet Ingredients

Ingredient	g/100 g
Wheat Gluten	15
Casein	30.5
Egg Whites	4
Cellulose	3
Vitamin Mix[1]	4
Mineral Mix[2]	4
Starch	26.5
Tocopherol-Stripped Soybean Oil	7
Tocopherol-Stripped Soy Lecithin	5
Stay C (Argent Chemical Laboratories)	1
α-Tocopherol acetate (E+ diets only)	0.05

[1] Containing the following (g/kg vitamin mix): vitamin A (500,000 IU/g), 0.15; vitamin D_3 (400,000 usp/ug), 6.2445; vitamin K, 0.025; thiamine, 0.15; riboflavin, 0.25; vitamin B_6, 0.125; pantothenic acid, 0.75; niacin, 1.25; biotin, 0.005; folate, 0.05; vitamin B_{12}, 0.0005; myoinositol, 6.25; PABA, 1; celufil (alpha cellulose), 983.75.

[2] Containing the following (g/kg mineral mix): calcium carbonate, 19.23; calcium phosphate dibasic ($2H_20$), 766.29; citric acid, 5.28; cupric carbonate, 0.36; ferric citrate, 2.99; magnesium oxide, 22.89; manganese carbonate, 5.65; sodium chloride, 28.02; disodium hydrogen phosphate, 11.89; zinc carbonate, 0.97; potassium phosphate dibasic, 74.16; potassium sulfate, 62.26; potassium iodide, 0.01.

Table 2

Primers for genes of interest

NCBI Gene ID#	Abbreviation[1]	Forward Primer	Reverse Primer
373125	AIF	5′-AAAGTCCGGAAAGAGGGTGT-3′	5′-GCCTGGAGCTCAGCATTAAC-3′
30400	β2M	5′-CCACTCCGAAAGTTCATGTGT-3′	5′-ATCTCCTTTCTCTGGGGTGAA-3′
58100	BAD	5′-CTGAAAGGAGAGCAACTGGG-3′	5′-CCGCATCAGACTCTTTGTGA-3′
57934	β-actin	5′-AAGCAGGAGTACGATGAGTC-3′	5′-TGGAGTCCTCAGATGCATTG-3′
317743	GAPDH	5′-GAATTCTGGGATACACGGAG-3′	5′-AAAGGGGTCACATCTACTCC-3′
326857	GCLc	5′-CTATCTGGAGAACATGGAGG-3′	5′-CATTTTCCTCTGTTGACCGG-3′
352928	GPx4a	5′-CCTTGGAAATGGCATCAAAT-3′	5′-CCACCACACTTGGATCCTG-3′
352929	GPx4b	5′-GAAGTGGATGAAGGAGCAGC-3′	5′-GCCGCAGCATACACACTCTA-3′
79381	GSTp1	5′-TTCAGTCCAACGCCATGC-3′	5′-ATGAGATCTGATCGCCAACC-3′
322732	LOX	5′-GGCACAGCCAAAAAGCTGAGCG-3′	5′-ATCGAGCGTCCTGGGGCAGA-3′
360149	NRF2	5′-CAGACGGAGGAGGAGCGGGA-3′	5′-GGCACTGCTGCAACTCTGGGA-3′
559087	PLA2gIV	5′-ATTTATCCTCGACCCCAACC-3′	5′-ACACCAGCATTGTTTGTCCA-3′
406370	PLA2gVI	5′-AAACTGCAGAGATGAGCCGT-3′	5′-GCCTGGTGTTTCTCCAACAT-3′
325906	TTP	5′-ATGGGAGACGCAAAGAAATG-3′	5′-TGCATGTGAATCCGTTGTTT-3′

[1] **AIF** (pdcd8), apoptosis-inducing factor (programmed cell death 8); **β2M**, beta-2-microglobulin; **BAD**, BCL2-antagonist of cell death; **β-actin**, beta-actin; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **GCLc**, glutamate-cysteine ligase, catalytic subunit; **GPx4a**, glutathione peroxidase 4a; **GPx4b**, glutathione peroxidase 4b; **GSTp1**, glutathione S-transferase pi; **LOX**, arachidonate 12-lipoxygenase; **NRF2**, nuclear factor (erythroid-derived 2)-like 2; **PLA2gIV**, phospholipase A2, calcium dependent; **PLA2gVI**, phospholipase A2, calcium independent; **TTP**, α-tocopherol transfer protein.

Table 3

Zebrafish vitamin E concentrations and body weights taken at various times between ~80 to ~300 days consuming experimental diets

Diet	Observations (N=)	α-Tocopherol (nmol/g)	γ-Tocopherol (nmol/g)	Body Weight (g)
E-	80	5 ± 3[a]	1.8 ± 1.0 [a]	379 ± 154
E+	84	369 ± 131 [b]	3.6 ± 2.4 [b]	334 ± 145
lab	80	362 ± 107 [b]	4.2 ± 2.2 [c]	334 ± 108

A significant diet effect (P<0.0001) was observed for both zebrafish α- and γ-tocopherol concentrations. Data in columns not bearing the same letter are significantly different (P<0.05). Time points are indicated in Figure 2.

Table 4

mRNA expression of in adult zebrafish liver from fish fed E-, E+ or lab diets

Gene Symbol*	Diet		
	E-	E+	Lab
BAD	56 ± 22	57 ± 23	100 ± 29
GCLc	76 ± 42	67 ± 29	100 ± 6
GSTp1	100 ± 48	70 ± 40	100 ± 20
GPX4b	64 ± 19	56 ± 31	100 ± 14
LOX	73 ± 26	77 ± 28	100 ± 42
NRF2	98 ± 42	85 ± 32	100 ± 20
TTP	64 ± 31	42 ± 27	100 ± 38

mRNA expression (mean ± SEM) in adult zebrafish livers from fish from each diet group (n=4 per group) was analyzed by qPCR. Genes are defined and primers shown in Table 2. Expression levels were normalized to β2M and are shown as fold change over the average of the lab diet control liver mRNA, set to 100. No significant differences was found for a diet effect for the indicated genes.