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Title: Embryogenesis is Dependent upon 12-lipoxygenase, 5-lipoxygenase, and α-tocopherol to Modulate Polyunsaturated Fatty Acid Status and the Production of Oxidized Fatty Acids in Zebrafish

Abstract approved:

Maret G. Traber

Arachidonic acid (ARA) and docosahexaenoic acid (DHA) are polyunsaturated fatty acids required for proper embryonic development, specifically neurodevelopment. However, little is known regarding their conversion to other metabolites during embryogenesis. The oxidation of ARA gives rise to the biologically active eicosanoids and the oxidation of DHA gives rise to the biologically active docosanoids. The oxidation of ARA and DHA occurs through enzymatic processes, via lipoxygenase (LOX), or non-enzymatic processes, via radical-mediated lipid peroxidation. We hypothesize that oxidation of ARA and DHA via LOX is required for proper embryonic development. Additionally, we hypothesize that α-tocopherol, a potent lipid soluble antioxidant, mediates the conversion of ARA and DHA to their respective oxidized metabolites. Using zebrafish as a model of vertebrate embryogenesis, we found that the selective knockdown of either 12-LOX or 5-LOX decreased the production of docosanoids, altered fatty acid homeostasis, and increased the incidence of malformations and mortality in embryos by 24 hours post fertilization. α-Tocopherol deficiency also increased the incidence of malformations and mortality during embryogenesis, and in its absence, increased oxidized metabolites of ARA and DHA and decreased fatty acids concentrations. Therefore, oxidized metabolites of ARA and DHA perform crucial functions during embryonic development, but the production of oxidized fatty acids must be balanced with antioxidant bioavailability for proper embryogenesis.

Embryogenesis is Dependent upon 12-lipoxygenase, 5-lipoxygenase, and α-tocopherol to Modulate Polyunsaturated Fatty Acid Status and the Production of Oxidized Fatty Acids in Zebrafish

by
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APPROVED:

Major Professor, representing Nutrition

Co-director of the School of Biological and Population Health Sciences

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Katherine M. Lebold, Author

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CONTRIBUTION OF AUTHORS

Jay S. Kirkwood and Alan W. Taylor were directly involved with method development, method validation, and data collection. Carrie L. Barton assisted with data collection and performed all animal husbandry. Galen W. Miller contributed to study design, data collection and interpretation. Robert L. Tanguay contributed to study design and data interpretation. Maret G. Traber was involved with the study design, data interpretation, and writing of all chapters contained herein.

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DEDICATION

This thesis is dedicated to Keddy and beets.
For your inspiration, energy, and passion.
For your guidance, strength, and love of life.

Embryogenesis is Dependent upon 12-lipoxygenase, 5-lipoxygenase, and α-tocopherol to Modulate Polyunsaturated Fatty Acid Status and the Production of Oxidized Fatty Acids in Zebrafish

Chapter 1: Introduction

Arachidonic acid (ARA, 20:4 ω-6) and docosahexaenoic acid (DHA, 22:6 ω-3) are polyunsaturated fatty acids (PUFAs) required for proper embryonic development, specifically neurodevelopment. This report addresses the importance of ARA and DHA, as well as the oxidized metabolites derived from these PUFAs, during embryonic development in the context of vitamin E deficiency using a zebrafish model. Zebrafish are widely used to study embryonic development and the use of zebrafish to study nutrient requirements and nutrient-nutrient interactions has been pioneered in our laboratory[1-3]. Strengths of this model organism include an easily manipulated paternal diet, dissociation of embryonic and maternal nutrient requirements since reproduction occurs through spawning, and noninvasive, whole organism observation facilitated by optically clear embryos. Furthermore, we have previously demonstrated that vitamin E-deficient adult zebrafish produce viable vitamin E-deficient embryos, which display morphological abnormalities by the end of primary organogenesis (48 hours post-fertilization [hpf])[2]. Enzymes involved in ARA and DHA metabolism have also been characterized in zebrafish[1,4-8].

I. Docosahexaenoic acid (DHA) and arachidonic acid (ARA) during fetal development

DHA is highly enriched in the central nervous system (CNS), comprising upwards of 50% of CNS PUFA content[9]. Rapid accretion of DHA within the CNS occurs during the last trimester of pregnancy in humans [10] and coincides with a time of maximal neurogenesis and synaptogenesis[11]. DHA supplementation during pregnancy has shown both positive[12-15] and null[16-20] effects on fetal neurodevelopment and cognitive outcomes, while DHA deficiency during pregnancy distinctly and adversely affects neurodevelopment. DHA deficiency inhibits fetal neurogenesis[21,22] and synaptogenesis[21], alters the synaptic proteome[23], serotoninergic neurotransmission[24], dopaminergic regulatory protein composition[25], neuronal phospholipid composition[26] and signaling[27], and impairs neuronal migration[28]. Maternal ω-3 PUFAs deficiency

reduces visual acuity in infant rhesus monkeys[29,30]. Adverse developmental outcomes caused by DHA inadequacy persist even after repletion with DHA[31,32], demonstrating long-lasting effects of embryonic DHA deficiency regardless of later restitution with an adequate diet. Notably, the dietary essential fatty acids, linoleic acid (LA, 18:2 ω-6) and α-linolenic acid (ALA, 18:3 ω-3) comprise less than 1% of neural PUFAs[33]. The fetal liver and brain are capable of synthesizing DHA from ALA[34,35]; however this synthetic capacity decreases over time[36] and in adult humans is limited such that less than 5% of ingested ALA is converted to DHA[37,38]. Additionally, greater amounts of DHA are accumulated within the developing brain when preformed DHA, as compared with its precursor ALA, is supplied to the developing fetus[39,40]. Indeed, the placenta preferentially transfers DHA over other PUFAs to the developing fetus[41,42]. Moreover, studies evaluating the efficacy of ALA supplementation to preterm infants demonstrate that endogenous DHA synthesis cannot match *in utero* accretion rates of preformed DHA[43]. Although dietary recommendations for and definitions of fetal DHA adequacy remain controversial, a recent consensus statement recommended pregnant woman consume at least 200 mg DHA daily[44] to ensure sufficient DHA delivery to the developing fetus.

ARA is the most abundant ω-6 neuronal fatty acid throughout gestation and postnatal development[10]. ARA is a component of cellular phospholipids and functions as a precursor for lipid secondary messengers involved in numerous cell signaling pathways. An appropriate balance between DHA and ARA is required during neonatal development, as infant formula supplemented with DHA, but lacking ARA, impairs infant growth[45]. Indeed, higher infant ARA concentrations are positively correlated with infant birth weight and length[46]. Conversely, ARA inadequacy is associated with delayed postnatal development and reduced growth[47,48]. As of 2005, recommendations on infant formula composition stated that ARA should be added in equivalent concentrations as DHA[49].

II. α-Tocopherol

A. Antioxidant function

α-Tocopherol (αTOC) is a potent lipid soluble antioxidant and is one of the eight naturally occurring isoforms of vitamin E (**FIG 1**), yet only αTOC meets human vitamin E requirements due to the action of the hepatic αTOC transfer protein. αTOC scavenges peroxyl radicals during the propagation of lipid peroxidation (**FIG 2**), and is therefore termed a chain-breaking antioxidant. αTOC is particularly enriched in neuronal tissue, where it is tenaciously retained during inadequate intake even after peripheral tissues become vitamin E depleted[50]. Overt vitamin E deficiency occurs rarely in humans, but does occur in patients with fat malabsorption syndromes or genetic defects in the hepatic αTOC transfer protein, as reviewed[51]. Patients with vitamin E deficiency present initially with a mild sensory neuropathy that advances to a spinocerebellar ataxia and a progressive, peripheral neuropathy caused by a dying back of large-caliber, sensory neurons[51]. αTOC protects PUFAs, notably DHA and ARA, and is postulated to co-localize with PUFA-enriched phospholipid domains of the cell membrane[52], which are highly susceptible to peroxidation. Indeed, αTOC requirements increase in parallel with dietary PUFA consumption or with an increasing index of fatty acid unsaturation[53]. Studies in experimental animals have demonstrated the importance of αTOC in protecting PUFAs: adult zebrafish fed a vitamin E deficient diet have reduced visceral percentages of total ω-6 and ω-3 PUFAs[1] and maternal feeding of fish oil reduced fetal brain αTOC concentrations in rats[47].

B. Inter-relationship with other nutrients

αTOC protects cellular membranes from lipid peroxidation in association with a larger antioxidant network (**FIG 2**). Once αTOC reduces lipid peroxyl radicals to lipid hydroperoxides, the selenium-dependent enzyme, phospholipid hydroperoxide glutathione peroxidase (GPx4) converts the hydroperoxides to the less toxic lipid hydroxides at the expense of glutathione. Ascorbate (vitamin C) recycles the αTOC radical, regenerating active αTOC. Subsequently, ascorbate is regenerated at the expense of glutathione. Experimental studies have shown that the maintenance of

this antioxidant network is crucial to protect cellular membranes against radical-mediated degradation. Vitamin E disappears faster from plasma in individuals who smoke, but vitamin C supplementation corrects the rapid αTOC disappearance[54,55]. In adult zebrafish, chronic vitamin E deficiency causes a secondary depletion of vitamin C, and concomitantly, a severe degeneration of skeletal muscle (Lebold et al, unpublished data). Taken together, this evidence demonstrates the *in vivo* interaction between these nutrients and the inter-dependence of each nutrient in the maintenance of antioxidant homeostasis and cellular redox status. These data additionally illustrate how concurrent or secondary nutrient inadequacies may augment pathology associated with primary nutrient deficiencies.

III. Lipid mediators derived from DHA and ARA

In 1922, αTOC was discovered because rats fed rancid fat failed to carry their offspring to term[56]. Since its discovery, αTOC's specific biologic functions, and the mechanism mediating its requirement for reproduction, remain unknown. Given its role in protecting PUFAs from lipid peroxidation, it is possible that αTOC is required during embryonic development specifically to protect ARA and DHA, or mediate the production and actions of lipid mediators derived from these PUFAs. Production of lipid mediators derived from ARA and DHA occurs through enzymatic peroxidation or non-enzymatic radical-mediated peroxidation. The three known enzymatic pathways that act upon PUFAs include the cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP450) pathways.

Metabolism of DHA or ARA leads to several classes of lipid mediators. Specifically, the oxidation of ARA gives rise to the biologically active hydroxyeicosatetraenoic acids (HETEs) and the eicosanoids, a class of lipids which encompasses the prostaglandins, prostacyclins, thromboxanes, leuokotrienes, lipoxins, and isoprostanes (**FIG 3**). Similarly, the oxidation of DHA gives rise to the docosanoids, which include the resolvins (D-series), neuroprotectins, and maresins, as well as intermediary monohydroxy lipids termed HDHAs. The synthesis of docosanoids requires the complex coordination of multiple enzymes, including LOXs and COXs[57].

A. Lipoxygenase function

LOXs are a family of enzymes that catalyze the dioxygenation of PUFAs to the corresponding hydroperoxy fatty acids. There are four major LOX isoforms: 5-LOX, platelet-type 12-LOX (12-LOX), leukocyte-type 12-LOX (12/15-LOX), and 15-LOX[58]. LOXs are non-heme containing enzymes which initiate the sterospecific addition of molecular oxygen to a *cis,cis*-1,4-pentadiene moiety of a PUFA through a free radical mechanism. To be fully activated, the iron in the LOX active-site must be oxidized from the inactive ferrous (Fe^{2+}) state to the active ferric (Fe^{3+}) state. Consequently, LOX activity is regulated by the cellular hydroperoxide tone, as the heterolytic cleavage of hydroperoxides in the presence of iron (ie Fenton reaction) provides the necessary electron acceptor to reduce the active-site iron[58]. As further evidence of the regulation of LOX by hydroperoxide levels, cells overexpressing GPx4, a peroxidase which utilizes glutathione to detoxify fatty acid hydroperoxides (**FIG 2**), have a diminished capacity to generate LOX products[59,60]. Incubating purified LOX *in vitro* with GPx4 and glutathione inhibits the metabolism of arachidonic acid by LOX[61], while the addition of exogenous hydroperoxy fatty acids restores LOX activity[61]. LOX activity also increases following GPx4 knockdown in cell culture[62,63]. LOX activity similarly increases following depletion of intracellular glutathione, such as occurs during glutamate toxicity[64-66], exposure to buthionine-sulfoximine (BSO,a glutathione synthesis inhibitor)[62,65], or cystine deprivation (which limits glutathione production)[67].

Following acquisition of a substrate, the LOX abstracts an allylic hydrogen, forming a carbon-centered radical and reducing the active-site iron back to the ferrous state. The carbon-centered radical then reacts with molecular oxygen, forming a peroxy radical. Subsequently, the peroxy radical oxidizes the iron, forming a peroxy-anion and re-activating the iron (Fe^{3+}). Finally, the peroxy-anion is protonated, creating the fatty acid hydroperoxide[58]. Although nonenzymatic lipid peroxidation occurs through the same mechanism, that process yields a racemic mixture of fatty acid hydroperoxides, as opposed to the stereospecific products created by the LOX reaction. Although the peroxidation of PUFAs by LOX proceeds via a free radical mechanism, due to the high fidelity of the enzyme, the radical is contained within the enzyme-substrate complex and few radical intermediates are released. However,

under certain conditions, specifically high substrate availability or low molecular oxygen concentrations[68], radicals may escape, leaving the iron in the inactive ferrous state and potentiating secondary, nonenzymatic, radical-mediated lipid peroxidation reactions. Indeed, previous experiments have demonstrated that radical-scavengers react with lipid allylic radicals present in LOX and LA incubations carried out at lower oxygen content[69]. In contrast to radicals generated during the LOX reaction, potentially damaging sources of radicals are the LOX products themselves. Incubations of LOX with either ω-6 or ω-3 PUFAs generated hydroxy-, alkoxy- and short chain carbon-centered radicals[70-74], suggesting that the fatty acid hydroperoxides are cleaved to form both a hydroxy radical and an alkoxy-radical containing fatty acid, which subsequently undergo β-scission to generate short chain carbon-centered radicals. Furthermore, the alkoxy-radical containing fatty acid may undergo intramolecular rearrangement, react with molecular oxygen, and generate superoxide as a product[70]. Superoxide is also generated through secondary reactions of LOX and/or LOX products with NADH or NADPH[75-77]. Thus, although LOX is important for generating biologically active lipid mediators, the inherent radical mechanism and the reactive hydroperoxy fatty acid products are sources of potentially damaging radicals.

B. Neuronal programmed cell death and α-tocopherol

Physiologically programmed cell death is a key function throughout embryonic development, regulating the formation and remodeling of complex multicellular tissues, but it must be closely regulated. The developmental processes regulated in part by apoptosis are numerous and will not be addressed here, but as examples, apoptosis mediates neural tube closure [78] and intra- and extraretinal synaptogenesis[79]. Under various conditions, PUFAs, LOX, and the antioxidant network have been implicated in the induction and control of apoptosis. Glutathione depletion has long-been recognized to induce neuronal cell death. Notably, glutathione depletion leads to an increase in 12-LOX activity which is a requisite step for neuronal apoptosis induced by glutathione depletion[64-66]. Furthermore, neuronal apoptosis following knockdown of GPx4 requires 12/15-LOX activity and induces apoptosis-inducing factor translocation to the mitochondrial nucleus[63]; notably, αTOC

entirely prevents 12/15-LOX mediated cell death[63]. In neuroblastoma cells, 15-LOX converts DHA to a series of hydroperoxy DHAs, including 4-HpDHA, 7-HpDHA, 14-HpDHA and 17-HpDHA[80]. Exposure to either DHA or 17-HpDHA, but not the monohydroxy DHA product 17-HDHA, potentiates apoptosis in neuroblastoma cells[80,81]. Notably, co-treatment of neuroblastoma cells with αTOC prevents DHA[81] or 17-HpDHA[80] -induced apoptosis. The production of hydroperoxy fatty acids clearly mediates neuronal cell death since DHA exposure reduces GPx4 protein levels and GPx4 knockdown (using siRNA) enhances DHA cytotoxicity[82]; importantly, αTOC treatment reverses enhanced DHA cytotoxicity following GPx4 knockdown[82]. Similarly, ARA depletes intracellular glutathione and induces apoptosis in cultured cortical neurons, which is attenuated by LOX inhibitors or the αTOC analog trolox[83], suggesting that ARA-derived 12-LOX products are responsible for apoptosis. Indeed, 12-HETE, the major ARA derived 12-LOX product, mimics ARA-induced apoptosis[83]. Given that neuronal tissue is highly enriched with DHA, it is notable that DHA hydroperoxides are more cytotoxic than LA- or ARA-derived hydroperoxides in neuroblastoma cells[84]. This evidence illustrates an important interaction between αTOC, ARA and DHA, LOX, and hydroperoxy fatty acids which mediates neuronal function; such an interaction may explain the requirement for αTOC during embryonic development.

C. Is fetal lethality caused by α-tocopherol deficiency mediated by 12/15-lipoxygeanse?

Clearly, αTOC plays an important role in the ARA/DHA-LOX-hydroperoxy fatty acid-cell death pathway in neuronal tissue *in vitro*. Less clear, however, is how or if this interaction impacts embryonic development. LOXs, as well as COXs and CYP450s, convert ARA to numerous lipid mediators that exert diverse roles during embryonic development. For example, knockdown of COX-1 in zebrafish embryos led to gastrulation arrest[85], while inhibition of COX-1 after completion of gastrulation caused defective vascular tube formation[86]. In mice, COX-2 regulates ovulation and embryonic implantation[87], however no effect of COX-2 knockdown was noted in zebrafish embryos[85,86]. In zebrafish embryos, inhibition of five lipoxygenase-activating protein (FLAP), a membrane protein required for 5-LOX function, resulted

in pericardial edema and reduced intersegmental vasculature and vessel/axial blood flow[88].

In order to prevent fetal resorption, vitamin E must be administered to the rodent mother on post-fertilization (pf) days 5 to 9[89,90]. Interestingly, this is the same critical period where the 12/15-LOX pathway appears to mediate implantation[91] and GPx4-knockout mice embryos are resorbed[92]. Zebrafish express 12/15-LOX within the developing notochord around 16-19 hours pf (hpf)[93], a time point corresponding to 8-10 days pf in mice. Neuronal growth-cone collapse also requires functional 12/15-LOX[94,95], as does hematopoietic stem cell function[96] and epidermal barrier formation[97]. Knockdown of 12/15-LOX in zebrafish embryos resulted in abnormal brain, eye, and tail development by 24 hpf and pericardial edema by 48 hpf[98]. Importantly, 5-LOX and COX inhibitors did not prevent neuronal cell death induced by ARA or DHA *in vitro*[63-65,83], only 12/15-LOX inhibitors and αTOC rescued cell death. Thus it appears that the mechanism by which αTOC interacts with LOX and ARA/DHA to protect neuronal cells is specific to 12/15-LOX and may be the mechanisms mediating the fetal lethality caused by αTOC deficiency.

IV. Hypothesis and Aims

We hypothesize that 12/15-LOX is required for normal embryonic development in zebrafish and that the production of hydroperoxy fatty acids is in careful balance with antioxidant networks. Therefore, αTOC deficiency will cause a depletion of ARA and DHA due to non-enzymatic lipid peroxidation, thereby limiting the availability of these crucial PUFAs for normal developmental processes. Moreover, inadequate αTOC concentrations will potentiate a secondary depletion of other antioxidants, specifically vitamin C and glutathione. Taken together, these factors will potentiate the activity of 12/15-LOX, thereby increasing the production of hydroperoxy fatty acids, further depleting ARA and DHA, and exacerbating the effects of αTOC deficiency. We therefore hypothesize that αTOC is required for proper embryonic development through three mechanisms:

1. αTOC prevents the non-enzymatic, radical-mediated lipid peroxidation of ARA and DHA, which are needed for proper embryonic development

2. αTOC modulates the cellular hydroperoxide tone, which in turn mediates the enzymatic production of ARA and DHA hydroperoxides

3. αTOC mediates the actions of hydroperoxy fatty acids derived from ARA and DHA, which in excess result in cell death

To test our hypotheses, we chose the embryonic zebrafish model, developed a novel method allowing for the simultaneous quantitation of vitamin E, ARA, and DHA in a single embryo, as well as a highly sensitive method for the detection of hydroperoxy and hydroxy PUFAs in zebrafish embryos.

Our aims are:

1. Develop and validate a novel method for the detection of PUFAs and αTOC in zebrafish embryos

2. Demonstrate the requirement of LOX enzymes for normal zebrafish embryonic development

3. Characterize changes in PUFAs caused by αTOC deficiency during the first 72 hours of zebrafish embryonic development

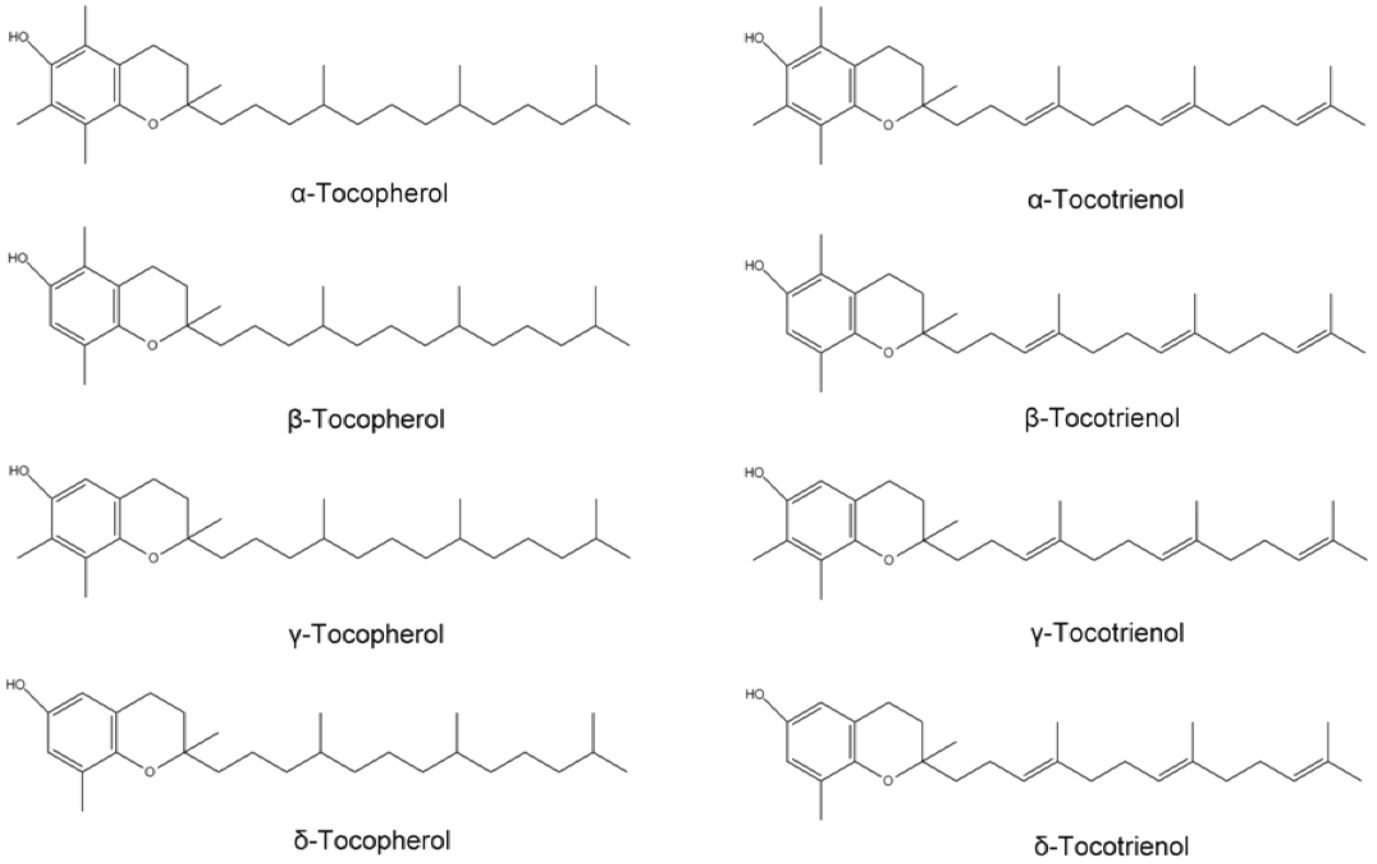

Figure 1. The eight naturally occurring isoforms of vitamin E. The tocopherols contain a saturated side-chain, whereas the tocotrienols contain an unsaturated side-chain. α-, β-, γ-, and δ-isoforms differ with regard to the number of methyl groups on the chromanol head. Not pictured are the eight stereoisomers of one isoform, ex. 2*R*4*R*8*R*-, *RSR*-, *RRS*-, *RSS*-, *SRR*-, *SSR*-, *SRS*-, or *SSS*-α-tocopherol.

Figure 2. Lipid perodixation and the antioxidant network. During the propagation stage of lipid peroxidation, a carbon-centered radical (R·) abstracts an allylic hydrogen from a neighboring, unsaturated fatty acid. Molecular oxygen reacts with the fatty acid radical, generating a peroxyl radical, which can be reduced by α-tocopherol, creating a hydroperoxy fatty acid and the α-tocopheryl radical. The hydroperoxy fatty acids are converted to hydroxy fatty acids via phospholipid hydroperoxide glutathione peroxidase (GPx4), using two glutathiones (GSH) as the reducing agents and creating oxidized gluthatione disulfide (GSSG). To replenish the α-tocopherol, the α-tocopheryl radical is reduced by ascorbate. The oxidized ascorbyl radical is subsequently reduced back to ascorbate via GSH.

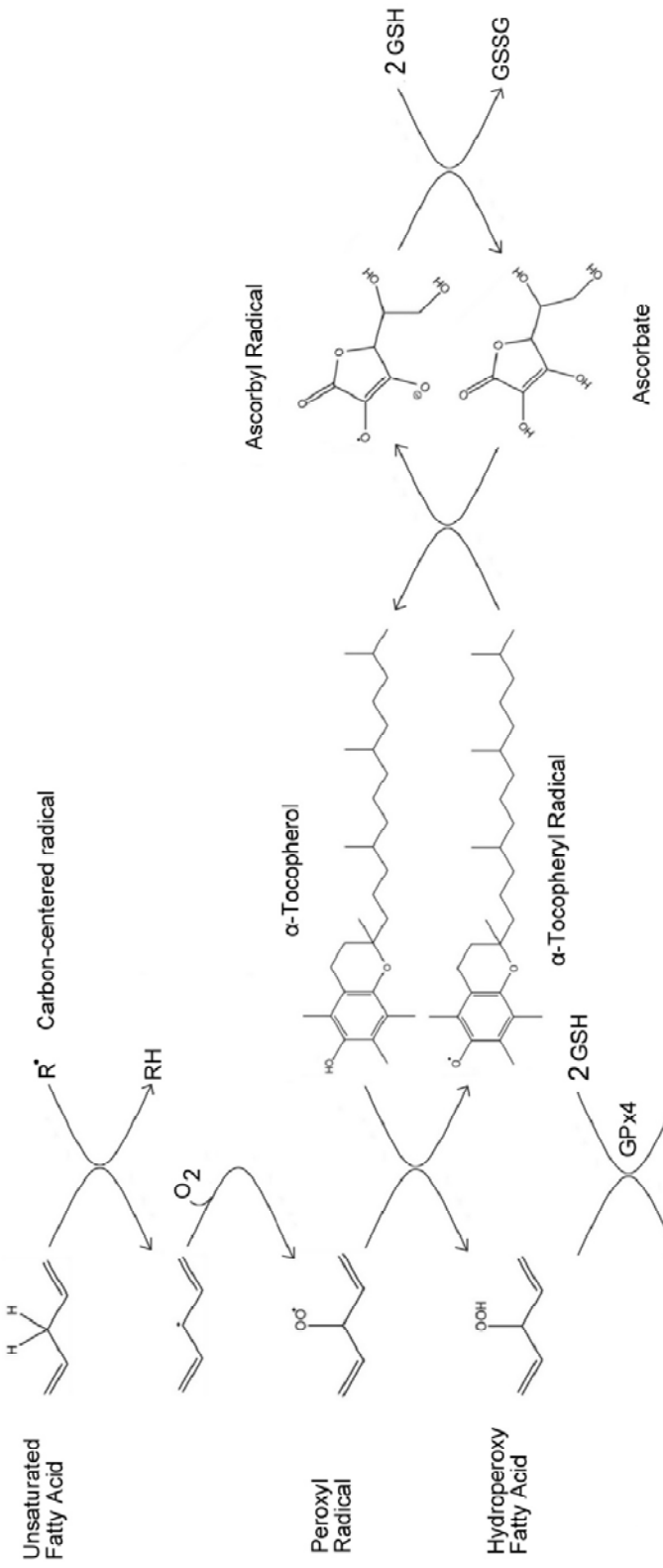

Figure 2. Lipid peroxidation and the antioxidant network.

Figure 3. Lipid mediators derived from arachidonic acid. Arachidonic acid (ARA) is cleaved from membrane phospholipids by phospholipase A2. The lipoxygenase (LOX) pathways convert ARA to 5-hydroperoxyeicosatetraenoic acid (5-HpETE) via 5-LOX, 12-hydroperoxyeicosatetraenoic acid (12-HpETE) via 12-LOX, or 15-hydroperoxyeicosatetraenoic acid (15-HpETE) via 15-LOX (not pictured). 12/15-LOX can generate 12- or 15-HpETE (not pictured). The HpETEs are reduced by a peroxidase to produce 5-HETE and 12-HETE. 5-LOX converts the peroxide of 5-HpETE to an epoxide, generating leukotriene A4 (LTA4). LTA4 is the precursor for leukotriene B4 (LTB4), lipoxins, and cysteinyl leukotrienes (CysLTs). The cyclooxygenase (COX) enzymes, which are either inducible (COX-2) or constitutively expressed (COX-1), convert ARA to prostaglandin G2 (PGG2), which is a precursor for other prostaglandins, prostacyclins, and thromboxanes. Non-enzymatic, radical-mediated peroxidation of ARA yields the isoprostane class of signaling molecules (8-F_2-isoprostane pictured). Finally, cytochrome P450s (CYP450) convert ARA to 20-HETE or the epoxyeicosatrienoic acids (-EETs; 8,9-EET pictured).

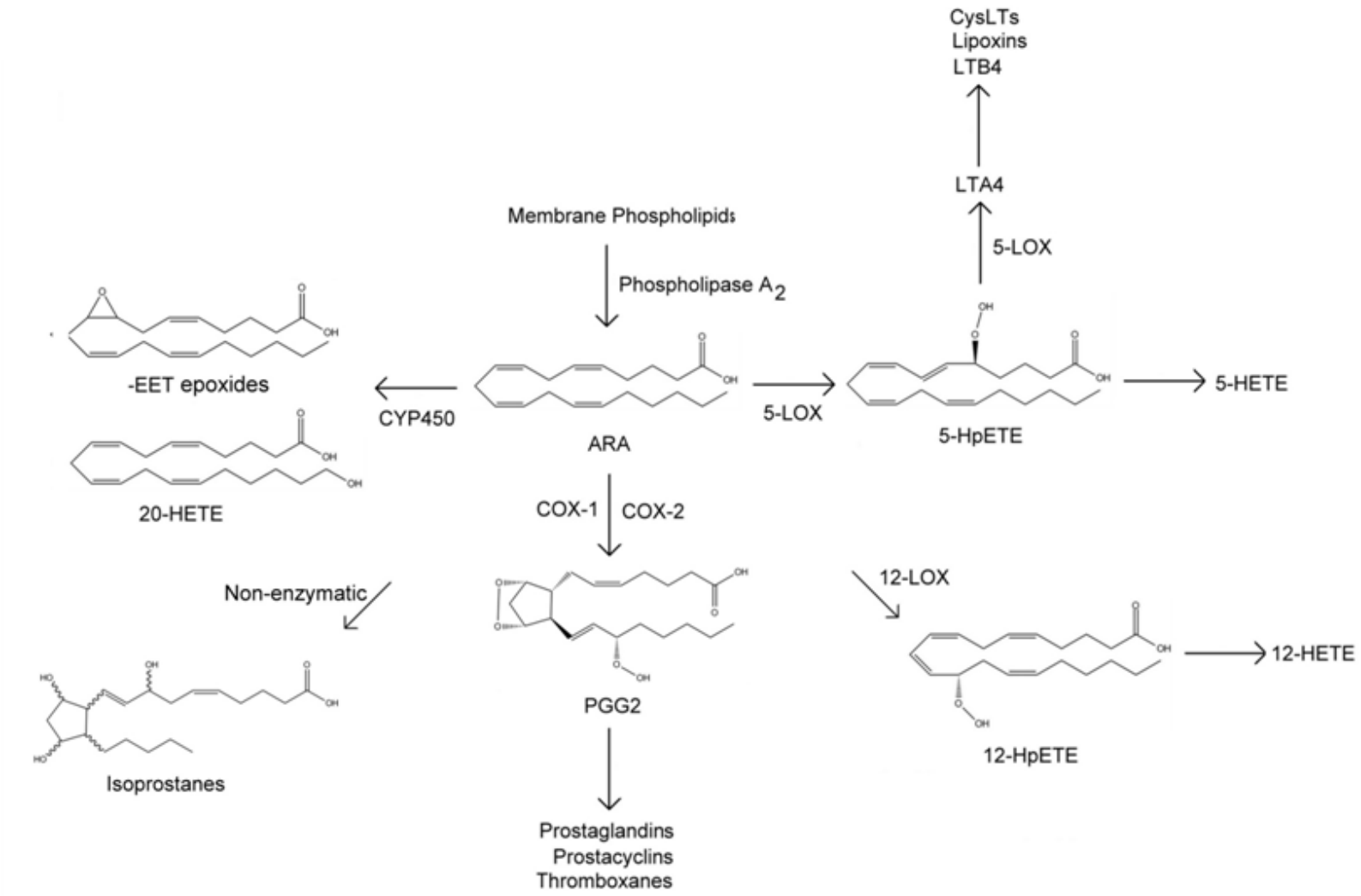

Figure 3. Lipid mediators derived from arachidonic acid.

Embryogenesis is dependent upon 12-lipoxygenase, 5-lipoxygenase, and α-tocopherol to modulate polyunsaturated fatty acid status and the production of oxidized fatty acids in zebrafish

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Chapter 2: Manuscript

I. Introduction

Arachidonic acid (ARA, 20:4 ω-6) and docosahexaenoic acid (DHA, 22:6 ω-3) are polyunsaturated fatty acids (PUFAs) required for proper embryonic development, specifically neurodevelopment. DHA is highly enriched in the central nervous system (CNS), comprising upwards of 50% of CNS PUFA content[9]. Rapid accretion of DHA within the CNS occurs during the last trimester of pregnancy in humans[10] and deficiency inhibits neurogenesis[5,6] and synaptogenesis[21]. DHA affects a wide-array of cellular processes by modulating phospholipid composition, membrane structure and function, eicosanoid synthesis, and gene expression[99]. ARA is the most abundant ω-6 neuronal fatty acid throughout gestation and postnatal development[10]. ARA is a component of cellular phospholipids and functions as a precursor for lipid secondary messengers involved in numerous cell signaling pathways. The oxidation of ARA gives rise to the biologically active eicosanoids and the oxidation of DHA gives rise to the docosanoids.

Lipoxygenases (LOXs) are a family of enzymes that catalyze the dioxygenation of PUFAs to the corresponding hydroperoxy fatty acids. There are four major isoforms of LOX: 5-LOX, platelet-type 12-LOX (12-LOX), eukocyte-type 12-LOX (12/15-LOX), and 15-LOX[58]. Characterization of LOX, hydroperoxy, and hydroxy fatty acid function during embryogenesis has been limited, but it appears that 12/15-LOX mediates implantation[91], neuronal growth-cone collapse[94,95], hematopoietic stem function[96], and epidermal barrier formation[97]. 12/15-LOX knockdown in zebrafish embryos resulted in abnormal brain, eye, and tail development by 24 hours post-fertilization (hpf)[98] and pericardial edema by 48 hpf. Less is known regarding the role of 5-LOX during development, but the inhibition of five lipoxygenase-activating protein (FLAP), a membrane protein required for 5-LOX function, resulted in pericardial edema and reduced intersegmental vasculature and vessel/axial blood flow in zebrafish embryos[88].

α-Tocopherol (αTOC) is a potent lipid soluble antioxidant which scavenges peroxyl radicals during the initiation and propagation of lipid peroxidation. αTOC is particularly enriched in neuronal tissue, where it is tenaciously retained during inadequate intake even after peripheral tissues become vitamin E depleted[50]. αTOC protects PUFAs, notably ARA and DHA, and is postulated to co-localize with PUFA-enriched phospholipid domains of the cell membrane[52], which are highly susceptible to peroxidation. Additionally, αTOC prevents neuronal cell death induced by enhanced 12/15-LOX activity[63]. In 1922, αTOC was discovered because rats fed rancid fat failed to carry their offspring to term[56]. Since its discovery, the specific biologic functions of vitamin E, and the mechanism mediating its requirement for successful reproduction, remain unknown. Given its role in protecting PUFAs from lipid peroxidation, it is possible that αTOC is required during embryonic development specifically to protect ARA and DHA, or mediate the production and actions of lipid mediators derived from these PUFAs.

We hypothesize that 12-LOX and 5-LOX are critical during embryonic development and that knockdown of either enzyme will perturb PUFA metabolism and the production of oxidized PUFAs. Furthermore, using selective knockdown of each LOX enzyme, and measuring the production of oxidized PUFAs, we hypothesize that we can determine the *in vivo* vertebrate synthetic pathway for oxidized products of DHA. Finally, we hypothesize that αTOC is required for embryogenesis to prevent unregulated peroxidation of DHA. To test these hypotheses, we developed a highly sensitive LC-MS method for the detection of PUFAs in developing zebrafish embryos. We also report the novel *in vivo* measurements of oxidized PUFAs produced endogenously by zebrafish embryos. Additionally, we demonstrate that αTOC modulates PUFA status throughout embryogenesis and that the essentiality of αTOC for proper reproduction may be independent of LOX function.

II. Methods

A. Fish Husbandry

Tropical 5D strain zebrafish 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 under standard laboratory conditions at 28.5°C with a 14 h light/10 h dark cycle. Embryos were obtained through natural group spawning, collected, and kept in standard fish water. Embryos used for analysis, described below, were euthanized by an overdose of tricaine.

B. Feeding Study

Beginning at 50 days post fertilization (dpf), zebrafish were fed either a defined diet (described below) or a conventional zebrafish diet (hereafter referred to as "Lab"), as previously described[1]. The lab diet is a mix of commercially available foods with undefined ingredients, thus it contains large amounts of fish oil and fish meal.

The defined diets were prepared in 300-g batches with added αTOC (E+, 500 mg RRR-α-tocopheryl acetate/kg diet, ADM, Decatur, IL) or without (E-) and stored at -20°C until fed to the zebrafish. The fatty acid compositions of the defined diets were described previously[1] and consisted primarily of palmitic, stearic, oleic, α-linolenic (ALA), and linoleic (LA) acid. The defined diets did not contain any polyunsaturated fatty acids (PUFA) with carbon chains longer than ALA or LA.

C. Knockdown Studies

Morpholinos (MO) (GeneTools LLC, Philomath, OR) were designed complimentary to the arachidonate 12-lipoxygenase (ALOX12; Ensembl ID: ENSDART00000101126) or the arachidonate 5-lipoxygenase (ALOX5; Ensembl ID: ENSDART00000079884) RNA sequence. ALOX12 MO sequence: ACACACTGATGTTCACTCACAGCTC; ALOX5 MO sequence: TGTGGTTAAAAGCTCCTTACCGCTC. Both MOs were designed to inhibit proper splicing of the mRNA transcript. MOs were dissolved in UltraPure DNase/RNase-free distilled water (Life Technologies, Grand Island, NY) and injected into 1-2 cell-stage lab embryos at a concentration of 1.0mM (ALOX12) or 0.5mM (ALOX5) in 3-5 nL injections. A standard zebrafish control MO (CON, 1.0mM or 0.5mM injection concentration, sequence: CCTCTTACCTCAGTTACAATTTATA) was used to control for the injection process. Phenol red (Sigma Aldrich, St. Louis,

MO) was added to verify injection location. A group of non-injected embryos (NON) were collected to control for spawn quality and embryo handling. At approximately 5 hours post fertilization (hpf), embryos were placed individually in 96 well plates. At 24 hpf, embryos were observed for malformations by stereomicroscopy and collected as described below for further analysis. To confirm knockdown, MO injected embryos (n=30) were collected at 24 hpf, RNA extracted as previously described [1], and RT-PCR performed using primers specifically designed to flank the MO-targeted exons and Platinum®Taq DNA polymerase (Life Technologies) per the manufacturer's directions. Primer sequences were as follows: ALOX12 F (AGCGAGAGGACGCTGCTGGA), ALOX12 R (CGCTCAGCTTTTTGGCTGTGCC), ALOX5 F (CACTGGCACTCAGTGGTTTG), ALOX5 R (CAGTACCAGTCGTCGTGCAT).

D. Lipoxygenase Inhibitor

To confirm MO-knockdown experiments, embryos were incubated with cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC, Enzo Life Sciences, Farmingdale, NY). Reportedly, CDC inhibits all isoforms of LOX (5-LOX, 12-LOX, 15-LOX, 12/15-LOX), but with differing selectivity; there are conflicting reports of which isoform is most potently inhibited[100,101]. CDC was dissolved in dimethyl sulfoxide (DMSO) and diluted in embryo medium (final DMSO concentration was held constant across dilutions at <1%). Lab embryos were de-chorionated[102] at 6 hpf, plated individually in 96-well plates, and exposed to varying CDC concentrations (NON, 0.01, 0.1, 1, 10, and 100 μM, n=48 embryos per concentration) and observed at 24 hpf to test for toxicity. For subsequent exposure studies, E- and E+ embryos (n=100 per diet) were de-chorionated, plated individually in 96-well plates in 100 μL of embryo medium containing CDC (10 μM), and observed for malformations at 24 hpf.

E. Sample preparation for α-tocopherol, fatty acid, and cholesterol analysis

At 24 hpf, MO injected embryos were collected for PUFA and αTOC analysis (n=10 embryos per sample) in 1.6 mL centrifuge tubes, excess water removed, and snap-frozen in liquid nitrogen. All samples were stored at -80°C until analyzed. Embryos

were added to 10 mL screw-top, round bottom, glass tubes with teflon-lined caps containing 2 mL 1% ascorbic acid (w/v) in ethanol and 1 mL Milli-Q water (Millipore, Billerica, MA). The following deuterium-labeled (d) internal standards were obtained from Cayman Chemical (Ann Harbor, MI), diluted in ethanol, and then added in amounts equivalent to endogenous levels of respective fatty acids: ALA-d_{14}, EPA-d_5, DHA-d_5, LA-d_4, and ARA-d_8. Delta-tocotrienol (δT3) was added as an internal standard for αTOC. Following addition of saturated KOH (300 µL), tubes were gently mixed, flushed with argon, and then placed in a water bath at 65°C for 30 min. After cooling to room temperature, the samples were adjusted to a pH of 7.5 ± 0.1 with 3 N HCl for solid phase extraction (described below).

F. Solid Phase Extraction

Strata-X-A 33u Polymeric Strong Anion Exchange cartridges (200 mg/3 mL, Phenomenex, Torrance, CA) loaded on a Zymark Rapid Trace SPE robot (Caliper Life Sciences, Hopkinton, MA) were conditioned with 3 mL methanol and then with 3 mL Milli-Q water. The samples were then loaded onto the prepared cartridges. The cartridges were washed with 3 mL 1:3 (v/v) methanol:water. Cholesterol was then eluted from the cartridges using 4 mL of methanol. After partial drying of the cartridges with three consecutive “washes” of 6 mL of air using the robots, αTOC was eluted from the cartridge with 4 mL acetonitrile. Finally, PUFAs were eluted from the cartridge with 4 mL of 5% formic acid in methanol-acetonitrile (5:47.5:47.5 v/v/v). Samples were dried under nitrogen. The cholesterol-containing extract was resuspended in 200 µL 1X Amplex Red Cholesterol Assay reaction buffer (Life Technologies, Carlsbad, CA) and measured as described below. The αTOC-containing extract was resuspended in 50:50 (v/v) methanol-ethanol and analyzed as described below. The PUFA-containing extract was resuspended in LC-MS grade methanol containing 0.01% (v/v) formic acid and analyzed as described below.

G. Cholesterol

Cholesterol (CHOL) was analyzed using the Amplex Red Cholesterol Assay Kit (Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions.

Specifically, cholesterol concentrations were determined fluorometrically using a SpectraMax Gemini XS microplate spectrofluorometer (Molecular Devices, Silicon Valley, CA) with an excitation wavelength of 545 nm and an emission wavelength of 590 nm and quantitated using authentic CHOL standards.

H. α-Tocopherol via HPLC-ECD

αTOC and δT3 were analyzed by high performance liquid chromatography (HPLC) with electrochemical detection based on a previously described method [103]. Briefly, the HPLC system consisted of a SCL-10A VP Shimadzu (Columbia, MD) system controller, two LC-10AD VP Shimadzu pumps, a SIL-10AD VP Shimadzu autoinjector with sample cooler, a Brownlee Spheri-5 RP-18 precolumn (5 micron 30 x 4.6 mm), an UltraSphere ODS (5 micron x 4.6mm x 25cm) column (Beckman, Brea, CA), and a LC-4C amperometric electrochemical detector (Bioanalytical Systems, West Lafayette, IN). The mobile phase consisted of 0.1% (w/v) lithium perchlorate and 98:2 (v/v) methanol-water and was run isocratically at 0.6 mL/min/pump for 15 min. αTOC concentrations were calculated from the peak area ratios of αTOC to δT3 using authentic compounds.

I. Fatty Acids via LC-MS

High pressure liquid chromatography was performed on a Shimadzu system (DGU-20A3 prominence degasser, two LC-20AD prominence pumps, CMB-20A prominence command bus module, and SIL-20AC HT prominence autosampler) coupled through an electrospray ionization source to a single quadrupole LC-MS 2010A mass spectrometer (Shimadzu, Columbia, MD) operated in negative single ion monitoring mode (see **TABLE 1** for analyte *m/z*). The acquisition time was set at 0.5 sec, the detector voltage at 1.5 kV, the CDL temperature at 230°C, the block temperature at 200°C, and the nitrogen nebulizing gas at 2.5 mL/min. Chromatographic separations were carried out on an Ascentis Express C8 column (15cm x 2.1mm x 2.7µm, Supelco, Bellefonte, PA) with matching guard column. Mobile phases consisted of Milli-Q water with 0.05% (v/v) acetic acid (A) and methanol (B) with a flow rate set at 0.2 mL/min. Elution gradient was as follows: 0 min, 80% B; 12 min, 85% B; 15 min,

85% B; 18 min, 100% B; 22 min, 100% B; 22 min, 80% B. The injection volume was 1 µL. Analyte concentrations were calculated from the peak area ratios of authentic compounds (obtained from Cayman Chemicals) to internal standards.

J. Precision experiments

Lab embryos (n=300) were collected at 5 hpf, homogenized in 3 mL 1% ascorbic acid (w/v) in ethanol, and stored at -80°C until analyzed. Six replicate aliquots (100 µL) were extracted and analyzed for PUFAs and αTOC (described above) on three different days. Within-run, between-day, and total imprecision were calculated according to the method of Krouwer and Rabinowitz[104,105].

K. Oxidized lipid extraction and UPLC-MS/MS

At 24 (n=200) or 36 hpf (n=100), embryos were collected in 1.6 mL tubes, water removed, and snap-frozen in liquid nitrogen. All samples were stored at -80°C until analysis. Embryos were transferred to 10 mL screw-top, round bottom, glass tubes with teflon-lined caps, homogenized in 3 mL ice-cold 66% LC-MS grade methanol containing internal standard (20-HETE d6, Cayman Chemical), and placed at -80°C for 1 hour. The embryo homogenate was then transferred to 1.6 mL polypropylene tubes and centrifuged at 3000 x g at 4°C for 15 minutes. The supernatant was collected and dried under nitrogen. Samples were resuspended in 100 µL LC-MS methanol with 0.1% formic acid (v/v) and transferred to injection vials. Ultra high-pressure liquid chromatography was performed on a Shimadzu Nexera system (Shimadzu, Columbia, MD) coupled to a hybrid quadruple-time of flight mass spectrometer (TripleTOF™ 5600, AB SCIEX). Chromatographic separations were carried out on a Brownlee Analytical DB AQ C18 column (100 x 2.1 mm, 1.9 µm, PerkinElmer). The flow rate was 0.35 ml/min and mobile phases consisted of water (A) and acetonitrile (B), both with 0.1% formic acid. The elution gradient was as follows: 0 min, 35% B; 2.5 min, 50% B; 7 min, 64.4% B; 9 min, 100% B; 12 min, 100% B; 12.5 min, 35% B; and 13.5 min, 35% B. Column temperature was held at 40 °C and the injection volume was 4 µL. Mass spectrometry was performed using an electrospray ionization source. The instrument was operated in high resolution

product ion mode and negative ion polarity. Product ion accumulation time was 0.12 s for each parent ion and collision energies were between -15 and -25 V. Scan range for each product ion experiment was 40-450 m/z. Ion source gas 1 and 2 and curtain gas (all nitrogen) were set at 50, 40, and 25, respectively. The source temperature was set at 500 °C and IonSpray voltage at 5.5/-5.5 kV. Two-minute auto calibrations were performed hourly.

L. Statistics

Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). For morpholino knockdown experiments, a one-way ANOVA with Bonferonni post-hoc test was used to analyze PUFA, αTOC, CHOL, and oxidized PUFA changes. A two-way ANOVA (morpholino x embryo status [normal, moribund, or dead]) was used to assess phenotype incidence following morpholino injections. Time-course E- and E+ PUFAs were analyzed using a repeated measures two-way ANOVA (diet x time). Time-course PUFAs in lab embryos were analyzed separately using an one-way repeated measures ANOVA. E- and E+ embryo oxidized PUFA data were analyzed using an unpaired t-test. Data were log-transformed when unequal variances were observed between groups, as confirmed by Bartlett's test for equal variances.

III. Results

A. Analysis of PUFAs via LC-MS

The optimized gradient conditions and chosen column for LC-MS allowed for the resolution of positional isomers with identical *m/z*, as confirmed using authentic compounds. For quantitation of specific PUFAs of interest, optimized separation was necessary to exclude gamma-linolenic acid (18:3 ω-6) from ALA (18:3 ω-3). Total run time equaled 30 minutes, including 8 minutes required for column equilibration. Typical retention times were as follows: LA 10.9 min, ARA 10.8 min, ALA 8.8 min, EPA 8.7 min, DHA 10.5 min. Calibration curves relating the amount of analyte injected on column to the area ratio of each PUFA to its respective deuterated analog

showed linear responses in the following ranges: LA 1-125 ng, ARA 1-160 ng, ALA 0.5-25 ng, EPA 0.4-50 ng, and DHA 0.5-200 ng. The lower limit of quantitation for each PUFA was approximately 0.2 ng injected on column. The lower limit of detection for each PUFA was approximately 0.1 ng injected on column.

To test precision, six aliquots of an embryo homogenate were analyzed in three separate batches over a 2 week time period. The within-day and between-day variance did not exceed 5% for any of the PUFAs or αTOC and are listed in **TABLE 2**. Using the solid phase extraction method, analyte recovery exceeded 70% for each PUFA, but was substantially less for αTOC (**TABLE 2**). At this time, we could not assess CHOL recovery since we lack labeled CHOL and a validated method for labeled CHOL measurements. As such, the high variance noted in CHOL measurements may be associated with variable or incomplete recoveries.

B. ALOX12 is required for normal embryonic development

The ALOX12 MO was designed to inhibit proper splicing of the ALOX12 mRNA transcript by binding to the exon-3/intron-4 junction (**FIG 4A**). Partial knockdown of the endogenous ALOX12 transcript was achieved with MO injection, as confirmed by gel electrophoresis of PCR products (**FIG 4B**). MO injections increased the incidence of mortality in embryos at 24 hpf from NON (0.7 ± 1.3%, $p<0.05$, **FIG 4C**). The incidence of mortality did not differ between CON (11.3 ± 2.6%) and ALOX12 (11.9 ± 2.3%) MO injected embryos. Knockdown of ALOX12 resulted in a significant increase in embryos with developmental defects (69.3 ± 1.7%, $p<0.05$) as compared with CON (21.1 ± 6.2%) or NON (0.6 ± 0.6%). Embryos injected with ALOX12 MO showed consistent malformations, characterized by bending and/or spiraling of the notochord (**FIG 4D** and **4E**). CON (**FIG 4F**) and NON (**FIG 4G** and **4H**) embryos appeared normal.

C. ALOX12 knockdown alters PUFA concentrations

Knockdown of ALOX12 changed PUFA concentrations in embryos at 24 hpf (**TABLE 3**). ARA concentrations were 10% greater ($p<0.05$) in ALOX12 embryos compared

with CON embryos. LA concentrations decreased by 12%, ALA decreased by 21%, and EPA decreased by 9% in ALOX12 embryos compared with CON ($p<0.05$ for each PUFA compared with CON). ALOX12 knockdown did not alter DHA concentrations, nor did it alter αTOC concentrations when compared with CON embryos. Unexpectedly, ALOX12 knockdown increased CHOL concentrations 24% ($p<0.05$) compared with CON embryos. Importantly, PUFAs, αTOC, or CHOL did not differ between CON and NON embryos.

D. ALOX12 knockdown decreases 12-HETE and docosanoid concentrations

Out of 24 oxidized PUFAs (**TABLE 1**), only 5-HETE, 12-HETE, 7-HDHA, 10-HDHA, 14-HDHA, and 17-HDHA were detectable in CON and NON zebrafish embryos at 24 hpf. Following ALOX12 knockdown, the following oxidized PUFAs were undetectable: 12-HETE, 10-HDHA, and 14-HDHA. Therefore, the values reported for ALOX12 embryos are the respective limits of detection for those 3 oxidized PUFAs (**TABLE 4**). Notably, ALOX12 knockdown significantly decreased 12-HETE, 14-HDHA, and 17-HDHA compared with CON embryos. ALOX12 knockdown did not change 5-HETE, 7-HDHA, or 17-HDHA concentrations.

E. ALOX5 is required for normal embryonic development

The ALOX5 MO was designed to inhibit proper splicing of the ALOX5 mRNA transcript by binding to the exon-1/intron-1 junction (**FIG 5A**). Partial knockdown of the endogenous ALOX5 transcript was achieved with MO injection, as confirmed by gel electrophoresis of ALOX5 PCR products (**FIG 5B**). ALOX5 knockdown (34.1 ± 7.3%, $p<0.05$, **FIG 5C**) increased the incidence of development defects in embryos compared with CON (3.8 ± 4.3%) and NON (1.2 ± 1.0%) embryos. Mortality also increased following injection with ALOX5 MO (17.3 ± 13.6%, $p<0.05$) compared with CON (0.5 ± 0.8%) and NON (0.0 ± 0.0%). The incidences of mortality and malformations were not significantly different between CON and NON embryos. The severity and type of developmental defects noted in ALOX5 embryos varied, but were consistently present across injection days. Corkscrew tails, mild pericardial edema,

and shortened heads predominated in malformed ALOX5 embryos at 24 hpf (**FIG 5D**), however, more severe defects were noted in a subset of embryos (**FIG 5E**).

F. ALOX5 knockdown alters PUFA concentrations

In distinct contrast to ALOX12 embryos, ALOX5 knockdown only affected ARA and EPA concentrations in embryos at 24 hpf (**TABLE 4**). ALOX5 knockdown increased ARA concentrations by 12% and EPA concentrations by 15% compared with CON embryos ($p<0.05$ for each fatty acid compared to CON). Knockdown of ALOX5 did not alter LA, ALA, DHA, αTOC, or CHOL levels compared with CON.

G. ALOX5 knockdown decreases 5-HETE and docosanoid concentrations

Only 5-HETE, 12-HETE, 7-HDHA, 10-HDHA, 14-HDHA, and 17-HDHA were detectable in ALOX5 embryos at 24 hpf. While there was a significant main effect of MO injection on specific oxidized PUFAs, none of the paired comparisons between ALOX5 and CON embryos reached statistical significance. Notably, ALOX5 knockdown tended to decrease 5-HETE, 7-HDHA, and 17-HDHA compared with CON embryos (**TABLE 6**).

H. α-Tocopherol deficiency alters PUFA concentrations and utilization during embryonic development

Embryos spawned from adult zebrafish fed the E- diet had 40-fold less αTOC compared with embryos obtained from adult zebrafish fed the E+ diet (**TABLE 7**). αTOC altered the absolute amounts of each PUFA present in embryos, as well as the magnitude of change in PUFAs during development (**TABLE 7**). E- embryos on average contained 0.4 ng more LA and 0.7 ng more ARA compared with E+ embryos at 3 hpf. During the first 72 hours of development, E- embryos lost 1.1 ng of LA and 0.8 ng of ARA, whereas amounts in E+ embryos decreased by only 0.8 ng and 0.4 ng, respectively. Despite a greater rate of decrease in ω-6 PUFAs during development, E- embryos still had higher absolute quantities of ω-6 PUFAs at 72 hpf compared with E+ embryos. No difference in ALA levels at 3 hpf or throughout development were observed between E- and E+ embryos. However, stark

differences were noted in EPA and DHA levels between E- and E+ embryos. E- embryos contained only half the amount of EPA as E+ embryos throughout the first 72 hours of development. While EPA levels in E+ embryos declined at a greater rate (0.3 ng during 72 hours) than E- embryos (0.1 ng during 72 hours), EPA concentrations in E- embryos remained half of those in E+ embryos at 72 hpf. Interestingly, at 3 hpf, E- embryos contain 0.8 ng more DHA than E+ embryos. However, by 72 hpf, E- embryos lost 1.2 ng of DHA while amounts in E+ embryos remained constant. We previously reported that αTOC deficiency in adult zebrafish decreased the ω-3 to ω-6 PUFA ratio [1]. Similarly, during the first 72 hours of development, the ratio of DHA to ARA remained low in E- embryos compared with E+ embryos. This reflects the declining DHA concentrations and significantly greater ARA concentrations in E- embryos as compared with E+ embryos. Finally, CHOL did not differ between E- and E+ embryos.

I. α-Tocopherol deficiency increases HETE and docosanoid concentrations

We have previously demonstrated that E- embryos appear normal at 36 hpf, but by 48 hpf, fifty percent of E- embryos display developmental defects (Miller et al, unpublished data). Thus, we measured oxidized PUFAs at 36 hpf to discern if altered PUFA status precedes observable malformations in E- embryos. Using authentic standards, we searched for 24 specific oxidized PUFAs (**TABLE 1**). Of the 24 analytes, only 5-HETE, 12-HETE, 7-HDHA, 10-HDHA, and 14-HDHA were detectable in E- and E+ embryos. E- embryos had significantly increased concentrations of 5-HETE, 7-HDHA, 10-HDHA, 17-HDHA, and a trending increase in 12-HETE compared with E+ embryos (**TABLE 8**). 14-HDHA was not changed in E- embryos compared with E+ embryos.

J. The LOX inhibitor recapitulates ALOX12 embryo malformations

To confirm the changes in PUFAs and oxidized PUFAs that occurred with MO knockdown, de-chorionated embryos were incubated with a LOX inhibitor (CDC). Embryos exposed to 10 μM CDC developed similar malformations by 24 hpf as ALOX12 embryos (**FIG 6B** and **6C**). Embryos incubated in embryo medium with

equivalent DMSO concentrations displayed no adverse developmental defects (**FIG 6D** and **6E**). Using less than 10 μM of CDC produced little to no malformations in embryos by 24 hpf (**FIG 6A**), whereas incubation of embryos with 100 μM CDC was 100% fatal.

K. Inhibition of LOX does not rescue α-tocopherol deficient embryos

Given that αTOC deficiency increased the production of 5-HETE and 12-HETE, we hypothesized that inhibition of LOX may rescue E- embryos. However, exposing de-chorionated E- embryos to 10 μM CDC increased malformations by 35% and mortality by 39% by 24 hpf (**FIG 7**). Incubation of E+ embryos with CDC also increased malformations and mortality by at least 30%.

IV. Discussion

A. ALOX12 and ALOX5 mediate PUFAs during embryogenesis

By 24 hpf, knockdown of ALOX12 or ALOX5 in zebrafish embryos led to distinct developmental defects (**FIG 4** and **FIG 5**) and altered concentrations of PUFAs (**TABLE 3** and **TABLE 5**) and oxidized PUFAs (**TABLE 4** and **6**). Our data demonstrate the essentiality of both LOXs and the production of oxidized PUFAs for embryonic development.

Expectedly, knockdown of ALOX12 reduced 12-HETE concentrations in embryos at 24 hpf. Interestingly, ALOX12 knockdown also reduced the concentrations of 10-HDHA and 14-HDHA. Previous *in vitro* work demonstrated that thrombin-activated human platelet 12-LOX produces 10-HDHA and 14-HDHA[106]. Additionally, 14-HDHA has been detected in incubations of porcine 12-LOX with DHA[107], in neuroblastoma cells cultured with exogenous DHA[80], and in murine epithelial wounds[107]. Reportedly, DHA is converted to maresin-1 via 12-LOX or 12/15-LOX through a 14-HpDHA intermediate[57]. To our knowledge, all previous data demonstrating the conversion of DHA to 10-HDHA or 14-HDHA has been *in vitro*, using soybean- or potato-purified LOXs, exogenously supplied DHA, or required the activation of cells using calcium ionophores, thrombin, or epithelia wounding. Our data is the first to demonstrate that

a vertebrate organism undergoing embryogenesis converts DHA to 10-HDHA and 14-HDHA via ALOX12 under normal physiological conditions. We cannot at this point discount the conversion of 10-HDHA and 14-HDHA to other metabolites, however reduced conversion of DHA to these HDHAs following ALOX12 knockdown appears the most likely mechanism to explain the decreased concentrations. Notably, ALOX12 knockdown did not affect 17-HDHA concentrations. DHA is reportedly converted to 17-HpDHA via 15-LOX, 12/15-LOX, or COX-2[57]. Neuroblastoma cells expressing 15-LOX generate 17-HpDHA when supplied with exogenous DHA, which can be prevented using a pan-LOX inhibitor[80]. 17-HpDHA is also the precursor for neuroprotectin D1 (NPD1/PD1; requires the conversion of the hydroperoxide to an epoxide by 15-LOX or 12/15-LOX) and the resolvin D-series (requires an epoxidation of 17-HpDHA at position 7,8 or 4,5 by 5-LOX)[57]. Zebrafish ALOX12 is classified as a 12/15-LOX, however the predominant HETE formed by ALOX12 is 12-HETE[4]. Therefore, zebrafish ALOX12 demonstrates little to no 15-LOX activity towards DHA and thus would not be expected to affect the production of 17-HDHA.

ALOX12 knockdown also altered PUFA concentrations in zebrafish embryos at 24 hpf. This is the first evidence to demonstrate that ALOX12 activity significantly impacts PUFA concentrations during embryogenesis. The noted increase in ARA following ALOX12 knockdown is presumably due to decreased synthesis of 12-HETE, as confirmed by a decrease in 12-HETE concentrations, as well as increased conversion of LA to ARA in an effort to saturate conversion of ARA to 12-HETE. Both mechanisms are likely since the magnitude of change in ARA (increase of 70 pg) is not matched by the magnitude of change in 12-HETE (decrease of approximately 10 pg). Less clear, however, is why ALOX12 knockdown decreased ALA and EPA. Increased PUFA synthesis would explain the noted decrease in ALA and EPA concentrations. Although DHA levels did not increase following ALOX12 knockdown, we did not measure intermediate PUFAs in the ω-3 PUFA synthesis pathway, and thus cannot exclude the possibility that ALA and EPA are converted to other PUFAs, such as docosapentaenoic acid (22:5 ω-3), or undergo β-oxidation. It is unlikely that the changes in ARA concentrations are solely responsible for altered LA, ALA, and EPA concentrations, since ALOX5 knockdown also increased ARA concentrations,

but did not affect LA and ALA and actually increased EPA concentrations. An alternate possibility is that the decrease in LA, ALA, and EPA observed following ALOX12 knockdown resulted from the decreased production of oxidized ARA and DHA. This finding suggests that 12-HETE, 10-HDHA, or 14-HDHA participate in the regulation of lipid metabolism. This hypothesis is supported by data concerning the interaction of oxidized fatty acids with peroxisome proliferator-activated receptors (PPARs). PPARs are transcriptional regulators with diverse roles in lipid biology. PPARγ is the master regulator of adipocyte differentiation and controls a number of genes involved in lipid metabolism[108], whereas PPARα is a major regulator of fatty acid oxidation (mitochondrial, peroxisomal and microsomal)[109]. Previous studies have demonstrated that LTA4, a 5-LOX product, is a potent PPARα agonist[110], 4-HDHA[111], 7-HDHA[112] and 17-HDHA[112] are PPARγ agonists, and the promotion of neuronal survival by NPD1/PD1 is mediated by PPARγ[113]. Additionally, PPAR gamma coactivator 1-alpha and 1-beta (PPARGC1a and PPARGC1b) are co-activators of the PPARs and regulate lipid homeostasis[114]. 7-HDHA, 10-HDHA, and 17-HDHA are increased in αTOC deficient embryos at 36 hpf (**TABLE 8**), a phenomenon that occurs concomitantly with increased mRNA expression of PPARGC1a and PPARGC1b (Miller et al, unpublished data). Further research is necessary to establish whether oxidized PUFAs are physiologically relevant PPAR agonists *in vivo* and if this is an additional mechanism by which ALOX12 can regulate PUFA homeostasis.

An unexpected finding was the increase in CHOL following ALOX knockdown. Previously, it has been reported that LOX impairs cholesterol efflux from macrophages[115,116]. However, we measured CHOL concentrations in the entire embryo, therefore, efflux or movement of CHOL would not impact the CHOL concentrations. As discussed above, oxidized PUFAs may be PPAR agonists; given that PPARs mediate cholesterol synthesis[117], this may be the mechanism by which ALOX12 affected cholesterol concentrations. Finally, the malformations noted in ALOX12 knockdown embryos were similar to those reported previously[98]. Haas et al[98] characterized ALOX12 activity and reported the phenotypic changes caused by ALOX12 knockdown, however, they were unable to measure endogenous levels of

12-HETE. After supplying an embryo homogenate with exogenous ARA, the authors demonstrated diminished 12-HETE production following ALOX12 knockdown[98]. Our study extends the work of Haas et al in that we measured endogenous concentrations of 12-HETE without supplying exogenous ARA *ex vivo*. Additionally, we measured previously unreported changes in docosanoids following selective MO knockdown of ALOX12.

The role of ALOX5 during zebrafish embryogenesisis is unknown. Expectedly, knockdown of ALOX5 tended to decrease 5-HETE. ALOX5 knockdown also decreased 7-HDHA and 17-HDHA. Reportedly, 17-HpDHA requires an epoxidation at position 7,8 by 5-LOX to generate RvD1 or RvD2[57]. Thus, it seems reasonable that DHA can serve as substrate for ALOX5 to generate 7-HDHA. Additionally, ALOX5 gene variants are associated with 17-HDHA production by human monocytes when stimulated with a calcium ionophore[118]. To our knowledge, this is the first direct evidence that the vertebrate ALOX5 converts DHA to 7-HDHA and 17-HDHA during embryogenesis under normal physiological conditions. ALOX5 knockdown also altered PUFA concentrations in zebrafish embryos at 24 hpf. This is the first evidence to demonstrate that ALOX5 activity significantly impacts PUFA concentrations during embryogenesis. The noted increase in ARA following ALOX5 knockdown is presumably due to decreased synthesis of 5-HETE, but similarly to ALOX12 knockdown, the magnitude of change in ARA does not parallel the magnitude of change in 5-HETE. It is interesting to note that EPA concentrations increased following ALOX5 knockdown, whereas they decreased following ALOX12 knockdown. Reportedly, EPA is a poor substrate for LOX reactions[99]. However, the proposed synthesis of the resolvin E-series (RvE1 and RvE2) involves an oxidation of EPA by 5-LOX[57]. Our data supports the idea that EPA is a substrate for ALOX5 during embryogenesis.

B. α-Tocopherol mediates PUFAs during embryogenesis

αTOC deficiency altered the amount and rate of decline of PUFAs, as well as the production of oxidized PUFA, during embryonic development (**TABLE 7** and **8**). In both E- and E+ embryos, the concentration of PUFAs declined as development

progressed. This decrease likely reflects PUFAs being converted to other metabolites or undergoing β-oxidation. Monroig et al[6] previously reported that LA and ALA decline during zebrafish development, whereas potential products of LA and ALA (sum of all other ω-6 and ω-3 PUFAs, respectively) increase during embryogenesis relative to total lipid content. αTOC deficiency increased the rate of decline in PUFAs, which could be a measure of PUFA utilization for metabolic processes or loss of PUFAs due to lipid peroxidation.

Notably, DHA levels declined in E- embryos and did not change in E+ embryos. Concurrently, HDHAs were elevated in E- embryos compared with E+ embryos. Radical-mediated lipid peroxidation could produce the HDHAs measured in E- embryos at 36 hpf. We did not use a chiral column to measure oxidized PUFAs, and thus cannot determine the stereochemistry of each HDHA and confirm whether enzymatic or non-enzymatic oxidation occurred. Not all HDHAs increased coordinately with αTOC deficiency, which is suggestive of a regulatory mechanism controlling the peroxidation of DHA. LOX activity is mediated by cellular hydroperoxide tone[58]. With αTOC deficiency, it is reasonable to assume that the cellular hydroperoxide tone increases, and consequently, that LOX activity would increase and account for the noted increase in HDHAs. Similarly, elevated 17-HDHA concentrations may reflect an up-regulation of the NPD1/PD1 synthesis pathway in an attempt to protect developing neuronal tissue from apoptosis induced by αTOC deficiency. However, LOX knockdown did not affect DHA levels and we were unable to detect any hydroperoxy or di- or trihydroxy DHA metabolites in zebrafish embryos. Regardless of the mechanism, this is the first *in vivo* evidence to demonstrate that αTOC deficiency specifically increases DHA peroxidation and increases the concentrations of specific, key lipid mediators.

Previously, we reported that the total percentage of ω-6 or ω-3 PUFAs did not differ between E- and E+ embryos collected at 0 hpf[1]. However, previous methods used by our laboratory precluded the absolute quantitation of specific PUFAs. It remains a matter of debate whether fatty acids should be expressed as ratios, percentages, or absolute amounts and how predictive each measure is of the health benefits or pathologies ascribed to PUFAs. Likely, the testable hypothesis and measurable

outcomes of a study will determine the most appropriate way to report PUFAs. For example, the ratio of ω-6 to ω-3 PUFAs influences the production of eicosanoids to a greater degree than the absolute amounts of PUFA[119]. Conversely, the conversion of LA and ALA to other PUFAs is mediated by the absolute quantity of each PUFA [120]. Given that we are measuring changes in specific PUFAs with respect to a dynamic process, ie embryonic development, it seems more reasonable to express PUFAs in absolute amounts. As a case in point, the DHA/ARA ratio was not only significantly less in E- compared with E+ embryos at 3 hpf, but during development DHA/ARA increased in E+ embryos and further decreased in E- embryos. Simply providing a DHA/ARA ratio would have masked that the observed differences in the ratio can largely be attributed to declining levels of DHA in E- embryos and constant DHA concentrations in E+ embryos.

C. α-Tocopherol and lipoxygenase

The parallel decrease in PUFAs and increase in oxidized PUFAs may contribute to the improper development of E- embryos. Thus we hypothesized that the inhibition of LOX activity may rescue E- embryos. However, inhibition of LOX activity using CDC increased mortality and malformations in E- embryos by 24 hpf. At this time, we do not have measures of PUFAs or oxidized PUFAs following CDC inhibition of LOX, and thus cannot make conclusions regarding PUFA status in these embryos. It is clear, however, that LOX activity is required for embryonic development even during αTOC deficiency.

D. Method development

Based on our previous measurements of PUFAs in zebrafish embryos[1], the LC-MS technique reported herein allowed us to reduce the number of embryos from 100 to 10 embryos per sample. The method is sufficiently sensitive to measure PUFAs in one embryo, but variances were reduced by increasing the number of embryos to 10 per sample (data not shown). The very small changes of PUFAs detected following either LOX knockdown or induced by αTOC deficiency speak to the sensitivity and precision of this new method. Additionally, the novel solid phase extraction method permitted measurements of PUFA, αTOC, and CHOL in the same embryo, whereas

previous methods used by our laboratory excluded the simultaneous measurement of these molecules. Every method has strengths and limitations; a significant strength of our method is the capacity to conduct absolute quantitation and recovery for each individual PUFA. However, by selectively analyzing specific PUFAs, we cannot make conclusions regarding global fatty acid and lipid metabolism throughout development.

E. Summary

In summary, we demonstrated that LOX activity and αTOC modulate PUFA and oxidized PUFA homeostasis during vertebrate embryogenesis using novel methodologies. Additionally, we have reported the highly novel findings that ALOX12 modulates 10- and 14-HDHA concentrations during embryogenesis, while ALOX5 modulates 7- and 17-HDHA concentrations during embryogenesis. Finally, we have the first evidence that αTOC deficiency increases the peroxidation of specific PUFAs and produces oxidized lipid mediators with biologic functions.

Figure 4. Knockdown of ALOX12 causes notochord malformations in embryos by 24 hpf. **A.** ALOX12 MO target. The ALOX12 MO binds to the exon-3/intron-3 junction, preventing proper splicing of the mRNA transcript. Numbered boxes represent exons, spanning lines are introns, and the red line indicates the ALOX12 MO. **B.** RT-PCR indicates partial knockdown of endogenous ALOX12 following MO injection. **C.** ALOX12 knockdown increases the incidence of malformations in embryos at 24 hpf. Data shown as mean percent incidence from three separate experiments (total number of embryos: NON [non-injected], n=294; CON, [injected with non-specific MO] n=448; ALOX12 [injected with ALOX12 MO], n=881). **D.** Representative picture of notochord malformation at 24 hpf due to ALOX12 knockdown. Asterisk denotes location of a bend and spiral in the notochord. **E.** Enlarged image of notochord malformation of embryo pictured in C. Asterisk denotes location of a bend and spiral in the notochord. **F.** CON MO embryos develop normally at 24 hpf. **G.** NON embryos develop normally at 24 hpf. **H.** Enlarged image of normal notochord of embryo pictured in F.

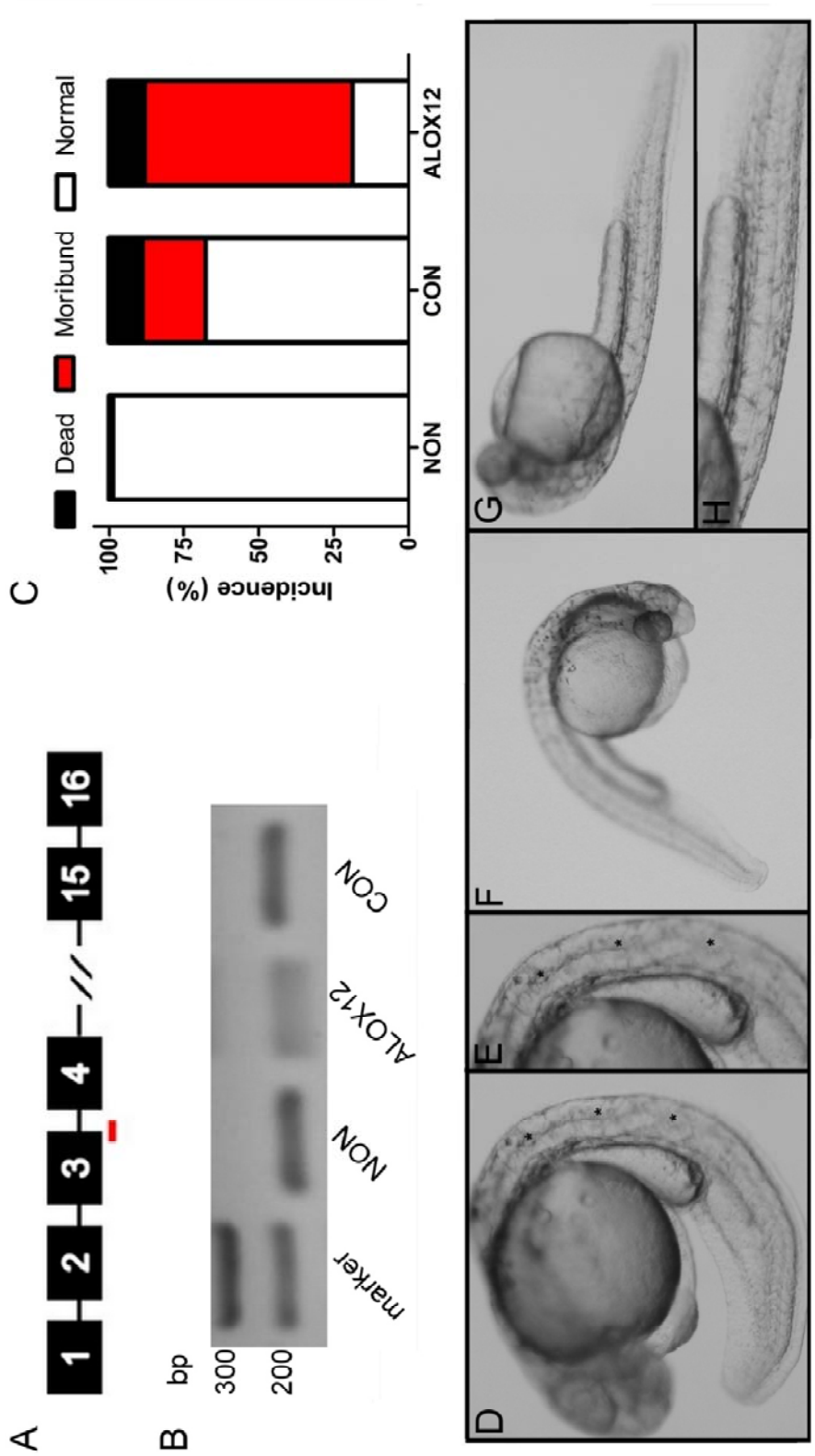

Figure 4. Knockdown of ALOX12 causes notochord malformations in embryos by 24 hpf

Figure 5. Knockdown of ALOX5 causes malformations in embryos by 24 hpf. **A.** ALOX5 MO target. The ALOX5 MO binds to the exon-1/intron-1 junction, preventing proper splicing of the mRNA transcript. Numbered boxes represent exons, spanning lines are introns, and the red line indicates the ALOX5 MO. **B.** RT-PCR indicates partial knockdown of endogenous ALOX5 expression following MO injection. **C.** ALOX5 knockdown increases the incidence of malformations in embryos at 24 hpf. Data shown as mean percent incidence from three separate experiments (total number of embryos: NON [non-injected], n=189; CON [injected with non-specific MO], n=189; ALOX5 [injected with ALOX5 MO], n=1136). **D.** Representative picture of malformations at 24 hpf due to ALOX5 knockdown. Arrow head points to short and flattened head. Asterisk denotes pericardial edema. Arrow points to bent and corkscrew tail. **E.** More severe malformations noted at 24 hpf due to ALOX5 knockdown.

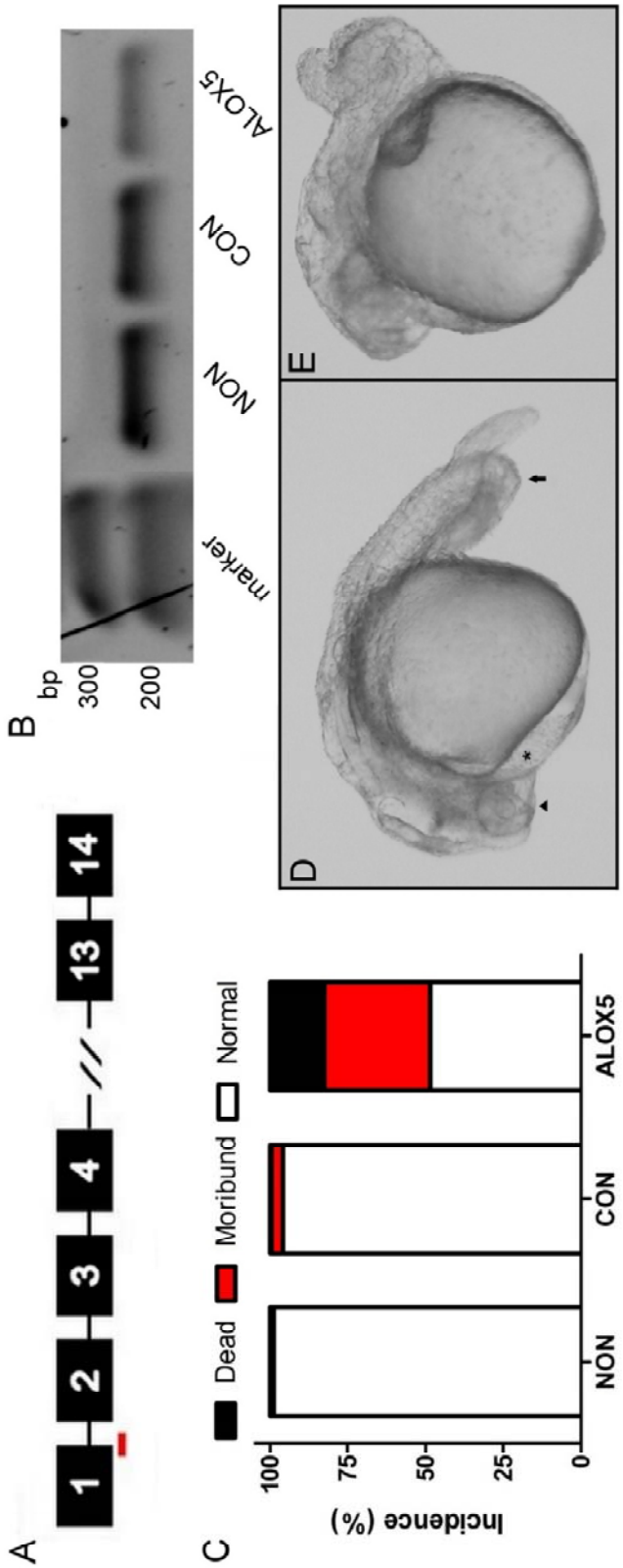

Figure 5. Knockdown of ALOX5 causes malformations in embryos by 24 hpf

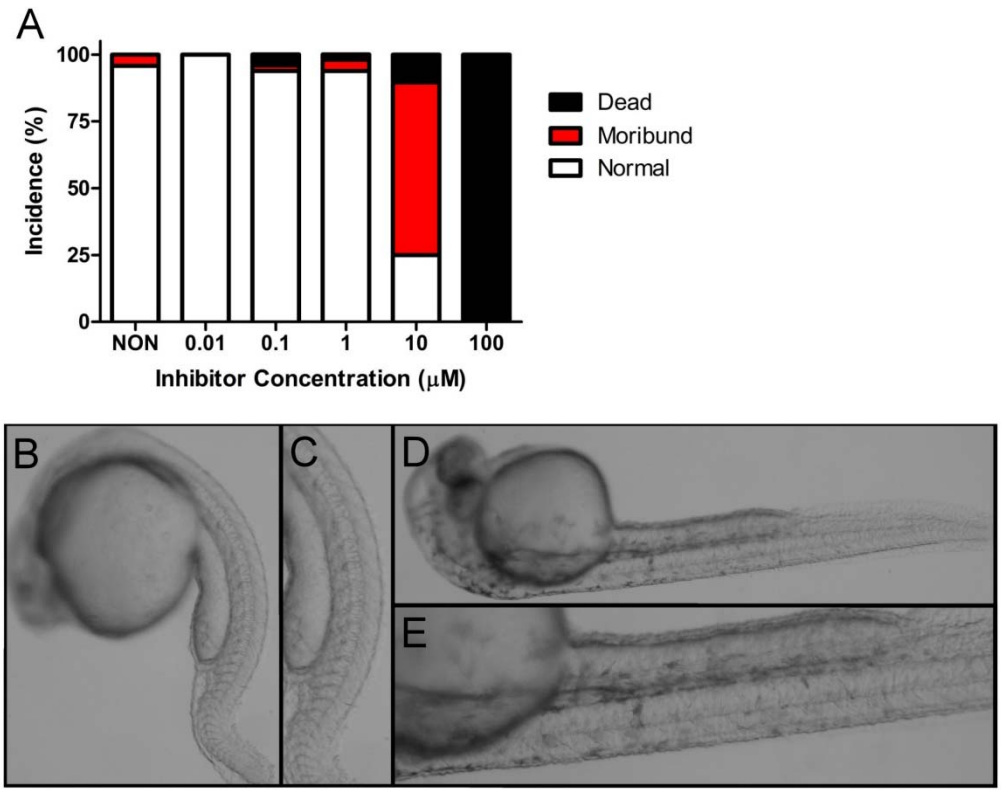

Figure 6. The LOX inhibitor recapitulates ALOX12 embryo malformations. **A.** Incidence of malformations noted in embryos incubated with the LOX inhibitor at 24hpf (n=48 embryos per concentration). **B.** Embryos incubated with the LOX inhibitor display bent tail and curved notochord malformations. **C**. Enlarged image of curved notochord of embryo pictured in B. **D.** Embryos incubated in control medium (NON) develop normally. **E.** Enlarged image of notochord of embryo pictured in D.

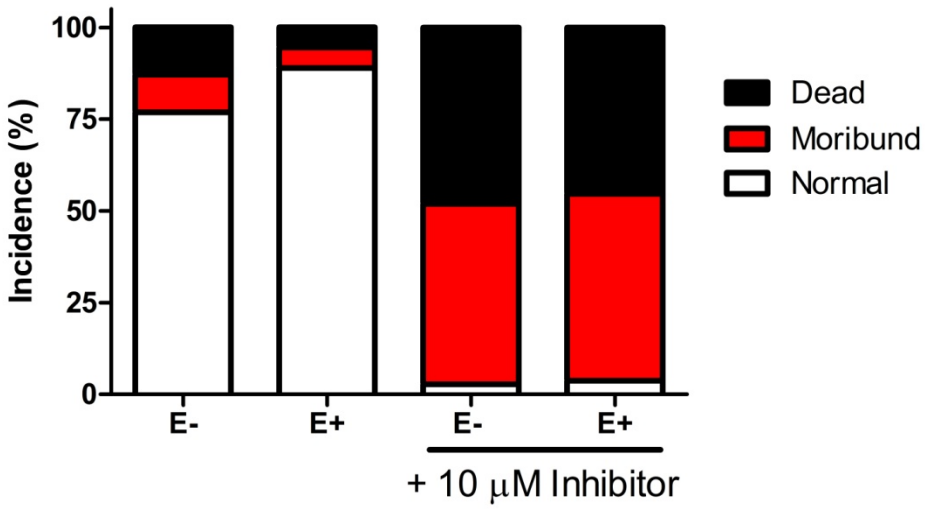

Figure 7. LOX **i**nhibition does not rescue α-tocopherol deficient embryos. The incidence of malformations and mortality increased by at least 30% in both E- (n=100) and E+ (n=100) embryos following incubations with the LOX inhibitor.

Table 1. PUFAs and oxidized PUFAs: abbreviations and detection characteristics

Analyte	Abbreviation
Fatty Acids	
Linoleic acid (18:2 ω-6)	LA
Linoleic acid-d4	LA-d4
Arachidonic acid (20:4 ω-6)	ARA
Arachidonic acid-d8	ARA-d8
α-Linolenic acid (18:3 ω-3)	ALA
α-Linolenic acid-d14	ALA-d14
Eicosapentaenoic acid (20:5 ω-3)	EPA
Eicosapentaenoic acid-d5	EPA-d5
Docosahexaenoic acid (22:6 ω-3)	DHA
Docosahexaenoic acid-d5	DHA-d5
Oxidized Fatty Acids	Abbreviation
Derived from DHA	
4-hydroxy-5E,7Z,10Z,13Z,16Z,19Z-DHA	4-HDHA
7-hydroxy-4Z,8E,10Z,13Z,16Z,19Z-DHA	7-HDHA
10-hydroxy-4Z,7Z,11E,13Z,16Z,19Z-DHA	10-HDHA
11-hydroxy-4Z,7Z,9E,13Z,16Z,19Z-DHA	11-HDHA
14-hydroxy-4Z,7Z,10Z,12E,16Z,19Z-DHA	14-HDHA
17-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-DHA	17-HDHA
17-hydroperoxy-4E,7Z,10Z,13Z,15Z,19Z-DHA	17-HpDHA
20-hydroxy-4Z,7Z,10Z,13Z,16Z,18E-DHA	20-HDHA
10,17-dihydroxy-4Z,7Z,11E,13Z,15E,19Z-DHA	NPD1/PD1
7,8,17-trihydroxy-4Z,9E,11E,13Z,15E,19Z-DHA	RvD1
7,16,17-trihydroxy-4Z,8E,10Z,12E,14E,19Z-DHA	RvD2
Derived from LA (octadecadienoic acid; ODE)	
9-hydroxy-10E,12Z-ODE	9-HODE
9-hydroperoxy-10E,12Z-ODE	9-HpODE
13-hydroxy-9Z,11E-ODE	13-HODE
13-hydroperoxy-9Z,11E-ODE	13-HpODE
Derived from ARA (eicosatetraenoic acid; ETE)	
5-hydroxy-6E,8Z,11Z,14Z-ETE	5-HETE
5-hydroperoxy-6E,8Z,11Z,14Z-ETE	5-HpETE
11-hydroxy-5Z,8Z,12E,14Z-ETE	11-HETE
12-hydroxy-5Z,8Z,10E,14Z-ETE	12-HETE
12-hydroperoxy-5Z,8Z,10E,14Z-ETE	12-HpETE
15-hydroxy-5Z,8Z,11Z,13E-ETE	15-HETE
15-hydroperoxy-5Z,8Z,11Z,13E-ETE	15-HpETE
20-hydroxy-5Z,8Z,11Z,14Z-ETE	20-HETE
5,12-dihydroxy-6Z,8E,10E,14Z-ETE	LTB4

Table 1. PUFAs and oxidized PUFAs: abbreviations and detection characteristics (continued)

Analyte	*m/z*
Fatty Acids	
Linoleic acid (18:2 ω-6)	279.2
Linoleic acid-d4	283.2
Arachidonic acid (20:4 ω-6)	303.2
Arachidonic acid-d8	311.2
α-Linolenic acid (18:3 ω-3)	277.2
α-Linolenic acid-d14	291.2
Eicosapentaenoic acid (20:5 ω-3)	301.2
Eicosapentaenoic acid-d5	306.2
Docosahexaenoic acid (22:6 ω-3)	327.2
Docosahexaenoic acid-d5	332.2
Oxidized Fatty Acids	*m/z* ⟶ *m/z*
Derived from DHA	
4-hydroxy-5E,7Z,10Z,13Z,16Z,19Z-DHA	343.2-101.025
7-hydroxy-4Z,8E,10Z,13Z,16Z,19Z-DHA	343.2-141.056
10-hydroxy-4Z,7Z,11E,13Z,16Z,19Z-DHA	343.2-153.092
11-hydroxy-4Z,7Z,9E,13Z,16Z,19Z-DHA	343.2-121.103
14-hydroxy-4Z,7Z,10Z,12E,16Z,19Z-DHA	343.2-205.124
17-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-DHA	343.2-201.165
17-hydroperoxy-4E,7Z,10Z,13Z,15Z,19Z-DHA	343.2-111.082
20-hydroxy-4Z,7Z,10Z,13Z,16Z,18E-DHA	343.2-133.101
10,17-dihydroxy-4Z,7Z,11E,13Z,15E,19Z-DHA	359.2-359.272
7,8,17-trihydroxy-4Z,9E,11E,13Z,15E,19Z-DHA	375.2-141.057
7,16,17-trihydroxy-4Z,8E,10Z,12E,14E,19Z-DHA	375.2-141.056
Derived from LA	
9-hydroxy-10E,12Z-octadecadienoic acid	295.2-171.103
9-hydroperoxy-10E,12Z-octadecadienoic acid	311.2-185.119
13-hydroxy-9Z,11E-octadecadienoic acid	295.2-195.140
13-hydroperoxy-9Z,11E-octadecadienoic acid	311.2-113.097
Derived from ARA	
5-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid	319.2-115.040
5-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid	335.2-167.109
11-hydroxy-5Z,8Z,12E,14Z-eicosatetraenoic acid	319.2-167.109
12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid	319.2-179.108
12-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid	335.2-189.166
15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid	319.2-219.140
15-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid	335.2-167.108
20-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid	319.2-149.133
5,12-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid	335.2-195.104

Table 2. Precision of PUFA, αTOC, and CHOL measurements in zebrafish embryos.

	LA	ARA	ALA	EPA	DHA
	ng/embryo	ng/embryo	ng/embryo	ng/embryo	ng/embryo
Mean ± SD[1]	2.14 ± 0.06	0.73 ± 0.04	0.19 ± 0.01	1.33 ± 0.05	21.9 ± 0.8
Within-day CV[2] (%)	3.0	2.8	4.5	3.4	3.1
Between-day CV (%)	0.0	2.4	1.8	0.0	1.4
Total CV (%)	3.0	3.7	4.9	3.4	3.4
Recovery (%)	77.4 ± 2.3	76.7 ± 1.8	75.0 ± 3.2	82.0 ± 4.8	80.1 ± 2.7

[1]Mean ± SD of six replicate aliquots (100 µL) extracted and analyzed on three different days
[2]CV=(SD/mean)*100

Table 2. Precision of PUFA, αTOC, and CHOL measurements in zebrafish embryos (continued)

	αTOC	CHOL
	pmol/embryo	nmol/embryo
Mean ± SD[1]	91.4 ± 5.7	10.3 ± 2.5
Within-day CV[2] (%)	3.9	24.9
Between-day CV (%)	3.1	0.0
Total CV (%)	5.0	24.9
Recovery (%)	56.9 ± 4.2	ND[3]

[1]Mean ± SD of six replicate aliquots (100 µL) extracted and analyzed on three different days
[2]CV=(SD/mean)*100
[3]Not determined

Table 3. PUFAs in ALOX12 knockdown embryos at 24 hpf.

	NON	CON	ALOX12	ANOVA[3]
	N=8	N=8	N=10	*P* value
PUFA (ng/embryo)[1,2]				
LA	1.9 ± 0.1[b]	2.0 ± 0.2[b]	1.7 ± 0.1[a]	0.0026
ARA	0.6 ± 0.0[b]	0.7 ± 0.0[b]	0.8 ± 0.0[a]	<0.0001
ALA	0.4 ± 0.0[b]	0.3 ± 0.1[b]	0.3 ± 0.1[a]	0.0047
EPA	1.5 ± 0.1[b]	1.4 ± 0.1[ab]	1.3 ± 0.1[a]	0.0165
DHA	21.4 ± 1.2	22.4 ± 0.9	21.6 ± 1.7	0.3306
αTOC (pmol/embryo)	85.0 ± 5.3	84.2 ± 5.6	83.2 ± 3.3	0.7357
CHOL (nmol/embryo)	10.9 ± 0.5[b]	10.9 ± 1.7[b]	14.4 ± 2.6[a]	0.0012

[1]Data shown as mean ± SD of ten embryos per N sample, collected over three separate injection days: NON (non-injected); CON (injected with non-specific MO); ALOX12 (injected with ALOX12 MO)

[2]Data in the same row not sharing the same letter are significantly different by at least $p<0.05$

[3]One-way ANOVA with a Bonferroni post-hoc test for paired comparisons

Table 4. Oxidized PUFAs in ALOX12 knockdown embryos at 24 hpf.

Oxidized PUFA[1,2] (pg/embryo)	NON N=3	CON N=3	ALOX12 N=3	ANOVA[3] *P* value
5-HETE	4.1 ± 1.5	8.4 ± 1.8	6.1 ± 4.7	0.2899
12-HETE	11.6 ± 3.2[b]	13.7 ± 3.9[b]	3.8 ± 0.0[a]	0.0015
7-HDHA	38.7 ± 20.5	94.4 ± 50.7	42.2 ± 55.4	0.2663
10-HDHA	4.3 ± 0.8[ab]	6.4 ± 1.3[b]	3.2 ± 0.0[a]	0.0083
14-HDHA	13.2 ± 3.3[c]	23.9 ± 2.8[b]	9.7 ± 0.0[c]	0.0011
17-HDHA	38.1 ± 20.2	95.1 ± 51.4	42.2 ± 57.1	0.2657

[1]Data shown as mean ± SD of 200 embryos per N sample, collected over three separate injection days: NON (non-injected); CON (injected with non-specific MO); ALOX12 (injected with ALOX12 MO)

[2]Data in the same row not sharing the same letter are significantly different by at least $p<0.05$

[3]One-way ANOVA with a Bonferroni post-hoc test for paired comparisons

Table 5. PUFAs in ALOX5 knockdown embryos at 24 hpf.

	NON	CON	ALOX5	ANOVA[3]
	N=8	N=8	N=8	*P* value
Fatty Acid (ng/embryo)[1,2]				
LA	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	0.4240
ARA	0.6 ± 0.0[b]	0.7 ± 0.0[b]	0.7 ± 0.0[a]	<0.0001
ALA	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.7535
EPA	1.5 ± 0.1[b]	1.4 ± 0.1[a]	1.6 ± 0.1[b]	0.0004
DHA	21.4 ± 1.2	21.5 ± 1.0	21.4 ± 0.6	0.8189
αTOC (pmol/embryo)	85.0 ± 5.3	82.5 ± 8.7	80.7 ± 8.1	0.5339
CHOL (nmol/embryo)	10.9 ± 0.5	10.3 ± 2.1	10.7 ± 1.5	0.4781

[1]Data shown as mean ± SD of ten embryos per N sample, collected over two separate injection days: NON (non-injected); CON (injected with non-specific MO); ALOX5 (injected with ALOX5 MO)

[2]Data in the same row not sharing the same letter are significantly different by at least $p<0.05$

[3]One-way ANOVA with a Bonferroni post-hoc test for paired comparisons

Table 6. Oxidized PUFAs in ALOX5 knockdown embryos at 24 hpf.

Oxidized PUFA[1,2] (pg/embryo)	NON N=3	CON N=3	ALOX5 N=3	ANOVA[3] *P* value
5-HETE	30.5 ± 13.3	61.2 ± 16.1	23.9 ± 19.5	0.0558
12-HETE	41.3 ± 30.1	43.0 ± 27.8	21.1 ± 5.1	0.4985
7-HDHA	186.7 ± 104.7	414.0 ± 125.2	154.8 ± 113.0	0.0466
10-HDHA	13.0 ± 10.2	15.5 ± 8.6	8.2 ± 1.1	0.4366
14-HDHA	52.8 ± 49.0	63.1 ± 39.6	29.7 ± 3.7	0.5502
17-HDHA	196.4 ± 106.4	434.5 ± 141.2	157.8 ± 121.7	0.0501

[1]Data shown as mean ± SD of 200 embryos per N sample, collected over three separate injection days: NON (non-injected); CON (injected with non-specific MO); ALOX5 (injected with ALOX5 MO)

[2]Data in the same row not sharing the same letter are significantly different by at least $p<0.05$

[3]One-way ANOVA with a Bonferroni post-hoc test for paired comparisons

Table 7. PUFAs in E- and E+ embryos during the first 72 hours of embryogenesis.

	LA	ARA	ALA	EPA	DHA
	ng/embryo	ng/embryo	ng/embryo	ng/embryo	ng/embryo
E-					
3 hpf	2.9 ± 0.1$^{a\#}$	4.0 ± 0.1$^{a\#}$	0.06 ± 0.0^{a}	0.05 ± 0.0$^{a\#}$	10.1 ± 0.3$^{ab\#}$
24 hpf	2.8 ± 0.3$^{a\$}$	4.2 ± 0.2$^{a\$}$	0.06 ± 0.0^{a}	0.05 ± 0.0$^{a\$}$	10.6 ± 0.3$^{a\$}$
48 hpf	2.4 ± 0.1^{b}	3.5 ± 0.1^{b*}	0.04 ± 0.0^{b}	0.05 ± 0.0^{a*}	9.3 ± 0.3^{bc}
72 hpf	1.8 ± 0.1^{c}	3.2 ± 0.1$^{c\%}$	0.02 ± 0.0^{c}	0.04 ± 0.0$^{b\%}$	8.9 ± 0.5^{c}
E+					
3 hpf	2.5 ± 0.0$^{a\#}$	3.3 ± 0.0$^{a\#}$	0.06 ± 0.0^{a}	0.10 ± 0.0$^{a\#}$	9.3 ± 0.3$^{a\#}$
24 hpf	2.4 ± 0.1$^{ab\$}$	3.2 ± 0.1$^{a\$}$	0.05 ± 0.0^{a}	0.09 ± 0.0$^{ab\$}$	9.3 ± 0.1$^{a\$}$
48 hpf	2.3 ± 0.1^{b}	3.3 ± 0.1^{a*}	0.04 ± 0.0^{b}	0.08 ± 0.0^{ab*}	9.5 ± 0.5^{a}
72 hpf	1.7 ± 0.0^{c}	2.9 ± 0.0$^{b\%}$	0.02 ± 0.0^{c}	0.07 ± 0.0$^{b\%}$	9.3 ± 0.1^{a}
ANOVA[1] *P* value	<0.0112	<0.0001	<0.3524	0.0024	0.0007
Main effect time	<0.0001	<0.0001	<0.0001	<0.0001	0.0020
Main effect diet	0.0024	<0.0001	0.7273	<0.0001	0.0134
Lab					
3 hpf	1.6 ± 0.1^{a}	0.88 ± 0.0^{a}	0.22 ± 0.0^{a}	1.3 ± 0.1^{a}	20.4 ± 0.1^{a}
24 hpf	1.6 ± 0.0^{ab}	0.81 ± 0.0^{ab}	0.23 ± 0.0^{ab}	1.3 ± 0.1^{a}	20.3 ± 0.3^{ab}
48 hpf	1.4 ± 0.1^{c}	0.80 ± 0.0^{b}	0.17 ± 0.0^{c}	1.2 ± 0.1^{a}	18.5 ± 0.4^{c}
72 hpf	1.1 ± 0.1^{d}	0.71 ± 0.0^{c}	0.12 ± 0.0^{d}	1.0 ± 0.1^{b}	17.1 ± 0.6^{d}
ANOVA[2] *P* value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Data shown as mean ± SD of ten embryos per N sample, N=4 samples per time point.
[1]Repeated measures two-way ANOVA (diet x time) with Bonferonni post-hoc test for paired comparisons
[2]One-way repeated measures ANOVA
For each fatty acid, time-points within a diet not sharing the same letter are significantly different by at least $p<0.05$
For each fatty acid, time-points between E- and E+ sharing the same symbol are significantly different by at least $p<0.05$

Table 7. PUFAs in E- and E+ embryos during the first 72 hours of embryogenesis (continued).

	DHA/ARA	αTOC	CHOL
	ng/ng/embryo	pmol/embryo	nmol/embryo
E-			
3 hpf	2.6 ± 0.1[a]	0.7 ± 0.1[a#]	12.0 ± 1.9
24 hpf	2.5 ± 0.1[a$]	1.0 ± 0.0[a$]	11.9 ± 0.5
48 hpf	2.6 ± 0.1[a*]	0.8 ± 0.0[a*]	12.4 ± 0.5
72 hpf	2.8 ± 0.1[b%]	0.4 ± 0.1[b%]	10.7 ± 1.2
E+			
3 hpf	2.7 ± 0.1[a]	40.4 ± 2.7[#]	14.3 ± 1.7[a]
24 hpf	2.9 ± 0.1[b$]	40.7 ± 5.0[$]	11.0 ± 3.1[ab]
48 hpf	2.8 ± 0.1[ab*]	42.0 ± 8.3*	10.3 ± 0.6[ab]
72 hpf	3.2 ± 0.1 [c%]	51.6 ± 7.2[%]	9.5 ± 1.8[b]
ANOVA[1] *P* value	0.0010	<0.0001	0.0891
Main effect time	<0.0001	0.0105	0.0193
Main effect diet	0.0005	<0.0001	0.6327
Lab			
3 hpf	23.1 ± 0.4	110.1 ± 4.5	12.4 ± 3.1
24 hpf	24.3 ± 0.2	111.1 ± 8.6	11.3 ± 2.4
48 hpf	23.1 ± 1.1	105.1 ± 3.3	10.2 ± 2.4
72 hpf	24.2 ± 1.2	110.0 ± 7.3	11.1 ± 2.1
ANOVA[2] *P* value	0.1567	0.6780	0.6246

Data shown as mean ± SD of ten embryos per N sample, N=4 samples per time point.

[1]Repeated measures two-way ANOVA (diet x time) with Bonferonni post-hoc test for paired comparisons

[2]One-way repeated measures ANOVA

For each fatty acid, time-points within a diet not sharing the same letter are significantly different by at least $p<0.05$

For each fatty acid, time-points between E- and E+ sharing the same symbol are significantly different by at least $p<0.05$

Table 8. Oxidized PUFAs in E- and E+ embryos at 36 hpf.

Hydroxy PUFA (ng/embryo)	E- N=3	E+ N=3	t-test *P* value
5-HETE	94.9 ± 12.0	40.4 ± 23.7	0.0099
12-HETE	199.9 ± 53.3	136.6 ± 49.9	0.1917
7-HDHA	52.9 ± 9.6	23.9 ± 9.4	0.0106
10-HDHA	9.9 ± 0.2	7.2 ± 0.7	0.0028
14-HDHA	23.8 ± 6.0	22.1 ± 10.7	0.6921
17-HDHA	50.1 ± 7.1	23.0 ± 5.6	0.0029

Data shown as mean ± SD of 100 embryos per N sample

Chapter 3: Implications and Future Directions

The purpose of this study was to determine the regulation and role of oxidized PUFA production during embryogenesis. We hypothesized that LOX and the oxidation of PUFAs are required for proper embryonic development. We demonstrated that the production of HDHAs occurs via vertebrate LOXs during embryogenesis under normal physiological conditions and that decreasing their production causes abnormal development. We also hypothesized that αTOC prevents the non-enzymatic, radical-mediated lipid peroxidation of DHA and ARA. To date, the specific interaction of DHA or ARA with αTOC was unknown and it was assumed that αTOC protected these PUFAs from peroxidation. Furthermore, the assessment of αTOC's role in lipid homeostasis has largely been limited by measuring non-specific markers of lipid peroxidation and degradation (ex malondialdehyde). We have demonstrated that αTOC particularly protects DHA during embryogenesis, that deficiency of αTOC decreases PUFA concentrations and augments production of specific oxidized PUFAs, which have pronounced biologic functions. Viewing oxidized PUFAs as only an outcome of LOX activity or αTOC deficiency is too simplistic; oxidized PUFAs have physiological roles and are not simply non-specific by-products of lipid peroxidation and lipid degradation.

The peroxidation of lipids, and in a broader sense oxidative stress, is generally considered detrimental to human health and is appropriately implicated in a myriad of diseases. However, our data challenges the concept that oxidative stress is purely a negative cellular event. We observed abnormal embryonic development in the absence, or in the presence of excess, oxidized PUFAs. As such, oxidative stress must be balanced with antioxidant status. Current guidelines recommend increasing ω-3 consumption during pregnancy and as a preventative/treatment measure for other diseases (ex: cardiovascular health [121]). The recommendations to increase PUFA intake are occurring concurrent with reports regarding the negative effects of αTOC on health outcomes (ex: prostate [122] and bone health[123]). Already, 96% of women do not meet the dietary recommended intake for vitamin E. It is concerning given our data demonstrating PUFA and αTOC are inter-dependent during development, that PUFA intake in humans may increase while αTOC intake may

decrease. Nutritional recommendations must take into account nutrient-nutrient interactions and whether or not supplementation with one nutrient during critical life stages, such as pregnancy and embryogenesis, may alter the requirements for another nutrient.

Our studies leave several questions unanswered. We are limited by observing one time point during development and need to assess changes in oxidized PUFAs prior to an observable phenotype to identify initiating mechanisms and potential intervention periods to prevent abnormal development. Notably, the sensitivity of our new method allowed us to detect changes in PUFA concentrations as a result of LOX knockdown or αTOC deficiency. However, we have yet to answer whether the magnitude of change in PUFAs and oxidized PUFAs is sufficient enough to elicit the observed developmental defects. As discussed above, it is also unclear how ALOX12 knockdown decreased specific PUFA concentrations and altered CHOL concentrations. Importantly, we carried out the characterization of ALOX12 and ALOX5 during development of lab embryos spawned from zebrafish fed a commercial diet filled with fish oil and fish meal. The zebrafish fed the defined diets must synthesize all PUFAs longer than ALA and LA[1]. As such, the E- and E+ embryos have a substantially different PUFA profile than lab embryos (**TABLE 7**). A different basal level of PUFAs could substantially alter the interaction between LOX, PUFAs, oxidized PUFAs, and αTOC during development. In light of these limitations, we propose these future studies:

1. Inject labeled PUFAs in conjunction with LOX knockdown to track the metabolism and trafficking of PUFAs
2. Inject specific oxidized PUFAs with LOX knockdown in an attempt to rescue embryos and determine which oxidized PUFAs are critical for embryonic development
3. Assess changes in oxidized PUFAs throughout embryogenesis
4. Complete validation of LOX inhibitor experiments with measurements of PUFAs and oxidized PUFAs
5. Measure gene changes of enzymes involved in lipid metabolism and cellular redox status

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