AN ABSTRACT OF THE DISSERTATION OF

Melissa Quin McDougall for the degree of Doctor of Philosophy in Nutrition presented on June 7, 2017.

Title: Assessment of the Neuropathological Consequences of Vitamin E Deficiency

Abstract approved: _____

Maret G. Traber

Cognitive impairment, or *cognitive decline*, a noticeable and measurable decline in cognitive abilities (*e.g.* memory and learning) that exceeds those attributed to normal aging, represents an early symptom of neurodegeneration and increased risk for progression to more severe dementias, such as Alzheimer's disease (AD). While the complex etiology of these conditions remains an area of active investigation, oxidative stress has been implicated as a primary factor in neurodegenerative disease pathogenesis. Zebrafish (*Danio rerio*) are a recognized model for studying the pathogenesis of cognitive deficits and the mechanisms underlying behavioral impairments, including the consequences of increased oxidative stress within the brain. The vertebrate brain is especially enriched in long-chain polyunsaturated lipids, such as ω-3 docosahexaenoic acid (DHA; 22:6 ω-3); therefore, lipid peroxidation is a likely contributor to neuropathology. Vitamin E (α-tocopherol; VitE), the body's most potent lipophilic antioxidant, was first discovered in 1922 as an essential nutrient for preventing fetal resorption in rodents, and has since been linked with embryonic and neurological health in both numerous animal (including my own work with zebrafish) and human studies. VitE deficiency increases early miscarriage risk in humans, which poses public health concerns since estimates of inadequate dietary VitE intakes exceed 80% of the global adult population. However, nearly a century after its initial discovery, the underlying biological rationale explaining VitE's essentiality for (neuro)development and brain function remains unknown.

The purpose of this project was to provide for an evidence-based assessment of metabolic interactions between VitE and specific membrane lipids to elucidate the biochemical basis underlying VitE's neurological function *in vivo* during neurodevelopment

and into adulthood. To accomplish this overall aim, I exploited a zebrafish model in which my lab group has pioneered the study of nutrition and dietary manipulation and the use of novel “omics” methodologies. I used both embryonic and adult conditions of VitE deficiency to publish compelling evidence that demonstrates the major role for VitE in the brain is to protect DHA and DHA-containing phospholipids (DHA-PLs) against oxidative stress, and without this antioxidant protection, ensuing secondary deficiencies in both DHA and choline coincide with increased morbidity, mortality, and/or cognitive impairments. Further, my work shows that VitE’s antioxidant activity is vital for maintaining the cellular antioxidant network, and dysregulation of such aberrantly alters energy metabolism by severely compromising mitochondrial function. The studies included in this work, when considered together, provide insight as to how inadequate VitE perturbs DHA, phospholipid, and choline metabolism, resulting in dysregulation of other metabolic pathways as well as epigenetic methylation reactions, and how disruption of these processes compromises neurological and cognitive outcomes during neurodevelopment as well as in later life.

My primary research goal was to help elucidate the mechanism(s) through which VitE contributes to lifetime brain health. To achieve this, I evaluated the consequences of inadequate VitE from the earliest stages of brain development through middle-age. My central hypothesis was that VitE protects DHA, a vital substrate for brain membrane phospholipid maintenance, and that dysregulation of DHA-PL status due to restricted dietary VitE severely perturbs critical events necessary for embryonic neurodevelopment that, ultimately, increase susceptibility for consequent, persistent cognitive impairments.

First, I performed phenotypic assessments and lipidomics analyses, as well as developed a method to measure PL turnover in zebrafish embryos using $H_2{}^{18}O$ labeling, to gain mechanistic insight on the organism-level effects of developmental α-tocopherol deficiency. I hypothesized that VitE is required by the developing embryonic brain to prevent depletion of highly polyunsaturated fatty acids, especially DHA, the loss of which I predicted would underlie abnormal morphological and behavioral outcomes. Therefore, I fed adult 5D zebrafish defined diets without (E-) or with added VitE (E+, 500 mg *RRR*-α-tocopheryl acetate/kg diet) for a minimum of 80 days, and then spawned them to obtain E- and E+ embryos. The E- compared with E+ embryos were behaviorally impaired at 96 hours post-fertilization (hpf), even in the absence of gross morphological defects. Evaluation of phospholipid (PL) and lysophospholipid (lyso-PL) composition using untargeted lipidomics in E- compared with E+ embryos at 24, 48, 72, and 120 hpf showed

that four PLs and three lyso-PLs containing DHA, including lysophosphatidylcholine (LPC 22:6, required for transport of DHA into the brain), were at lower concentrations in E- at all time-points. Additionally, $H_2{}^{18}O$ labeling experiments revealed enhanced turnover of LPC 22:6 and three other DHA-containing PLs in the E- compared with the E+ embryos, suggesting that increased membrane remodeling is a result of PL depletion. Overall, these data indicate that VitE deficiency in the zebrafish embryo causes the specific depletion and increased turnover of DHA-containing PL and lyso-PLs, which may compromise DHA delivery to the brain and thereby contribute to the functional impairments observed in E- embryos.

Next, I investigated the underlying mechanisms causing developmental VitE deficiency-induced mortality in E- embryos using targeted metabolomics analyses embryos over five days of development, which coincided with their increased morbidity and death. VitE deficiency resulted in peroxidation of DHA, depleting DHA-PLs, especially phosphatidylcholine, which also caused choline depletion. This increased lipid peroxidation increased NADPH oxidation as well, which depleted glucose by shunting it to the pentose phosphate pathway. Using bioenergetic profiling analyses, I also found that VitE deficiency was associated with mitochondrial dysfunction with concomitant impairment of energy homeostasis. The observed morbidity and mortality outcomes could be attenuated, but not fully reversed, by glucose injection into VitE-deficient embryos at developmental day one. These studies together suggest that embryonic VitE deficiency in vertebrates leads to a metabolic reprogramming that adversely affects methyl donor status and cellular energy homeostasis, with ultimately lethal outcomes.

I then shifted my focus to address outcomes of chronic VitE deficiency and to probe more thoroughly brain-specific consequences. I investigated behavioral perturbations due to isolated, chronic VitE deficiency in adult zebrafish fed diets that were either VitE-deficient (E- group) or sufficient (E+ group) for up to 18-months of age. I hypothesized that E- adult zebrafish would display significant cognitive impairments associated with elevated lipid peroxidation and additional metabolic disruptions in the brain. Using assays of both associative (avoidance conditioning) and non-associative (habituation) learning, I found E- adults were learning impaired compared with E+ fish, and that these functional deficits occurred concomitantly with the following observations in adult E- brains: decreased concentrations and increased peroxidation of polyunsaturated fatty acids (*e.g.* DHA), altered brain phospholipid and lysophospholipid composition, dysregulation of the cellular antioxidant network, and perturbed energy (glucose/ketone), phosphatidylcholine, and

choline/methyl-donor metabolism. Collectively, these data show that chronic VitE deficiency could lead to cognitive dysfunction through multiple potential mechanisms, including decreases in DHA, antioxidants, glucose, and choline, as well as corresponding dysfunction in related metabolic pathways (*e.g.* energy/NAD(P)H and methyl-donor metabolism) within the brain.

Finally, given the outcomes of my embryo studies demonstrating that increased lipid peroxidation in E– embryos perturbs their cellular antioxidant network, which ultimately disrupts aerobic energy metabolism, causing a significant decrease in whole-body (and, presumably, brain) glucose levels, and thus adversely impacts neurobehavioral outcomes, I investigated whether these consequences could be reversed via dietary remediation. Previous pilot studies showed that mortality and behavioral impairments are avoided with proactive VitE repletion, as an α-tocopherol emulsion administered into the yolk of 0 hpf E– embryos entirely prevented mortality and morbidity outcomes. However, remediation of VitE deficiency-induced (*i.e.* secondary) nutrient deficiencies only partially rescues E– embryos, as observed following glucose supplementation into the yolk at one day of age (24 hpf; after established VitE deficiency but prior to glucose depletion). Together, this data suggests the effects of developmental VitE deficiency may be *prevented*, but not necessarily *reversed*. I hypothesized, therefore, that deleterious outcomes of embryonic VitE deficiency cannot be ameliorated fully though later supplementation with VitE and other depleted nutrients (*e.g.* DHA and choline), and that long-term cognitive defects will persist in E– compared with E+ embryos despite dietary intervention.

To test this hypothesis, I selected normal appearing E– or E+ embryos, then fed them a complete diet for 7 days and analyzed them for behavioral, biochemical, and morphological changes. **I** evaluated the embryo groups for up to 12 days post-fertilization (dpf). The E– group suffered significantly increased morbidity and mortality as well as altered DNA methylation status through 5 dpf when compared to E+ larvae, but upon feeding with a VitE-adequate diet from 5-12 dpf both the E– and E+ groups survived and grew normally; the DNA methylation profile also was similar between groups by 12 dpf. However, 12 dpf E– larvae still had behavioral defects. These observations coincided with sustained VitE deficiency in the E– vs. E+ larvae, despite adequate dietary supplementation. I also found continued DHA depletion and significantly increased lipid peroxidation in E– vs. E+ larvae. Further, targeted metabolomics analyses revealed persistent dysregulation of the cellular antioxidant network, the CDP-choline pathway, and

glucose metabolism. While anaerobic processes were increased, aerobic metabolism was decreased in the E– vs. E+ larvae, potentially indicating mitochondrial damage and aberrant reliance on aerobic glycolysis (“Warburg effect”) in the E- group. Taken together, these outcomes indicate embryonic VitE deficiency causes lasting behavioral impairments due to persistent lipid peroxidation and metabolic perturbations that are not resolved via later dietary VitE supplementation.

Collectively, the findings from these completed studies provide mechanistic evidence to explain VitE’s essentiality for human neurodevelopment and adult brain function, and yield new insights regarding the impact early-life VitE deficiency has on embryonic (neuro)development as well as on the inception of cognitive decline and ensuing neurological disorders. These outcomes may be used to support continued research investigating and promoting the importance of adequate VitE for optimal brain health throughout life.

Assessment of the Neuropathological Consequences of Vitamin E Deficiency

by

Melissa Quin McDougall

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented June 7, 2017

Commencement June 2017

Doctor of Philosophy dissertation of Melissa Quin McDougall presented on June 7, 2017.

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

Melissa Quin McDougall, Author

ACKNOWLEDGEMENTS

This section should be the easiest to write, but I am finding it the most difficult because the written word truly cannot express the overwhelming gratitude I have for all the individuals who have supported and guided me throughout my years of graduate school. To all the persons named and unnamed herein, you have my deepest appreciation, and any positive attributes I have today are, in part, due to your influence in my life. First, to my major advisor, Dr. Maret Traber, you are a phenomenal scientist and the first true academic and professional mentor I have ever had. I consider you a visionary and am consistently impressed with your intellect, integrity, tenacity, and ingenuity. Your instruction and assistance these past years has exceeded my greatest expectations; I cannot tell you how thankful I am to have met you and to have benefited from your mentorship. Next, to the remaining members of my doctoral committee, Drs. Donald Jump, Kathy Magnusson, Robert Tanguay, and Theresa Filtz, your support has also been pivotal. Each of you deserves much more expression of sincere gratitude than I can grant in a mere, all-inclusive acknowledgements section.

To Scott Leonard, you are the embodiment of the word “helpful.” You are solely responsible for preserving my sanity on many occasions. To Jaewoo Choi, I had no idea (and did not care) what the term “lipidomics” meant prior to meeting you. Thank you for facilitating my continued scientific education. To Carrie Barton, Greg Gonnerman, Jane La Du, Mike Simonich, Lisa Truong, and all other SARL support staff, you answered more e-mails and cries for help as I navigated my zebrafish research than I can comprehend. I appreciate all of you being so considerate and patient, no matter how trivial the question. To the Nutrition Graduate Program faculty, most notably Dr. Urszula Iwaniec, your instruction and coaching in academic, professional, and personal realms has been incredible. To all the IGERT and CHAR faculty, LPI and OSU community – “thank you” does not suffice, but please know that I value the contributions you have made to my time as a doctoral student.

To my family, friends, and loved ones – especially my parents, Shawn and Angela McDougall, as well as my aunt, Rosemary Maichel – when I attempt to think of words to offer you to convey how integral, how foundational, how absolutely critical you are and have always been, in your own unique capacity, to my life – I am at a loss. I love you; perhaps there is nothing more meaningful that needs to be written than those three words.

CONTRIBUTION OF AUTHORS

The authors contributions were as follows:

Chapter One: MQM wrote.

Chapter Two: MQM, MGT, and RLT contributed to study concept, research design, data gathering and interpretation, manuscript preparation, and revision. JC assisted with lipidomics sample preparation, data gathering, and data analysis. JFS contributed to research design. LT performed statistical analyses for behavior assays.

Chapter Three: MQM, MGT, and RLT contributed to study concept, research design, data gathering and interpretation, manuscript preparation, and revision. JC assisted with lipidomics and metabolomics sample preparation, data gathering, and data analysis. HKK (The Catholic University of Korea) assisted with metabolomics data analysis. GB contributed to metabolomics statistical analysis. JFS contributed to research design. EC (University of Southern California) assisted with data interpretation and manuscript preparation.

Chapter Four: MQM, MGT, KM, and RLT contributed to study concept, research design, data gathering and interpretation, manuscript preparation, and revision. JC assisted with lipidomics and metabolomics data gathering and data analysis. LT performed data analysis for behavioral assays and assisted with behavior assay data interpretation.

Chapter Five: MQM, MGT, and RLT contributed to study concept, research design, data gathering and interpretation, manuscript preparation, and revision. JC assisted with lipidomics and metabolomics data gathering and data analysis. LT performed data analysis for behavioral assays and assisted with behavior assay data interpretation.

Chapter Six: MQM wrote.

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DEDICATION:

This dissertation is dedicated to Sharon Louise Bruno. Nana, even though you did not live to see me work towards and finally obtain my doctorate, I know you were with me – or more accurately, *within* me – every dogged step of the way. Not a Wednesday goes by that I do carry bitter-sweet memories of you in my mind and in my heart.

CHAPTER ONE:

INTRODUCTION

This introductory chapter begins with a brief background on dementia and cognitive decline, with a focus on how nutrition, especially during the perinatal neurodevelopmental period, may play an important role in the inception and progression of neurodegenerative pathology. Next, I provide general information for the three key nutrients implicated in my studies: vitamin E, docosahexaenoic acid, and choline. I then give a short overview of relevant biological activities of these nutrients to highlight general, potentially significant mechanisms via which they may affect brain development and function into mid-adulthood. A description of the zebrafish model system follows. Finally, I discuss in more detail how vitamin E, docosahexaenoic acid, and choline specifically relate both to lifetime neurological health as well as to one another, to provide appropriate context and rationale for appreciating the hypotheses and aims of my research.

Dementia and Cognitive Decline: Definitions and Prevalence

Dementia refers to a group of progressive age-related, debilitating neurological conditions [1, 2], that tend to be associated with severe, ultimately fatal neurodegenerative diseases in the elderly [3]. These conditions comprise one of the most rapidly increasing as well as socially and economically devastating causes of death in industrialized nations [4]. The incidence of dementia is expected to quadruple over the coming decades [3]; in the United States alone, the annual healthcare costs related to this trend are likely to exceed $1 trillion by the year 2050 [5]. Despite advances in understanding the pathophysiology of age-related dementia, no pharmaceutical agent has been developed to successfully treat the condition, making it one of the most pressing clinical and public health problems in the world today [6]. Given that most dementia coincides with irreversible structural [7, 8] and synaptic [9] neuronal damage, interventions that begin after symptoms present are expected to achieve negligible, if any, therapeutic benefit. It is, however, conceivable that preventing or delaying the causes of cognitive decline and ensuing dementia represent the most valid and effective alternatives to treatment, and research has shifted accordingly towards targeting and modifying factors that may trigger the inception of neuropathology [10].

Recent reports estimate that one-third of elderly Americans die with some form of age-related dementia [3]. Most of these conditions are associated with significant functional losses and underlying neurodegeneration that may begin to develop

decades before diagnostic symptoms are fully present. *Cognitive decline*, the experience of abnormal memory loss and increased confusion, affects over 13% of individuals 45 years and older in the U.S. and is linked with progression to dementia and associated neurodegenerative diseases [11]. Dementia pathogenesis may, in fact, be a life-long process that originates *in utero* during brain development [10, 12]. Both poor maternal and perinatal nutrition are significant risk factors for cognitive deficits and consequent dementia in adulthood [13], as healthy neurodevelopment to support optimal lifetime neurological function requires sufficient nutrient availability during critical periods throughout gestation and early life.

Dementia Pathogenesis: Neurodevelopmental Exposures

Dementia is not caused by a single etiologic entity, but instead results from the interplay between multiple genetic and environmental neurological exposures throughout life that may begin during fetal neurodevelopment [14, 15]. Early-life exposure to one or several risk factors may represent a fundamental, primary step in determining dementia susceptibility by predisposing affected individuals to clinical insurgence of the disease in adulthood through a variety of mechanisms. Such triggers include poor maternal and perinatal nutrition [12, 16, 17]. Adequate fetal nutritional support is key for two primary reasons: first, fetal neurodevelopment is a highly metabolically taxing process [18, 19], so optimal brain development is extremely sensitive to the sufficient availability of specific nutrients required for neuronal metabolism, proliferation, and differentiation [14], as well as the genetic orchestration of essential neurophysiological milestones (*e.g.* closure of the neural tube) [20] that occur during critical developmental periods [21, 22]. Second, nutrition is a factor that can be willfully altered, which presents an incredible opportunity to improve human cognitive health outcomes – and enhance future life trajectories – by leveraging knowledge of which nutrients, and how much, to provide at which time(s) to support optimal neurodevelopment and, ultimately, lifetime brain function.

Critical and sensitive periods during brain development are salient opportunities for environmental stimuli to shape the brain. These periods are typically characterized by a high degree of neuronal plasticity [23]. They occur early in life, apparently spanning fetal and early postnatal development, and the events that occur during these periods influence brain function across the lifespan [24, 25]. At a human/organism level, what happens during such times differentiates individuals and

can determine how resilient or compromised a person is in old age. There is general agreement that *critical periods* refer to time periods when the brain's response to environmental stimuli (either the presence/absence of necessary stimuli or exposure to noxious stimuli) results in irreversible long-term neurobehavioral effects [26, 27]. Such defined critical periods seem particularly present during the embryologic and anatomic development of the fetal and early postnatal brain.

Environmental factors clearly impact the brain differentially across the lifespan, and can be conceptualized as demonstrating sensitive (and possibly, critical) periods where they exert more influence [12, 15, 28, 29]. Perinatal nutrition represents an important environmental factor that exhibits both critical and sensitive periods of influence [27]. Development is a time of rapid growth and differentiation. Since the brain is not a homogenous organ, the requirements for metabolites/nutrients that support development are distributed regionally to the areas of most rapid growth at a given time in life [15, 29, 30]. Brain regions (*e.g.* hippocampus or prefrontal cortex) begin as highly undifferentiated, yet pluripotent regions [18, 23, 31, 32]. In their nascent form, they have poor specificity of function, but are more capable of recovery from insults than the older, more highly differentiated brain [23]. This capacity for recovery represents one definition of *plasticity*. As development of a brain region progresses, it becomes more specialized functionally but loses a significant amount of its ability to recover from insults: such loss can be characterized as a loss of potential plasticity [14, 33-36]. The period of rapid development during which a region undergoes this maturational process and is most impressionable (positively or negatively) is coincident with critical or sensitive periods that ultimately may dictate the brain's capacity or "reserve" for synaptic plasticity throughout life.

Specifically, the greater potential inherent in phases of early cell determination and early neuronal connectivity confers an enhanced need for sufficient energy substrates, appropriate environmental stimuli, and sequential, well-orchestrated patterns of gene expression [14, 34]. For instance, the inception of a critical or sensitive period of regional brain development is often marked by elevated metabolic demand because of the considerable energy cost of growth – indeed, upwards of 60% of fetal oxygen and glucose is consumed by the brain [34]. A developing area is at risk when any of these three aspects are compromised. In the same way that the neonatal brain is more vulnerable to the positive or negative effects of non-nutritional factors

(*e.g*. maternal stress; for a review, see [27]), it also may be more responsive to nutritional manipulation. There are two fundamental mechanisms by which a nutrient's status early in life can affect neural plasticity during neurodevelopment and, subsequently, have an impact on long-term brain function: first, structural deficits induced during development may persist into adulthood [37, 38], and second, modifications of gene expression during neurodevelopment may perturb brain function in later life [17, 39, 40]. While a comprehensive review of these overarching processes is beyond the scope of the present work, each will be discussed as they pertain to specific nutrients the research reported herein has found to be essential for optimal fetal (neuro)development and long-term cognitive health – the antioxidant vitamin E, the ω-3 polyunsaturated fatty acid docosahexaenoic acid, and the methyl-donor nutrient choline.

Vitamin E (α-Tocopherol)

Vitamin E (VitE) refers to a family of eight hydrophobic plant compounds comprised of tocopherols and tocotrienols. Each member is distinguished by the methylation pattern on its chromanol ring as well as the saturation of its phytyl side-chain. A common feature to all vitamers is a strongly electrophilic hydroxyl group on the chroman ring, capable of effectively quenching carbon-centered lipid radicals, which provides VitE with potent antioxidant function [41]. Naturally occurring VitE, most concentrated in various nuts, oils, seeds, and some leafy green vegetables [42], contains 2′, 4′ and 8′ chiral centers in the phytyl side-chain that are in the *R* configuration (*e.g. RRR*-α-tocopherol), while synthetic VitE, the form found in many dietary supplements, is a racemic mixture of both *R* and *S* stereoisomers in each of these positions [43]. The various vitamers are similarly absorbed in the intestine, incorporated into chylomicra, and delivered to the liver within hours of ingestion under normal circumstances [44], but two critical hepatic activities create a physiological preference for *RRR*-α-tocopherol: the α-transfer protein (α-TTP), which facilitates selective incorporation of *RRR*-α-tocopherol from the liver into circulating lipoproteins for deposition into tissues [45, 46], and a CYP450 enzyme (most likely CYPF4F2 [47], which selectively degrades all other forms of the vitamin. Despite the heterogeneity of tocols ingested by humans, only *RRR*-α-tocopherol is appreciably enriched in plasma and tissues [44], and accordingly is the most biologically active form of VitE; it also is the only form considered for determining the recommended dietary intake values by

the Institutes of Medicine [48]. Thus, throughout this document, the term “VitE” will henceforth refer to *RRR*-α-tocopherol alone, unless otherwise indicated.

VitE terminates propagation of lipid peroxidation, thereby functioning as a lipid-soluble “chain-breaking” antioxidant to prevent cellular membrane damage [44, 49, 50]. Beyond its direct antioxidant function, VitE has been implicated in the regulation of genes related to lipid uptake and degradation, expression of extracellular proteins, inflammation, cell signaling and maintenance of the cell cycle [51-53]. In most studies, the noted gene effects are not directly attributed to VitE signaling, but instead result from changes in membrane environment that mediate cell signaling [44], as VitE has been found to influence membrane fluidity and maintenance [54-56].

VitE is particularly enriched in neuronal tissue, where it is aggressively retained during periods of inadequate dietary intake, even after peripheral tissues become depleted [45, 57]. Overt VitE deficiency rarely occurs in humans, but is frequently reported in patients with fat malabsorption syndromes or genetic defects in α-TTP, as reviewed [43, 58]. VitE-deficient individuals initially present with mild sensory neuropathy that advances to spinocerebellar ataxia and progressive, peripheral neuropathy caused by a dying back of large-caliber, sensory neurons [59-61]. Additional evidence emphasizes the neurological significance of VitE as an antioxidant: VitE’s biological half-life in the brain is distinctively slow, suggesting tissue-specific mechanisms that actively sequester VitE [45, 62]; expression of α-TTP, required for VitE trafficking to and within the brain [58] is markedly elevated in brain samples from patients afflicted with oxidative stress-related neurodegenerative diseases like “Ataxia with vitamin E deficiency” (AVED, due to loss-of-function mutations in the α-TTP protein) and Alzheimer’s disease (AD) [63]; selective α-TTP expression in the cerebellum is regulated both by oxidative stress and VitE status [46, 64, 65]; and the functional deficits that accompany heritable VitE deficiency (AVED) are solely neurological [66-68]. These deficits generally are attributed to inadequate antioxidant protection, *i.e.* the accumulation of oxidative damage leading to compromised integrity and function of neuronal phospholipid membranes, since VitE protect neurons from oxidative stress *in vivo* and *in vitro* [64, 69-71]. Elevated markers of lipid peroxidation (oxidative stress), including both oxidized phospholipids [72] and isoprostanes (autoxidized polyunsaturated fatty acid [PUFA] derivatives) [73] also have been reported in brain samples obtained from dietary as well as genetic rodent

and zebrafish models of chronic VitE deficiency [61, 74]. Collectively, this data shows VitE's action as a lipophilic antioxidant within the brain is critical for optimal neurological function.

Dietary intake recommendations for VitE (as α-tocopherol) first were established in the United States by the Institute of Medicine in 2000 [48]. The Recommended Daily Allowance (RDA) of 15 mg VitE for both male and female adults (age 19 and over) was derived from the amount needed to prevent peroxide-induced erythrocyte hemolysis in VitE-deficient patients, observed at 12 μmol/L serum VitE, as determined from a limited number of dietary depletion/repletion studies performed in the 1950s and 60s using data from a small sample of institutionalized male psychiatric patients [75, 76]. In subjects fed with a VitE-deficient diet for over six years, an intake of 12 mg VitE per day was sufficient to achieve this serum VitE concentration, which was then defined as the estimated average requirement (EAR) and became the basis for extrapolation to the current RDA. A daily intake of 12 mg VitE was also considered sufficient to protect dietary polyunsaturated fatty acids from lipid peroxidation [48]. Importantly, because hemolysis has been reported in children with severe VitE deficiency [48, 77], the preventive effect of VitE against oxidative damage-induced hemolysis was considered a clinically relevant *in vitro* analysis of VitE status, which means that the latest RDA continues to be based on the prevention of deficiency symptoms rather than on health promotion and prevention of chronic disease. It is also noteworthy that increased intake recommendations to support prenatal development during pregnancy do not yet exist – this may be highly concerning, given that: (i) VitE was first discovered due to its necessity for preventing fetal resorption in rodents [78], (ii) intake data suggests women, particularly pregnant women, do not consume adequate VitE [42, 79]; and (iii) VitE-deficient pregnant women have an increased risk of early miscarriage [80].

Docosahexaenoic Acid (DHA)

Docosahexaenoic acid (DHA; 22:6ω-3) is the most abundant (ω-3) fatty acid in the mammalian brain, comprising upwards of 50% of nervous tissue PUFA content [81-84]. Levels in brain membrane lipids are altered by the type and amount of fatty acids in the diet, and with life stage, increasing with development and decreasing with aging [85-87]. Mammals obtain DHA either from the diet as DHA itself, or as its essential ω-3 fatty acid precursor, α-linolenic acid (ALA,18:3ω-3), and/or as

intermediates between ALA and DHA, such as eicosapentaenoic acid (EPA, 20:5ω-3). Synthesis of DHA occurs in algae and animals, but not plants. Accordingly, DHA is uniquely absent from all vegetable fats and oils, including nuts, grains, and seeds [88]; it is also of negligible presence in ruminant fats, including milk and dairy products [89]. The richest dietary sources of DHA are fish and sea foods, although recent “fortified” egg products provide lower, but appreciable, sources of DHA [90]. Primary dietary sources of precursor ALA are flax and canola oils as well as other nuts and seeds like walnuts, though these sources are not usually consumed consistently or in large quantities within the Western diet [79].

Humans can synthesize DHA from ALA in a pathway that requires sequential activity of two desaturases (FADS1, FADS2) and two elongase enzymes (ELOVL2, ELVOL5) in the mitochondria, followed by a β-oxidation reaction (or “retroconversion”) that occurs in the peroxisome; the Δ6-desaturase activity of FADS2 constitutes the rate-limiting step of this process [91]. The synthesis of ω-3 and ω-6 fatty acids utilizes the same enzymes, thus these two types of PUFAs are in metabolic competition, though the pathway does favor metabolism of ω-3 fatty acids over other PUFAs – specifically, over ω-6 fatty acid conversion of linoleic acid (LA, 18:2ω-6) to arachidonic acid (ARA, 20:4ω-6) [92-94].The overall conversion process from ALA to DHA is very low [94-96]; some studies suggest it is limited such that less than 5% of ingested ALA is converted to DHA in adult humans [97, 98]. Such minimal endogenous synthesis is, in large part, attributed to feedback inhibition, as the activities of both FADS and ELOVL enzymes in particular are regulated by dietary PUFA status, the majority of which comes from ω-6 intake in most human populations [91-94]. Importantly, the fetal liver and brain also are capable of synthesizing DHA from ALA [99, 100]; however, this synthetic capacity decreases over time [101]. Consequently, greater amounts of DHA accumulate within the developing brain when preformed DHA, as compared with its precursor ALA, is supplied to the growing fetus [102, 103], and the placenta preferentially transfers DHA over other PUFAs from maternal to fetal circulation [104, 105]. Relatedly, ALA supplementation to preterm infants likely does not support adequate DHA needs, as reports show endogenous DHA synthesis cannot match *in utero* accretion rates of preformed DHA [106].

DHA is an important building block of neuronal membranes. The lipid bilayer of neuronal membranes consists of phospholipids, with DHA, ARA, and EPA as their

main components. Three compounds are important for the membrane formation in the Kennedy cycle (cytidine diphosphosphate [CDP]-choline pathway) [107, 108]: a uridine source, a fatty acids source, and a choline source [109, 110]. Other phospholipids also are synthesized via the Kennedy cycle and incorporate PUFAs, such as phosphatidylethanolamine (PE) that uses ethanolamine instead of choline [111]. Phosphatidylserine (PS) exchanges a serine molecule for choline in phosphatidylcholine (PC) or ethanolamine in PE [108, 112, 113]. Notably, the dietary essential fatty acids, LA and ALA (sources of ω-3) comprise less than 1% of neural PUFAs [114]. Studies in humans and animal models demonstrate that many neuronal abnormalities induced by developmental DHA deficiency, including but not limited to attention disorders, depression, schizophrenia, autism, and anxiety [85, 115-117], may be attributed to reduced neurotransmission processes, especially of the dopaminergic and serotonergic systems, that are caused by disruption of membrane fluidity and related membrane receptor functions [118-121] Further, DHA and its metabolites are involved in regulating brain gene expression, such as that of cAMP response element binding protein (CREB)[122, 123], which plays an important role in long term potentiation, a key mechanism for memory formation and learning, as well as expression of genes in the retinoid signaling pathways regulating synaptic plasticity, learning, and memory involving the retinoic acid receptor (RAR), retinoid X receptor (RXR), and peroxisome proliferator-activated receptor (PPAR) [120].

Derivatives of DHA metabolism, aptly named “docosanoids”, also are neuroprotective (see [124] for extensive review); among the most well-characterized is neuroprotectin D1 (NPD1), generated from the selective oxygenation of DHA by 15-lipoxygenase (15-LOX) [125]. NPD1 induces homeostatic/pro-survival signaling in response to cellular and systemic insults [120, 126] by NPD1 up-regulating antiapoptotic proteins (Bcl-2 and Bcl-xL) and down-regulating proapoptotic proteins (Bax and Bad) in response to cellular oxidative stress and cytokine activation, leading to an overall pro-survival transcriptome [127, 128]. NPD1 elicits neuroprotection in brain ischemia-reperfusion and in oxidative-stressed retinal cells [127, 129, 130] as well as inhibits retinal ganglion cell death [131], thereby serving as a mediator that executes the protective bioactivity of DHA in the nervous system. Notably, deficiency of NPD1 and of the enzyme involved in its formation, 15-LOX, has been observed in AD brain tissue, and NPD1 has been shown to influence beta-amyloid precursor

protein (βAPP) processing by decreases amyloid-beta 42 release [128]. Many DHA "oxylipins" (as well as those from other PUFAs, as reviewed [132]) also exert neurological functions through complex cell signaling pathways [120, 133]. Additionally, free radical–mediated DHA peroxidation products, such as neuroprostanes, accumulate during ischemia and neurodegeneration [134-137]. These oxidation products in turn may form protein adducts and other cytotoxic molecules that promote further free radical injury in the brain [1, 138-141].

Although dietary recommendations for fetal DHA adequacy are controversial at present, the most widely-accepted consensus statements recommend pregnant and lactating woman to consume at least 200-300 mg DHA daily to ensure sufficient DHA delivery to the developing fetus [142, 143]. Clinical data also is in favor of regular dietary DHA intake during at least the first six months of life and suggest that DHA should be added in formulas at the level generally found in human breast-milk, approximately (0.2-0.3 weight % of total fatty acids) [144, 145]. Of note, ARA, the most abundant ω-6 neuronal fatty acid throughout gestation and postnatal development [19] also is required during the perinatal period, as infant formula supplemented with DHA, but lacking ARA, impairs infant growth [146] and adversely impacts neurodevelopment [147]. While ARA is not the focus of the present work, its neurological significance during early life cannot be ignored, as argued in several recent reviews [146, 148, 149]. Finally, because the available evidence from clinical and epidemiological studies is equivocal concerning adequate dietary DHA (as well as total PUFA) to support neurocognitive function and health in old age [150-152], there are no present recommendations in place regarding intake levels to prevent or postpone cognitive decline and dementia pathogenesis [153].

Choline

Choline, a water-soluble micronutrient generally categorized with the B-complex vitamins, serves as the starting material for several important metabolites that play essential roles in fetal development, particularly for the brain. As a major constituent of cellular membranes, the phospholipid phosphatidylcholine (PC) is required for cell division and growth, with subsequent effects on brain structure and function [154, 155]. A period of particularly rapid growth of the human brain begins during the third trimester of pregnancy and continues up to approximately five years of age [156]. Sphingomyelin, a PC-derived phospholipid, is also abundant in nervous

tissue, as is required for myelination of nerve fibers (axons) in both the central and peripheral nervous system [157, 158]. Myelination facilitates electrical impulses, protects and insulates nerve fibers, and is essential for proper nervous system development. The most dramatic changes in myelination occur between mid-gestation and the end of the second year of life [158]. Further, the phospholipids PC and sphingomyelin yield important cellular signaling molecules including diacylglycerol, ceramide, and platelet activating factor [159], all of which have putative roles in fetal development [160]. Choline also is required for biosynthesis of acetylcholine, a key neurotransmitter for myriad brain functions, including regulation of neuronal proliferation, differentiation, migration, maturation, plasticity, survival, and synapse formation [155, 161]. Large amounts of acetylcholine are produced and accumulate in the placenta, where it functions as a signaling molecule to influence cellular differentiation and proliferation, as well as parturition [162].

The choline derivative betaine acts as the primary methyl donor in the liver, kidney, embryonic stem cells, and the lens of the eye, and accounts for approximately 60% of the methyl moieties required for DNA methylation, synthesis, and repair in mammals [163]; it is used for remethylation of homocysteine to the amino acid methionine [162, 164, 165]. Following the activation of methionine to S-adenosyl-methionine (SAM), the betaine-derived methyl group can be used by DNA methyltransferases (DNMTs) for methylation of cytosine bases that precede guanosine (CpG dinucleotides). In turn, methylation of CpG dinucleotides influences gene transcription and chromosomal stability [30, 163]. By influencing SAM production, choline/betaine status has effects on methyl group availability in brain (and other tissues), thereby influencing organ-specific genome expression and fetal programming [166]. Maternal choline supply during pregnancy has been shown to modify the epigenome of fetal liver [39] and brain [111, 167, 168] in animals, as well as the placenta and fetal cord leukocytes in humans [169].

In the liver, the major site of choline metabolism, choline may proceed through either the CDP-choline pathway to form PC, or it can be oxidized to betaine and partake in one-carbon metabolism. At low cellular free-choline concentrations, free-choline is preferentially incorporated into phospholipids [30, 155]. However, as cellular concentrations rise, the majority of free-choline is converted to betaine within hepatocyte mitochondria [170]. Of the more than sixty methyltransferases that acquire

methyl groups from SAM, phosphatidylethanolamine N-methyltransferase (PEMT), guanadinoacetate N-methyltransferase, and glycine N-methlytransferase are among the greatest consumers[171]. PEMT catalyzes the sequential methylation of PE forming PC and S-adenosyl-homocysteine (SAH). Although betaine can serve as the original source of the methyl groups, this pathway enables the formation of a new choline moiety from ethanolamine. The PEMT pathway produces approximately 30% of PC [172] and is most active under conditions of choline deficiency [173]. While both the PEMT and CDP-choline pathways produce PC, the fatty acid composition of the resulting PC molecule differs; PEMT-derived PC are enriched in long-chain PUFAs, such as DHA [174, 175]. Since the expression of PEMT is low or absent in placental tissue and fetal liver, maternal choline supply is critical to provide adequate PC for fetal development [176]. This heavy reliance on maternal sources is met, in part, through induction of the maternal hepatic PEMT pathway [173, 177]. Estrogen response elements are present in the PEMT gene promoter; binding of estrogen, which rises during gestation, induces PEMT expression and enhances PC synthesis [178].

Unique alterations to the maternal plasma PC composition occur during pregnancy with an increase of DHA at the *sn*-2 position in PC molecules from week 22 to term [173, 177]. As discussed previously, acquisition of DHA by the fetal brain is vital for its optimal growth, development, and maturation [179-181]. This adaptation in maternal PC fatty acid composition may arise from changes to the fatty acid composition of PE, the substrate of PEMT, as well as a preference for PE species containing DHA and ARA, as demonstrated in pregnant rats [182]. Indeed, PEMT deficiency in rodents is associated with reduced plasma concentrations of DHA and ARA [173]. The increase in PC-DHA occurring by mid-gestation may be a mechanism to ensure adequate supply of DHA to the fetus, and, more specifically, the fetal brain [155, 183].

The embryonic central nervous system is particularly sensitive to choline availability. Studies performed in neurulating mouse embryos during early gestation (embryonic day 9) show that inhibitors of choline uptake and metabolism cause developmental defects that affect the neural tube and face [184]. In human beings, low maternal dietary choline intake (<290 mg/day) increases the risk for having a baby with a neural tube defect by approximately two-fold [154]. Genetic variants residing

within choline metabolizing genes modulate neural tube defect risk as well [185], so women with genetic variants that increase choline requirements may be particularly susceptible to choline inadequacy during pregnancy. Furthermore, population data from the United States demonstrates that women eating diets low in choline content (about 150 mg/day) are at a significantly greater risk for having a baby with a neural tube defect (four-fold higher risk[186] than are women eating diets with adequate (>350 mg/day) choline content, indicating that higher levels of choline are required to support optimal fetal brain development [30]. Based on the most recent National Health and Nutrition Examination Survey 2009-2012 data, the average choline intake among American women of child-bearing age is 250-278 mg/day, and approximately 97-99% have usual intakes below the choline Adequate Intake (AI) level of 425 mg/day [187]; further, past population-level data from the United States suggests that pregnant women have a mean choline intake of 338 mg/day, and over 90% fall below the established AI level [179, 180, 188]. In fact, the need for choline during pregnancy likely far exceeds intake recommendations [162].

Similar to VitE, moderate choline insufficiency probably is quite pervasive, though routine laboratory tests of choline status and clear symptoms of marginal deficiency are lacking [113, 189]. Current population data suggest over 75% of American adults consume inadequate choline; moreover, older adults are especially at risk [187]. These estimate match past reports in other Western nations [188, 190, 191]. Given the significance of acetylcholine (as well as methylation-dependent epigenetic processes, discussed below) in neurological function and the etiology of dementia (see [192, 193] for reviews of the cholinergic system and AD pathophysiology), researchers have accordingly begun to investigate whether choline affects cognitive function in later life.

VitE and the Cellular Antioxidant Network

The function of VitE traditionally is ascribed primarily [44], though not always exclusively [52], to the nutrient's antioxidant activity within a greater cellular antioxidant network [194]. This concept is based on an extensive literature documenting tocopherol's efficacy in neutralizing unstable lipid peroxy-radicals generated from polyunsaturated fatty acids [49, 195]. The water-soluble antioxidant ascorbic acid (vitamin C) is thought to ‘recycle’ the oxidized tocopheroxyl radical created during this reaction back to tocopherol [196, 197]; vitamin C is then regenerated at the expense

of glutathione, and, ultimately, the reducing equivalent(s) NADH and/or NADPH, either of which may serve as the final electron donor in this network. Concomitantly, once VitE reduces lipid peroxyl radicals to lipid hydroperoxides, the selenium-dependent enzyme, phospholipid hydroperoxide glutathione peroxidase (GPx4) converts the hydroperoxides to less toxic lipid hydroxides, also at the expense of glutathione. Experimental studies demonstrate that the maintenance of this antioxidant network is crucial for protection of membrane phospholipids against radical-mediated degradation [198]. This is especially true for highly aerobic tissues like the brain, given that mitochondria represent a primary site of free-radical (or "reactive oxygen species") production [199, 200].

Oxidative stress and disruption of the cellular antioxidant network contributes to the pathophysiology of many neurological diseases [135, 201-206], including neurodevelopmental disorders like Down's syndrome [207, 208]; it follows, then, that links have been observed between VitE-deficiency and these same diseases [209-213] For example, the amniotic fluid of pregnant mothers carrying babies with Down's syndrome is lower in VitE than in control patients [214]. Conversely, VitE supplementation during gestation improves behavior and cognitive abilities, and decreases associated lipid peroxidation in Down's syndrome animal models [215]. Lower levels of VitE also have been observed in the cerebral-spinal fluid of individuals with mild cognitive impairment [216], cognitive decline progressing to dementia [217], as well as AD patients [210, 218, 219]. At the cellular level, studies suggest VitE depletion inhibits clearing of the amyloid-β protein, resulting in further accumulation of amyloid-β and increased pathological plaque formation in the brain [220, 221]; many others also demonstrate a relationship between cognitive impairment, increased oxidative stress and/or lipid peroxidation, and decreased VitE levels in nervous tissue [73, 222, 223]. Such investigations concerning VitE-deficiency in relation to brain function most frequently focus on VitE's protection of specific PUFAs with known significance for neurological health; namely, DHA.

Nutrition and Epigenetics

Many of the diverse cognitive effects of nutrition are mediated by changes in gene expression and associated regulatory networks, with numerous gene variants adding a further level of complexity [224-227]. This involves effects on cell membranes, enzymes, neurotransmitters, metabolism, neurogenesis, and synaptic

plasticity [224]. Energy status, for example, influences numerous hormones and growth factors that act as nutritional sensors to influence the brain via changes in gene expression [228]; such molecules include glucocorticoids, thyroid hormones, insulin, and brain-derived neurotropic factor (BDNF), which are involved in myriad cell signaling systems and neural networks that modulate brain metabolism, development, and function. The epigenome provides a critical layer of regulation during neurodevelopment, and perinatal nutrition is one of the many epigenetic regulators that modify gene expression during this period [15, 229]. An understanding of epigenetic influences on gene expression throughout life is therefore central to elucidating of the role of nutrition in accelerated cognitive decline and dementia. The term *epigenetics* means “above genes” and includes mechanisms that involve chemical marking of chromatin, the form in which DNA is packaged with histone proteins in the cell nucleus. While traditionally used to reference strictly heritable changes, it also is used more loosely to indicate alterations that occur within the lifespan of an individual. This review discusses “epigenetics” in the latter context.

Epigenetic marks can induce chromatin remodeling and related changes in gene expression through multiple highly dynamic, sophisticated mechanisms. These include changes in DNA methylation and hydroxymethylation, histone modifications, non-protein-coding RNAs (ncRNAs), RNA editing, and telomere control [230], all of which enable cell-specific and age-related gene expression, and are central to normal brain development, structure, and function. Thus, epigenetic signals have a pivotal role in synaptic plasticity, learning, and memory across the lifespan [224]. While an exhaustive discussion of all such mechanisms is beyond the context of this review, DNA (and histone) methylation is relevant to both choline function and cellular redox homeostasis, and thus deserves mention. This introduction will focus on DNA methylation in particular, but growing evidence shows that histone methylation also is similarly significant for epigenetic regulation of gene expression [166, 230].

Briefly, DNA methylation is a process involving addition of a methyl group to the cytosine bases mostly located at cytosine–phosphate–guanine sites (CpG sites) that are present in the 5′-untranslated regions of gene promoters [231]. DNA methylation results in gene silencing by the recruitment of methyl CpG-binding transcriptional repressors and by interfering with the DNA binding of transcriptional activators. Hypomethylated DNA, therefore, is associated with “active” chromatin.

DNA methylation affects transcription at the promoter region by preventing the binding of transcription factors to the gene [232, 233]. Recent studies show a further modification on the methylated CpG, the hydroxylation of 5-mC to 5-hydroxymethyl cytosine (5-hmC), which may make the gene less prone to transcription than those in the methylated state [234]. Oxidative stress results in hydroxymethylation of DNA, especially in neurons [235].

Importantly, DNA methylation is essential for optimal development and function of multiple brain regions; for example, this process controls long-term memory formation in the hippocampus [236]. Recent findings also show that DNA methylation has a key role in establishing the gene expression potential of diverse hypothalamic cell types [163, 237]. Moreover, DNA methylation is crucial for synaptic plasticity [229], learning [238], memory [239], modulation of neuronal gene expression [240], neuronal survival, and repair [241]; studies also show reduction in global DNA methylation correlates with a decline in learning and memory functions [242]. Although most investigations focus on perinatal methyl-donor nutrients (*e.g.* folate, choline) and epigenetic outcomes [225, 243], emerging findings suggest that fatty acids, in particular PUFAs like DHA, also modify the fetal epigenome [244] via DNA methylation [245]. The zebrafish, an established model for DNA methylation-toxicology studies [246, 247], represents an attractive system for research focused instead on the above regarding developmental nutritional status [248].

The Zebrafish Model

Early stages of vertebrate development are remarkably well-conserved; therefore, embryonic zebrafish (*Danio rerio*) studies are highly translatable to human health outcomes [249-251]. Importantly, the critical milestones of vertebrate brain development (*e.g.* neurulation) are similar between zebrafish and humans [252]. Embryonic neurodevelopment in the zebrafish is a rapid, highly dynamic process that progresses from the zygote stage (0 hpf) through gastrulation (5.25-10 hpf) and the appearance of neuromeres, to formation and closure of the neural tube by the end of the segmentation period (10-24 hpf). The brain and peripheral nervous system develop further as general organogenesis continues and the embryo passes through the pharyngeal period (24-48 hpf) and grows into a larva; by 48-72 hpf, when the embryo hatches from the chorion, membranous structures such as the blood-brain barrier begin to form and increase in structural integrity [253]. At 120 hpf, the larval

zebrafish has a fully-functional nervous system and can execute complex behaviors including reactions to external stimuli and active avoidance [252]. Overall, the zebrafish is rapidly increasing in popularity as a valuable research tool [254, 255], and both embryos and adults are extensively used to model many human-related conditions, including neurological disorders [256-262], and gene-environment interactions [246, 263].

Inter-Related Neurological Significance of VitE, DHA, and Choline

The importance of VitE for brain development was made evident at the beginning of the twentieth century when Evans and Burr described paralytic offspring from rats deprived of dietary VitE [264]. Several relevant reports followed, linking VitE deficiency with sensory neuron ataxia in humans whose symptoms could be reversed via VitE supplementation [265, 266], and, more recently, by reports of increased miscarriage due to maternal VitE deficiency [80]. The neurological significance of VitE is also emphasized by its association with dementia and neurodegenerative disorders [267]. Low levels of VitE in the brain are evident in patients with diagnosed dementia [216] and mild cognitive impairment [268]. Moreover, a reduced risk of progressing from cognitive decline to dementia has been observed in subjects with high VitE status [218] and following VitE supplementation [269]. In fact, at a recent International Conference on Nutrition and the Brain in Washington DC, July 2013, an expert panel included “increased VitE intake from food sources” among seven dietary and lifestyle guidelines for the prevention of neurodegenerative diseases that were developed based on the best available evidence [270]. However, VitE’s potential as an agent to prevent or slow cognitive decline and ensuing dementia remains controversial [271, 272], primarily because the mechanism(s) underlying its neurological essentiality are still unknown. This paucity of evidence showing VitE’s specific physiological role in the human brain has led to the specious assumption that VitE has a neutral or even negative impact on health [273], despite ample evidence of the contrary [43]. Further, although over 90% of American adults do not consume the estimated average requirement (EAR) of 12 mg/day dietary VitE [42, 191], little has been done to address the potential long-term consequences of such pervasive deficiency due to the lack of a mechanistic rationale for VitE’s necessity.

My studies in both adult and embryonic zebrafish indicate that the physiological link between brain health and adequate VitE relates to the vitamin’s role as the body’s

most potent lipophilic antioxidant [49], whereby it protects highly unsaturated membrane lipids that are vulnerable to peroxidation [44]. Foremost among these is DHA, as it is highly concentrated in nervous tissue and is critical for neurodevelopment [18, 274, 275] as well as for preservation of lifetime neurological function [117, 276]. Given that the developing brain consumes 60% of fetal oxygen [34], this environment makes adequate antioxidant status especially crucial for protection of membrane lipids. My findings in zebrafish embryos show that insufficient VitE not only leads to increased DHA peroxidation, but also to depletion of membrane DHA-containing phospholipid species (DHA-PLs), which subsequently disrupts phospholipid remodeling and decreases availability of choline, which is critical for brain development [30]. Two of these depleted DHA-PL species, DHA-PC (38:6) and DHA-PC (40:6), are depleted in humans who develop neurodegenerative disease [277], suggesting a causative relationship between disrupted early-life DHA-PL status and later dementia risk.

Relatedly, perturbed choline metabolism is highly relevant to the study of lifespan dementia etiology [224]. Disturbed one-carbon metabolism in early life causes neurological abnormalities [238, 239], impacts DHA status [16, 40], and also has a role in pathological epigenetic alterations that increase susceptibility for neurodegenerative disease [225, 228]. Finally, previous studies from the Traber laboratory have demonstrated that VitE deficiency in zebrafish causes defects during neural tube formation, resulting in impaired brain formation and increased mortality [278]. These collective discoveries suggest that VitE, by influencing embryonic DHA and choline metabolism, is critical for normal neurodevelopmental events that occur in humans prior to when a woman may be able to confirm her pregnancy (19-21 days for human fetuses [279], 12-15 hours post fertilization [hpf] for zebrafish embryos [252]).

At present, there is insufficient knowledge regarding VitE's physiological significance as a lipophilic antioxidant. This is especially true regarding VitE's function in the brain, an organ highly enriched in lipid species, like DHA, that are vulnerable to peroxidation. DHA comprises 35% of the phospholipid composition at the synaptic membrane [280], emphasizing its significance for synaptogenesis and neural plasticity. Thus, DHA-PLs play an integral role in neuroanatomy and physiology, from primary structural constituents ("lipid rafts") of the neural membrane [281] to precursors for a complex variety of lipid derivatives, such as docosanoids, that exert

anti-inflammatory, neuroprotective effects [282]. The importance of adequate DHA during neurodevelopment rests on the observation that rapid DHA accretion within the brain occurs during the last trimester of pregnancy [19] and occurs during a time of maximal human neurogenesis and synaptogenesis [283]. Sufficient prenatal DHA also supports normal behavioral outcomes by influencing development of signaling networks, such as the glutamatergic system [284]. Microarray data from VitE-deficient zebrafish embryos reveals significantly increased expression of genes involved in these signaling pathways (*e.g.* synaptotagmin, BDNF), suggesting that insufficient VitE during development impacts these same neural processes [285]. In addition, developmental VitE deficiency leads to rapid DHA depletion [286], and the embryonic zebrafish brain's requirement for VitE coincides with increased synthesis of DHA, as evidenced by elevated expression of enzymes required for PUFA elongation, *Elovl4* [287] and *Elovl5* [288], in the head/brain region. Together, the data indicate an interaction between VitE and DHA in the brain during a critical developmental period, but the nature of this interaction, as its relevance to long-term neurological outcomes, is poorly understood.

DHA status, and, more specifically the preservation of sufficient brain DHA and DHA-PLs, has established consequence for brain health beyond the neurodevelopmental period. The brain phospholipid milieu is highly dynamic, and aberrant changes in its composition have been linked to neurodegenerative disorders such as AD [281, 289, 290]. Moreover, AD neurons display abnormally low levels of DHA, as well as increased susceptibility to lipid peroxidation from endogenously produced free radicals [282, 290]. Recent research also shows that a low plasma level of the phospholipid species DHA-PC is a highly predictive biomarker for conversion to AD in humans [277]. The abundant PUFA content of neural tissue, coupled with the brain's high oxygen requirement, supports the significance of lipid peroxidation in the etiology of neurodegenerative disease [118]. Indeed, the notable reductions in PUFA levels as well as peroxidability and unsaturation indices in AD brains are consistent with the progressive generation of detrimental lipoperoxides implicated in AD pathogenesis [277, 291]. Dietary DHA supplementation may slow AD progression [120], but the therapeutic efficacy of such interventions likely is compromised when the brain lacks the antioxidant capacity to protect newly synthesized DHA-PLs [124, 292].

Most, though not all [51] research suggests VitE's primary physiological action is as a potent antioxidant [44, 49], and, importantly, biological modeling shows VitE co-localizes with PUFA- enriched phospholipid domains of the cell membrane [56]. This evidence indicates that VitE can specifically protects DHA-PLs in the brain from pathogenic peroxidation, as occurs in AD [293]. Its ability to prevent oxidative damage of neural membrane PUFA (e.g. DHA) [204], neuron death [212], β-amyloid deposition [294], and age-associated declines in memory and learning [217, 295] further highlight a vital role for VitE and its protection of brain DHA for maintaining lifetime neurological function. Lack of adequate VitE in the adult also leads to behavioral deficits in motor coordination and cognitive processes that are normalized after VitE supplementation [59, 61]. Moreover, recent animal studies from the Traber laboratory show that dietary VitE protects endogenous DHA: for example, when feeding adult zebrafish defined diets that require them to synthesize long-chain PUFAs from their respective precursors, VitE-deficient fish (though, intriguingly, not their fertilized eggs) have decreased percentages of total ω-3 PUFAs compared with VitE-sufficient fish [278, 296], suggesting that VitE preserves these long-chain lipids. While these studies establish a biological relationship between DHA and VitE, it still is unknown the extent to which preservation of brain DHA is dependent upon adequate brain VitE. Whether there is an especially critical temporality to this interaction also requires exploration, as little data exists for concomitant assessment of both DHA and VitE status considering immediate or long-term neurological outcomes. It may be that lack of VitE, during neurodevelopment and/or throughout the lifespan, contributes to the heterogeneity of research evaluating the cognitive benefits of increased DHA intake in later life.

Of significance with regard to the growing need for further elucidation of life-long interactions between brain VitE and DHA, the Traber laboratory's previous work with zebrafish demonstrates that VitE's antioxidant capacity is critical to facilitate the nervous system in the developing embryo [286, 297, 298] and during aging [72, 74], which indicates that VitE is required for both neurodevelopment as well as for maintenance of the adult brain. Moreover, VitE deficiency causes depletion of certain DHA-PLs (e.g. DHA-PC 38:6) in zebrafish brains [72], analogous to their observed depletion in AD pathology [277]. These results support the hypothesis that VitE is required because it preserves endogenous DHA, but additional research is needed to

elucidate the mechanism underlying VitE's essentiality and the role VitE plays in facilitating healthy neurological outcomes. Though the literature is replete with examples of DHA's neurological benefits [87, 120, 133, 275, 276, 299, 300]; less well understood are the adverse consequences of DHA depletion due to inadequate antioxidant protection and increased lipid peroxidation. Accordingly, numerous human studies [76, 301] show that the VitE requirement is related directly to PUFA intake and increases proportionally with greater PUFA consumption [292], as well as with PUFA chain length and unsaturation [302].

Increased DHA peroxidation alone likely does not explain the adverse consequences of VitE deficiency. Indeed, my studies show another serious perturbation in embryonic metabolism following induced by inadequate VitE: namely, depletion of choline, which potentially leads to alterations in the supply of methyl donors, methylation reactions, and potentially changes in epigenetic targets. Choline, as a key member of one-carbon metabolism methylation pathways, plays a central role in modulating gene expression through epigenetic methylation processes [167]. Neonatal methyl-donor deficiency (*e.g.* of choline and/or folate) causes dysregulation of global DNA methylation in brains of developing mice and is associated with impaired neurodevelopment and behavior. Since 95% of bodily choline is found as the phospholipid PC in most tissues [108] and PC species are depleted in VitE-deficient zebrafish embryos and adult brains, it follows that disrupted phospholipid metabolism due to VitE deficiency causes secondary depletion of choline.

The above neurological implications can be explained by the "latent early-life associated regulation" (LEARn) model, which postulates that factors, such as neonatal nutrition, modify expression levels of AD-associated genes in a long-term fashion, beginning *in utero* during neurodevelopment [227]. Such latent changes are maintained by epigenetic alterations in the promoter regions of genes, such as changes in DNA methylation, DNA oxidation, and chromatin organization, but these modifications do not have pathological results until significantly later in life, following the accumulation of additional environmental insults, like oxidative damage, incurred as the individual ages. Interestingly, adult AD patients often display high homocysteine and low methyl donor levels [303], suggesting a dysregulation in the SAM cycle required for epigenetic regulation through DNA methylation. It is also worth noting that expression of several AD-related genes, including APP and β-APP cleaving enzyme

(BACE) genes, are regulated via methylation of their promoters [304]. Finally, evidence in animal models has linked maternal methyl-donor deficiencies with decreased DHA status, enhanced oxidative stress, and neurodegeneration [15, 16, 40], providing a rationale to evaluate the neurophysiological interdependencies between VitE, DHA, and choline within the context of AD pathogenesis. To do so requires an *in vivo* system amenable to lifetime dietary manipulations, in which long-term consequences of developmental exposures may be readily assessed in the adult. The zebrafish model provides such a system.

The chronic inadequate VitE intakes in the US suggests that lack of this critical nutrient could be a potential cause of abnormal neurodevelopment and, potentially, contribute to the increased prevalence of neurodegenerative disease in later life [3]. However, the 2015 USDA Dietary Guidelines Committee [79] failed to endorse VitE as a "nutrient of public health concern," likely due to the lack of a mechanistic rationale supporting its functional role in promoting human health. Implicit within this present debate is the absence of a clearly defined mechanism of action showing the actual ramifications of VitE inadequacy to support its physiological essentiality. Without a more integrated and comprehensive explanation, it is difficult to provide a mechanistic basis for why the human brain requires VitE during development. Thus, the studies I conducted herein addressed the unique concept that defects in the VitE-deficient, developing embryo are not solely the result of increased lipid peroxidation, but, importantly, also are highly dependent upon resulting, *secondary* nutrient deficiencies, including increased depletion of DHA and choline. I utilized a novel zebrafish model of both developmental (embryonic) and chronic (adult) VitE deficiency to elucidate the mechanistic relationship between these nutrients and the inception of neurological disease.

Summary

The purpose of this project was to provide an evidence-based reassessment of the status quo of VitE by shifting focus towards metabolic interactions between VitE and specific membrane lipids to elucidate the biochemical basis underlying VitE's neurological function *in vivo* during neurodevelopment and into adulthood. My experiments are innovative in that I exploited a zebrafish model for the study of nutrition and dietary manipulation and the use of novel "omics" methodologies. Further, this model allowed for assessment of lasting effects of developmental VitE

deficiency via evaluation of embryonic zebrafish grown to the juvenile stage. I have published compelling evidence that provides the scientific underpinnings for my working hypothesis that the major role for VitE in the brain is to protect DHA-PLs against oxidative stress [72, 305-307]. The studies included in the following chapters describe and expand these findings, thereby providing insight as to how inadequate VitE perturbs DHA, phospholipid, and choline metabolism, resulting in dysregulation of other metabolic pathways and mitochondrial function, as well as epigenetic methylation reactions, and how disruption of these processes compromises functional and behavioral neurological outcomes.

HYPOTHESIS AND SPECIFIC AIMS

The overall goal of this work was to elucidate the mechanism(s) through which VitE contributes to lifetime brain health. To achieve this, I utilized both developmental (embryonic) and chronic (adult) models of dietary VitE deficiency, which allowed me to evaluate the consequences of inadequate VitE from the earliest stages of brain development through middle-age. My central hypothesis was that VitE protects DHA, a vital substrate for brain membrane phospholipid maintenance, and that dysregulation of DHA-PL status due to restricted dietary VitE severely perturbs critical events necessary for embryonic neurodevelopment that, ultimately, increase susceptibility for consequent, persistent cognitive impairments. I proposed three Specific Aims to provide mechanistic support for this global hypothesis:

AIM 1: Elucidate the underlying mechanism(s) by which VitE deficiency impairs neurodevelopment in VitE-deficient (E–) vs. VitE-sufficient (E+) zebrafish embryos.

Hypothesis: Inadequate embryonic VitE perturbs DHA and DHA-PL status, which leads to dysregulation of membrane phospholipid remodeling and consequent metabolic changes that together severely disrupt neurodevelopment, thereby resulting in behavioral abnormalities as well as increased embryonic morbidity and mortality.

AIM 2: Determine the extent to which chronic dietary VitE deficiency compromises neurological outcomes in E- vs. E+ adult zebrafish.

Hypothesis: Inadequate dietary VitE during adulthood causes depletion of brain DHA and DHA-PLs, which leads to nervous system aberrations that result in significant functional and cognitive impairments.

AIM 3: Evaluate the lifetime neurological consequences of developmental VitE deficiency in a cohort of E- vs. E+ zebrafish embryos raised to the larval stage.

Hypothesis: The dysregulation of biochemical processes and resulting neurological perturbations due to inadequate VitE during critical periods of neurodevelopment are manifest beyond the embryonic period and are irreversible despite ensuing VitE and DHA repletion.

The outcomes of these completed aims provide mechanistic evidence regarding VitE's essentiality for human neurodevelopment and adult brain function, and yield new insights regarding the role early-life VitE deficiency may have in the etiology of cognitive decline and ensuing neurodegenerative disease.

CHAPTER TWO:

Lipidomics and ^{18}O-water labeling techniques reveal increased remodeling of DHA-containing membrane phospholipids associated with abnormal locomotor responses in α-tocopherol deficient zebrafish (*Danio rerio*) embryos.

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2016 Aug; 8:165-74.
doi: 10.1016/j.redox.2016.01.0

Abstract

We hypothesized that vitamin E (α-tocopherol; VitE) is required by the developing embryonic brain to prevent depletion of highly polyunsaturated fatty acids, especially docosahexaenoic acid (DHA, 22:6), the loss of which we predicted would underlie abnormal morphological and behavioral outcomes. Therefore, we fed adult 5D zebrafish (*Danio rerio*) defined diets without (E-) or with added α-tocopherol (E+, 500 mg RRR-α-tocopheryl acetate/kg diet) for a minimum of 80 days, and then spawned them to obtain E- and E+ embryos. The E- compared with E+ embryos were 82% less responsive (p<0.01) to a light/dark stimulus at 96 hours post-fertilization (hpf), demonstrating impaired locomotor behavior, even in the absence of gross morphological defects. Evaluation of phospholipid (PL) and lysophospholipid (lyso-PL) composition using untargeted lipidomics in E- compared with E+ embryos at 24, 48, 72, and 120 hpf showed that four PLs and three lyso-PLs containing docosahexaenoic acid (DHA), including lysophosphatidylcholine (LPC 22:6, required for transport of DHA into the brain, p<0.001), were at lower concentrations in E- at all time-points. Additionally, $H_2{}^{18}O$ labeling experiments revealed enhanced turnover of LPC 22:6 (p<0.001) and three other DHA-containing PLs in the E- compared with the E+ embryos, suggesting that increased membrane remodeling is a result of PL depletion. Together, these data indicate that VitE deficiency in the zebrafish embryo causes the specific depletion and increased turnover of DHA-containing PL and lyso-PLs, which may compromise DHA delivery to the brain and thereby contribute to the functional impairments observed in E- embryos.

Introduction

The zebrafish model presents a highly useful and translational experimental system for investigating the impact of VitE status on neurodevelopment, as zebrafish and humans have similar dietary antioxidant requirements [308]. VitE's requirement during development may be due primarily to its fundamental role in facilitating healthy brain formation because knockdown of the α-tocopherol transfer protein in zebrafish embryos causes lethal head and/or brain malformations [297]. Further, we have observed that VitE deficient (E-) zebrafish embryos, when compared to VitE sufficient (E+) embryos, begin to develop morphologic abnormalities at 48 hours post-fertilization (hpf) [297], the severity of which increase from 48-72 hpf with simultaneous increases in E- embryo mortality.

We propose that that the physiological link between brain health and adequate VitE relates to the vitamin's role as the body's most potent lipophilic antioxidant [49], whereby it protects highly unsaturated membrane lipids that are vulnerable to peroxidation [44]. Foremost among these in terms of susceptibility to peroxidation is docosahexaenoic acid (DHA; 22:6) [309], a long-chain ω-3 poly-unsaturated fatty acid (PUFA) that is highly concentrated in nervous tissue [18] and is critical for neurodevelopment [115, 275], as well as for preservation of lifetime neurological function [117, 276]. Given that the developing brain consumes 60% of fetal oxygen [34], this environment makes adequate antioxidant status especially crucial for protection of vital membrane lipids like DHA.

We recently demonstrated that VitE deficiency significantly altered the PL composition of the brain in adult E- zebrafish by causing the specific depletion of PL species containing DHA [72]. Intriguingly, we also found that VitE deficiency disrupts brain lysophospholipid (lyso-PL) status, resulting in an overall depletion of lipid species by approximately 60% [72]. Lyso-PLs are substrates for the synthesis, remodeling, and repair of membrane PLs [310, 311]; thus, the results from our adult studies suggest these processes are compromised in the E- brain. One depleted lyso-PL species in particular, lysophosphatidylcholine (LPC) 22:6, also is essential for normal DHA delivery to the brain [312, 313], where evidence shows that DHA is required for function and maintenance of neural membranes, such as the blood-brain barrier [314, 315]. In addition, inadequate brain DHA has been found to compromise membrane integrity within the developing zebrafish [316], leading to brain deformities and early mortality.

In light of our lipidomics data from adult E- zebrafish brains [72] as well as our past studies showing morphological defects that coincide with the rapid depletion of DHA during development in E- embryos [278, 286], we hypothesized that embryonic VitE deficiency compromises brain development by decreasing the supply of DHA to the embryonic brain, which perturbs DHA-containing PL and lyso-PL (specifically, LPC 22:6) status and, hence, processes of membrane PL remodeling. Further, given the numerous reports of adverse behavioral outcomes due to embryonic DHA deficiency, we also postulated that the disruption of membrane DHA content may correlate with the onset of behavioral abnormalities in the developing embryonic zebrafish. Herein, we exploited our high-throughput embryonic assessments and lipidomics techniques,

as well as developed a method to measure PL turnover in zebrafish embryos using $H_2{}^{18}O$ labeling, to gain mechanistic insight on the organism-level effects of developmental VitE deficiency.

Materials and Methods

Materials

1,2-ditridecanoyl-*sn*-glycero-3-phosphocholine (DT-PC) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and used without further purification. $H_2{}^{18}O$ (^{18}O, 97%) was obtained from Cambridge Isotope Laboratories (Tewksbury, MA). Butylated hydroxytoluene (BHT) was obtained from TCI America (Portland, OR).

Zebrafish husbandry and diets

The Institutional Animal Care and Use Committee (IACUC) of Oregon State University approved this protocol (ACUP Number: 4344). Tropical 5D strain zebrafish were housed in the Sinnhuber Aquatic Research Laboratory. Adults 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 (Instant Ocean®) to create a salinity of 600 microsiemens. Sodium bicarbonate was added as needed to adjust the pH to 7.4.

At 55 days post-fertilization (dpf), zebrafish were randomly allocated to one of two diet groups, VitE deficient (E-) or VitE sufficient (E+), and fed one of the defined diets for the duration of the study [296]. The defined diets, which contained only fatty acids with 18 or fewer carbons and 2 or 3 double bonds [278, 296], were prepared with the vitamin C source as StayC (500 mg/kg, Argent Chemical Laboratories Inc., Redmond, WA) and without (E-) or with added VitE (E+, 500 mg *RRR*-α-tocopheryl acetate/kg diet, ADM, Decatur, IL), as described previously [286, 296]. Diets were stored at -20°C until fed to the adult zebrafish.

E- and E+ embryos were obtained from adult fish fed either the E- or E+ diet, respectively, for a minimum of 80 days. Embryos were obtained through natural group spawning, collected, and kept in standard embryo media (EM; prepared as described [308] from 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 4.2 mM $NaHCO_3$; pH 7.2-7.4). Embryos used for biochemical analysis, described below, were euthanized by cold exposure (placed on ice of a minimum of 30 min.) prior to sampling. Note that embryos are not fed prior to 120 hours post fertilization (hpf), as each is a complete unit containing only those

nutrients present in the yolk when eggs are laid, which the embryo fully utilizes by ~120 hpf. For the experiments described herein, the E+ embryos are considered the control condition.

Vitamin E analyses

Using high pressure liquid chromatography with electrochemical detection (HPLC-ECD), VitE was measured both in diet samples and embryos, as described [317]; ascorbic acid content in diet was measured using HPLC-ECD as described [318]. Measured VitE concentrations in the E- and E+ diets were 0.45 ± 0.01 and 369 ± 2 mg/kg (*n*=3 replicate samples measured for each diet), respectively; vitamin C was 143±16 mg ascorbic acid/kg. This level of dietary vitamin C has been found to be adequate for the zebrafish [74, 319]. Measured VitE concentrations in the E- and E+ embryos at 24 hpf were 3.4 ± 0.1 and 105 ± 3 pmol/embryo, respectively, similar to previous reports [278, 286].

Evaluation of phenotypic and developmental progress

At 24 hpf, embryos were assessed for viability, developmental progression and spontaneous movements (earliest behavior in zebrafish), using the zebrafish acquisition and analysis program (ZAAP). ZAAP is a custom program designed to inventory, acquire, and manage zebrafish data, and was used to collect 18 developmental endpoints, as either present or absent (i.e. binary responses were recorded, described below) [320]. Developmental progression is considered perturbed if zebrafish are more than 12 hours delayed compared to control animals. Spontaneous movements are assessed over a 2-min. period and are considered perturbed if there is a lack of embryonic contractions and/or movement. At 96 hpf, larval morphology (body axis, eye, snout, jaw, otic vesicle, notochord, heart, brain, somite, fin, yolk sac, trunk, circulation, pigment, and swim bladder) was evaluated and recorded and behavioral endpoints (motility, tactile response) were thoroughly evaluated *in vivo*. If the embryo was dead at either 24 or by 96 hpf, the non-mortality endpoints were not included in the evaluations.

Behavioral assessments

Locomotor activity was measured using Viewpoint Zebrabox [321, 322] in a total of *n*=128 embryos per diet group. Briefly, at 96 hpf, the plates containing the embryos were placed in a Viewpoint ZebraBox (software version 3.0, Viewpoint Life Sciences, Lyon, France). Embryo locomotor activity was assessed using the “tracking”

setting during alternating periods of light and dark, a modification of [258]. Embryos subjected to this test typically move less during the light periods and more during dark periods, and behavioral differences can be determined by comparing distances moved during the light and/or dark periods. Locomotor activity in response to the light/dark transition was tracked during 3 min. periods of alternating light and dark for a total of 24 min. The integration time was set to 6 seconds to increase statistical power. A high definition camera (30 frames/second) tracked the total movement (swim distance) in response to the multiple light-dark transitions.

Zebrafish embryo lipidomics analyses

At 12 hpf, E- and E+ embryos were transferred one embryo per well into 96 well plates containing 100 µL EM per well. Following 24, 48, 72, and 120 hpf, embryos (15 per replicate, *n*=4 replicates per group) were transferred to 1.5 mL Eppendorf tubes, covered with EM, and kept on ice for 30 min. to euthanize the animals. EM was carefully removed to prevent loss of embryos and samples were stored at -80^{o} C overnight. To extract embryos for lipidomics analyses, solvent (300 µL, 25:10:65 v/v/v methylene chloride: isopropanol: methanol, with 50 µg/mL butylated hydroxytoluene [BHT]) and internal standard (0.5 µg/µL, 1,2-ditridecanoyl-*sn*-glycero-3-phosphocholine [DT-PC, PC 26:0] in methanol) were added. Next, sample extracts were homogenized with 0.5 mm zirconium oxide beads (Next Advance, Inc., Averill Park, NY, product #ZrOB05) using a counter-top bullet blender for 6 min.; then, following a 15-min. incubation on ice, the extracts were centrifuged at 4 ^{o}C at 15,000×*g* for 13 min. Aliquots (200 µL) of the upper layer were transferred individually to new tubes and stored at -80^{o} C until analysis.

$H_2{}^{18}O$ incorporation by embryos

To optimize $H_2{}^{18}O$ labeling into lipids, a cohort of laboratory embryos (*n*=180; from adult 5D zebrafish not subjected to dietary manipulation) were incubated with increasing concentrations of $H_2{}^{18}O$ (0% to 50% v/v in EM) from 48 to 72 hpf (*n*=15 embryos per replicate, 2 replicates per concentration) to determine the $H_2{}^{18}O$ concentration that yielded the greatest label incorporation. Incubation for 24 hours (48-72 hpf) with a 40% v/v concentration of $H_2{}^{18}O$ provided optimal labeling of PL species (*Supplementary Figure 1*). Embryos showed no signs of toxicity when assessed using a phenotypic screen (ZAAP), described above, following exposure to any concentration of the $H_2{}^{18}O$ label used in the present experiment. The outcome of this

pilot trial informed the $H_2{}^{18}O$ labeling study with E- and E+ embryos, in which a cohort of 48 hpf E- and E+ embryos (*n*=120/group) was incubated with either 40% v/v $H_2{}^{18}O$ in EM (*n*=60/group) or with 40% v/v reverse-osmosis water (H_2O) in EM (*n*=60/group) from 48 to 72 hpf. At 72 hpf, embryos used for the labeling study were extracted as described above for lipidomics sample preparation.

UPLC-TOF-MS/MS analyses

Lipidomics analyses of the embryo extracts were carried out to identify lipids, which changed because of the dietary manipulation. Liquid chromatography/mass spectrometry (LC/MS) was performed using a UPLC with a 1.8 µm particle 100 × 2.1 mm id HSS T3 column (Waters, Milford, MA) coupled to a hybrid quadrupole time-of-flight mass spectrometer (UPLC-TOF-MS/MS; SCIEX 5600) operated in information dependent MS/MS acquisition mode, as described [72]. Data were generated from the UPLC separation of each lipid extract using TOF accurate mass detection and MS/MS fragment characterization. Data was imported into PeakView software (Version 1.2, SCIEX) for relative quantification and lipid identification. Lipid species were confirmed by validated UPLC retention times, high resolution MS, MS/MS fragmentation, and isotopic distribution using the PeakView database. Peak intensities were normalized using DT-PC (internal standard) intensities and then used for relative quantification between E+ and E- embryo samples and expressed per embryo based on the numbers of embryos extracted.

For assessment of $H_2{}^{18}O$ labeling, extracts were analyzed using the UPLC-TOF-MS/MS by following the lipidomics protocol. The incorporation of the $H_2{}^{18}O$ label was determined by comparing $[M+H]^+$ and $[M+2+H]^+$ mass spectral peak intensities from isotopic distributions of individual lipid species. Mass spectral peak identities were confirmed as described above, using validated UPLC retention times, high resolution MS, and MS/MS fragmentation. The ratio of $[M+2+H]^+/[M+H]^+$ was used as a metric to measure the extent of $H_2{}^{18}O$ label incorporation (minus the ratio found for unlabeled-H_2O incubated embryos).

Statistical analyses

Heatmaps of lipidomics data were generated using Metaboanalyst software [323]. Sample intensities were normalized against the intensity of internal standard (DT-PC) and then scaled with Pareto scaling. All lipidomics statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad, La Jolla, CA). Multiple,

unpaired student's *t*-tests with Sidak-Bonferroni post-test for multiple comparisons ($p<0.05$) were performed for datasets from each time-point (24, 48, 72, and 120 hpf), for each individual lipid species to identify differences between specific lipids in E- vs. E+ embryos. Lipid species that were significantly different for at least three of the four time-points between the E- and E+ diet conditions were then analyzed using two-way ANOVA comparisons (diet vs. time) with either a Bonferroni's or Tukey's post-test for multiple comparisons (as recommended by the software) to determine significant ($p<0.05$) differences during the developmental time-course.

For analysis of significance of the $H_2{}^{18}O$ incorporation, data from embryos incubated with the $H_2{}^{18}O$ label were analyzed using repeated measures two-way ANOVA with a Tukey's or Sidak's post-test (p <0.05; as recommended by the software) for multiple comparisons.

Statistical analyses for morphological and behavioral endpoints were performed using code developed in R (R Developmental Core Team 2014 http://www.R-project.org). For morphological assessments at 24 and 96 hpf, binary responses were recorded as either absent (0) or present (1) for each of the 18 endpoints.

For statistical analyses of behavior, raw data files were processed using custom R scripts (R Developmental Core Team 2014, http://www.R-project.org) with methodologies based on those previously described [324], to average the total distance traveled for each integration time-point, then the area under the curve was computed. The overall area under the cover was compared to the control (E+ defined diet) using a combination of percent change (30% difference from the control) and a Kolmogorov-Smirnov test ($p<0.05$).

Results

Embryonic VitE deficiency leads to behavioral impairments in E- embryos

Morphological assessments in the present study were in accord with past observations [278]. Approximately 75-88% of the E- embryo cohorts evaluated either died or developed morphological abnormalities after hatching from the chorion, between 48-72 hpf. The most prevalent abnormalities noted at 72 hpf were pericardial and yolk sac edema, as found previously [278]. By 120 hpf, the majority of E- embryos also demonstrated failure to inflate the swim bladder (**Figure 1**), even when other abnormalities were absent. Despite this high level of morbidity and mortality in the E-

condition, a sub-set of each spawn (12-25% on average) remained morphologically normal when compared to E+ embryos at 96 hpf, and appeared to sustain no developmental abnormalities. Therefore, we performed behavioral assessments to determine if these “normal” E- embryos suffered from behavioral impairments that were not evident in the phenotypic screen.

We evaluated the behavioral impact of developmental VitE deficiency with a high-throughput locomotor response assay and a modification of the protocol previously described [258]. Any dead or malformed embryos at 96 hpf were excluded from the data analysis. E- embryos were 82% less active in the dark phases than were E+ embryos (**Figure 2**). Of note, the included E- embryos displayed normal motility and tactile responses during the phenotypic screen that were comparable to evaluations of the E+ condition, indicating E- embryos have a functional motor system.

Untargeted lipidomics analysis shows significant changes in lipid profiles between E- and E+ embryos

Based on our recent finding that VitE deficiency results in significant changes in the phospholipid (PL) and lysophospholipid (lyso-PL) composition and status of brains from E- adult zebrafish [72], we hypothesized that embryonic VitE deficiency also would alter PL and lyso-PL levels in E- embryos. To examine alterations in lipid distribution and relative abundances between E- and E+ embryos, we used an UPLC-TOF-MS/MS lipidomics technique. Four PL classes were identified in embryo extracts, including 115 individually validated PLs and lyso-PLs. PL classes were detected in either positive (phosphatidylcholine [PC], phosphatidylethanolamine [PE], lysophosphatidylcholine [LPC], lysophosphatidylethanolamine [LPE]) or in negative ion modes (phosphatidylserine [PS] and lysophosphatidylserine [LPS], phosphatidylinositol [PI], and lysophosphatidylinositol [LPI]); PC and PE were detected in both ion modes.

Identities of all PL and lyso-PLs were confirmed using exact mass matching and MS/MS fragmentation patterns; for example, PE species generated the characteristic losses of 43 and 140 Da (positive ion mode) known to be associated with the PE head group, while PS species showed -87 and -184 Da losses (negative ion mode). Significant changes in the PL and lyso-PL profiles of E- compared to E+ embryos were evident at each developmental time-point (**Figure 3**). A global evaluation of these differences revealed that, while E- embryos tended to have

relatively higher levels of many PLs and lyso-PLs during early development (24-48 hpf), levels of these same lipids were markedly lower in the E- than in the E+ condition by 120 hpf.

Embryonic VitE deficiency causes the specific depletion of phospholipid and lysophospholipid species containing DHA moieties

We hypothesized that E- embryos would contain lower levels of DHA-containing PLs and lyso-PLs (e.g. PC 38:6), as past investigations found the overall depletion of DHA in developing E- embryos [286]. Comprehensive analyses of the time-course lipidomics dataset showed that, while levels of only four out of the 75 identified PLs were significantly different between the E- and E+ embryos at all four developmental time-points, each contained DHA (22:6) and each was lower in the E- condition by 48 hpf (**Figure 4**). Further, in-depth evaluation of differences in the lyso-PL composition between E- and E+ embryos demonstrated similar findings. Of the 40 identified lyso-PLs, levels of only six were significantly different during multiple (three out of four) developmental time-points, and the three lyso-PLs showing the most significant differences between E- and E+ conditions contained DHA (22:6) (**Figure 5**).

Increased $H_2{}^{18}O$ label incorporation demonstrates enhanced membrane phospholipid remodeling in E- embryos

The ^{18}O-labeling technique measures ester hydrolysis of parent PLs in the presence of $H_2{}^{18}O$, which results in the incorporation of ^{18}O into the carboxyl group of the resulting lyso-PL fatty acid; upon reacylation, the ^{18}O is incorporated into the newly formed ester bond of the new PL [107, 325], as outlined for PC (**Figure 6**). The time-dependent increase in the incorporation of the ^{18}O label into PL species can, therefore, be taken as a metric of acyl turnover, and, subsequently, processes of PL remodeling and repair [326]. Thus, we divided a cohort of 48 hpf E- and E+ embryos (*n*=120/group), then incubated half with either 40% v/v $H_2{}^{18}O$ (*n*=60/group) or with 40% v/v H_2O (*n*=60/group) for 24 hours from 48 to 72 hpf. This time-frame was chosen because it coincided with the onset of morbidity and mortality outcomes in E- embryos, which we hypothesized may be due to underlying perturbations in PL remodeling and/or repair mechanisms. Label incorporation was significantly elevated in E- embryos in four PC lipids specifically containing long-chain polyunsaturated fatty-acids, particularly DHA (22:6) (**Figure 7A and B)**. The concomitant increases in $H_2{}^{18}O$

labeling of both LPC 16:0 and LPC 22:6 in E- embryos (**Figure 7C and D**), show these lyso-PCs are involved during the remodeling of PC 38:6.

Discussion

Our discoveries regarding the effects of VitE deficiency during zebrafish embryonic development show that E- embryos have such severe morphologic defects that the survivors display altered behaviors. Moreover, increasing defect severity is accompanied by depletion of a relatively limited set of PLs and lyso-PLs containing DHA, which causes increased PL remodeling.

In agreement with our past reports [278], E- embryos begin to accumulate morphological abnormalities between 48 and 72 hpf and most embryos are severely malformed or dead by 120 hpf. Most E- embryos surviving to 120 hpf fail to inflate the swim bladder, even when other developmental deformities are absent (**Figure 1A)**. We do not at this time know if such an observation represents a defective swim bladder or merely a delay in its inflation; however, both are portents of poor survival outcomes during later stages of development.

Developmental VitE deficiency may compromise brain function, as apparently "normal" E- embryos are 82% less active during dark phases than are E+ embryos (**Figure 2**), suggesting a significantly impaired neurobehavioral response [258, 327]. The E- embryos demonstrated a normal, robust tactile (touch) response comparable to that of E+ embryos when subjected to a phenotypic screen, indicating that the reason underlying the impaired behavioral response is associated with compromised brain, rather than muscle, function. It also is possible that the E- embryos suffer compromised visual acuity; however, eye morphology and movements in the E- group were apparently normal during the phenotypic screen. Given that the E- embryos *did* demonstrate greater locomotor activity during the dark when compared to the light phases, we believe that compromised visual acuity was not the main cause of the impaired behavior observed in the E- condition. Instead, we argue that the perturbed behavior in E- embryos is primarily due to consequences of inadequate brain VitE and the subsequent changes in the brain PL and lyso-PL composition.

Our lipidomics data supports the hypothesis that the behavioral abnormalities in the E- group are, at least in part, a consequence of disrupted PL and lyso-PL status during development. While significant differences between E- and E+ embryos are noticeable in the global PL and lyso-PL pool at each developmental time-point (**Figure**

3), we found that only four PLs, all containing DHA, are significantly decreased throughout development from 24 to 120 hpf in E- when compared to E+ embryos (**Figure 4**). Two of these, PC 38:6 (also lower by 30% in adult E- vs. E+ zebrafish brains [72]) and PC 40:6, are plasma biomarkers that are depleted in humans who progress to dementia [277], raising the provocative possibility that lower levels of these PLs cause compromised neurodevelopment.

Relatedly, we found that six lyso-PLs are significantly altered in E- embryos during the developmental time-course (**Figure 5**). Of these, three contained DHA (LPC 22:6, LPE 22:6, and LPS 22:6) and were lower in the E- condition by 120 hpf, despite being higher in E- embryos at 24 hpf. Moreover, while levels of the DHA-containing lyso-PLs *increased* during development in E+ embryos, all *decreased* (rather substantially in the cases of LPC 22:6 and LPE 22:6) in E- embryos, suggesting developmental VitE deficiency leads to the rapid depletion of available DHA-containing lyso-PLs, in agreement with observed depletion of total DHA in developing E- embryos [286], for maintenance of membrane DHA-containing PLs. This may be particularly distressing to the brain, which sequesters substantial amounts of DHA during neurodevelopment [280], especially as LPC 22:6, the preferred transport and uptake form of DHA across the blood-brain barrier [315, 328]. It is possible that the decrease in LPC 22:6 during development in the E- embryos represents an impaired ability to provide adequate DHA to the brain, and that by 96 hpf (the time at which behavior was assessed) this may adversely affect neurological function. Similarly, as the major storage form of DHA in nervous tissue is within membrane PE lipids [329, 330], lower LPE 22:6 levels in the E- embryos suggests a simultaneous decrease in the brain DHA reservoir that also could underlie abnormal behavior.

Other changes in the lyso-PL composition of the E- embryos are worthy of comment: for example, LPI 20:4, the only inositol-containing PL to be significantly different between E- and E+ embryos, contains arachidonic acid (ARA, 20:4 ω-6). An important function of ARA is phospholipid-mediated signal transduction. In the brain, phospholipase A_2 (PLA_2), which removes ARA from the *sn*-2 position of membrane PLs, is activated by glutamatergic, serotonergic, cholinergic, and dopaminergic signaling, releasing ARA as a secondary messenger [124, 331]. ARA also is the major substrate for synthesis of bioactive eicosanoids, endogenous stimulators of myriad cellular responses [132], such as inflammation, that act through signal transduction

pathways mediated by inositol PLs [332, 333].The relevance of altered levels of PI lipids and the perturbed brain function as well as morphological deformities observed in E- embryos represents, therefore, an area that warrants additional research.

It should be noted that not all lyso-PLs were depleted in E- embryos. Levels of LPC 16:0 were higher in the E- than in the E+ condition throughout development. The increase in LPC 16:0 during development in the E- group, along with the reported concomitant decrease in LPC 22:6 (**Figure 5**), supports our hypothesis that membrane turnover of DHA-containing PLs (especially DHA 38:6, which contains both 16:0 and 22:6 fatty acyl chains) is enhanced in E- embryos. Hypothetically, if DHA is depleted by lipid peroxidation, then the saturated lyso-PLs (e.g. LPC 16:0) would be expected following PLA_2 action [334, 335].

This evidence agrees with the outcomes of our $H_2{}^{18}O$ labeling experiment, which revealed that E- embryos had greater incorporation of the $H_2{}^{18}O$ label into several DHA-containing PLs (**Figure 7**), as well as LPC 22:6, a required substrate for membrane PC remodeling. In the E- group, $H_2{}^{18}O$ incorporation also was elevated significantly in LPC 16:0 (**Figure 7C and D**), further supporting our hypothesis that PC 38:6 (and by extension other DHA-containing PLs) is depleted from the membrane PL pool due to increased turnover. In addition, LPC 22:6 label incorporation was not increased significantly in the E+ embryos (**Figure 7C**), indicating that in the presence of adequate VitE, the DHA-containing lyso-PL reserve (with DHA presumably at the *sn*-2 position for incorporation into remodeled membrane PLs) remains stable. While our 24-hour incubation time and high $H_2{}^{18}O$ label concentration protocol may reveal only the most gross changes in PL remodeling processes due to VitE deficiency, the fact that we found such selective labeling of DHA-containing PLs, species that also were decreased in the E- condition, makes the outcomes of this experiment all the more significant.

The alterations in both PL and lyso-PL levels and label incorporation we observed in the E- relative to the E+ embryos are, potentially, the result of a depletion of DHA-containing lipids and an increased requirement for DHA to remodel and repair affected PLs. Hypothetically, with respect to our recent finding of increased DHA peroxidation in adult E- zebrafish brains, [72] insufficient VitE concentrations during development may allow lipid peroxidation to deplete not only brain DHA-PLs, but DHA throughout the body, thereby limiting DHA delivery (e.g. via LPC 22:6) to the brain. We

have evidence of increased DHA peroxidation in E- embryos [286], suggesting that this is the cause of the selective depletion and enhanced remodeling of DHA-containing lipids reported in the present study. While we did search our lipidomics data specifically for oxidized DHA (and other PUFA) derivatives, the amount of sample material proved to be too small to allow for accurate identification and quantification of these oxidized lipids. Larger sample sizes and more targeted analyses will be necessary to confirm that greater lipid peroxidation in the E- condition leads to the ensuing depletion and enhanced remodeling of DHA-containing PLs that we observed.

In summary, the results of this study show that embryonic VitE deficiency causes: 1) morphological abnormalities, 2) an impaired locomotor response suggestive of compromised neurological function, 3) significant changes to the PL and lyso-PL profile of developing zebrafish embryos, and 4) a selective decrease in DHA-containing PL and lyso-PL species during the developmental time-course that matches evidence of their enhanced turnover in E- embryos. Our data demonstrate that critical lipids are protected by VitE and suggest that their depletion may be involved in the mechanism(s) leading to observed behavioral defects.

Acknowledgements

The authors thank Carrie L. Barton, Greg D. Gonnerman and Scott W. Leonard for providing excellent technical assistance. This work was supported by National Institutes of Health Grants S10RR027878, NICHD HD062109 (M.G.T. and R.L.T.), and NIEHS ES000210. M.Q.M is supported in part by National Science Foundation Grant DGE 0965820.

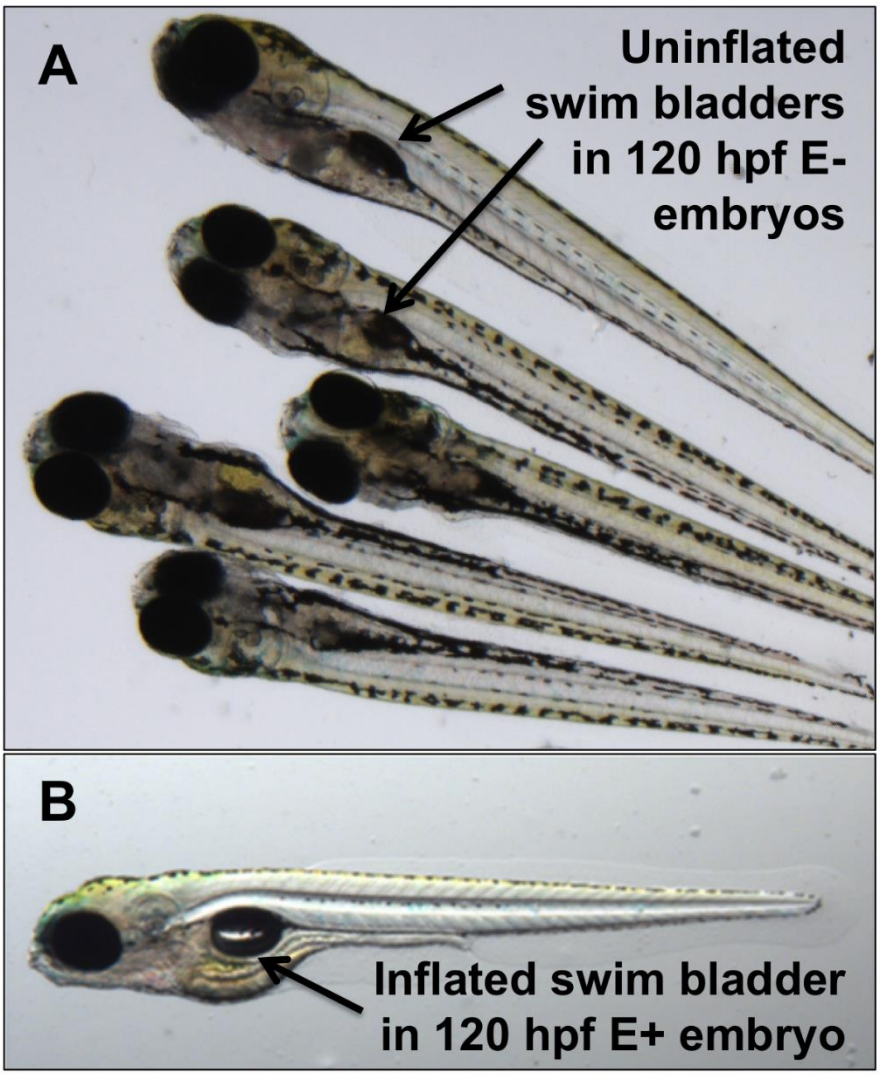


Figure 1. E- embryos have morphological defects and increased mortality compared to E+ embryos. Representative pictures from the two diet groups at 120 hours post-fertilization (hpf). A. Uninflated swim bladders in elsewise morphologically normal E- embryos that survived to this stage. B. An E+ embryo provided for comparison. Morphological evaluations were made using the zebrafish acquisition and analysis program (ZAAP). Phenotypic differences in E- embryos became evident between 48 and 72 hpf, with most mortalities occurring by 72 hpf. By 120 hpf, 75-88% of E- embryos from each spawn were malformed or dead.

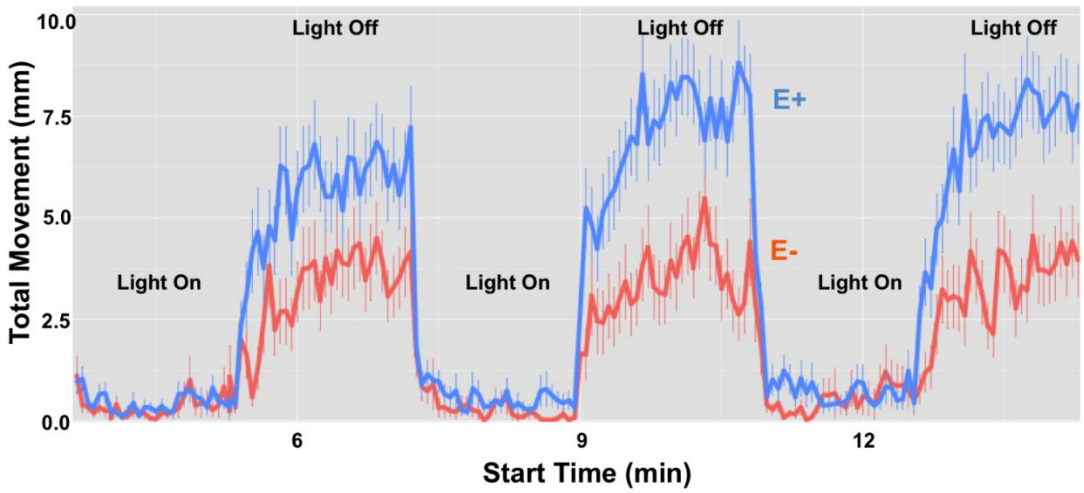


Figure 2. E- embryos have impaired behavior compared to E+ embryos when assessed using a locomotor response assay. Embryos were analyzed in 96-well plates, one embryo per well with 128 embryos per diet condition. Locomotor activity following a series of light/dark stimuli (one stimulus every 3 min. for 24 min. total) was measured as movement (mm) over time (seconds); criteria for statistical significance between conditions was an area-under-curve (AUC) difference of >30% and $p<0.05$ (Kolmogorov-Smirnov test). At 96 hpf, E- embryos (red) were 82% less responsive to the light than were E+ embryos (blue) (E- AUC:459; E+ AUC:2468; $p<0.01$). Dead or malformed embryos were removed prior to data analysis (apparently normal embryos: *n*=54 E-; *n*=94 E+). The abnormal behavioral response in the E- embryos is indicative of underlying perturbations in neurological processes, as there were no observed differences in swimming behavior between E- and E+ conditions to suggest motor impairments in the former.

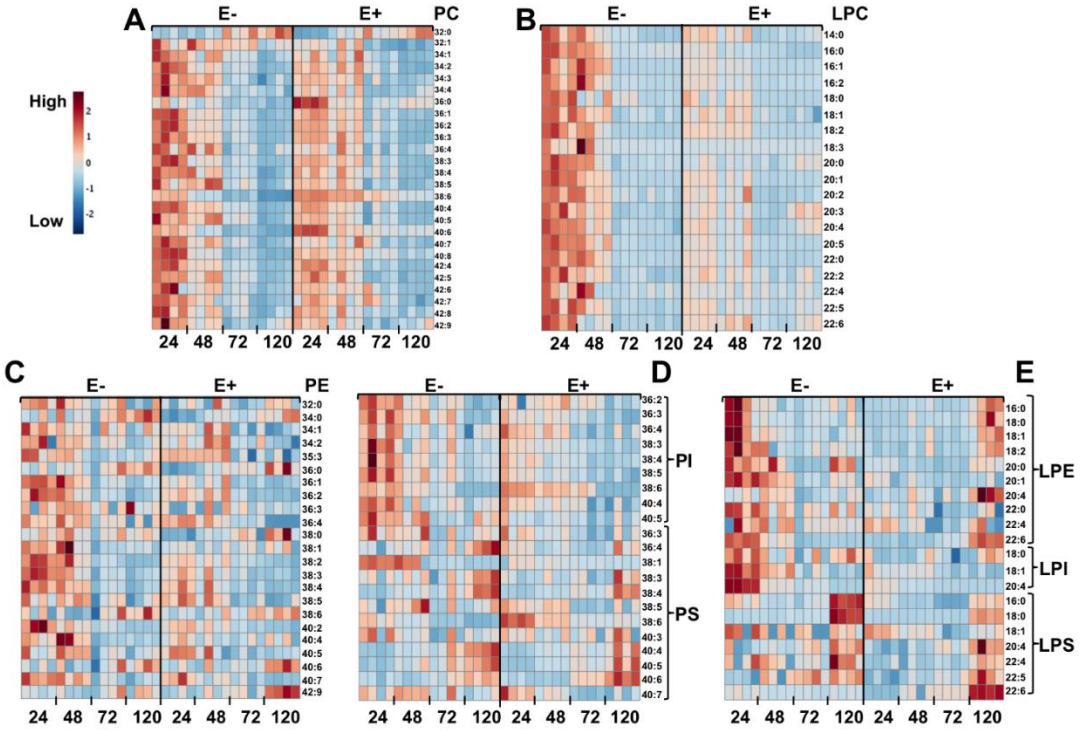


Figure 3. E- and E+ embryos display significantly different PL and lyso-PL composition profiles during development. Heatmaps of identified PL or lyso-PL are shown. Sample intensities were normalized against an internal standard (DT-PC 13:0/13:0) and then scaled with Pareto scaling. Figures were generated using Metaboanalyst software. Heatmap trends (high [red], low [blue]) indicate that the E- embryos contain, overall, greater quantities of lyso-PL species early in development (24 hpf) when compared to E+ embryos, but many of these species, notably those containing DHA (22:6), are markedly depleted by 120 hpf. PL trends are highly varied; however, E- embryos contain lesser quantities of PL species with DHA (22:6). A. PC, phosphatidylcholine; B. LPC, lysophosphatidylcholine; C. PE, phosphatidyl-ethanolamine; D. PI, phosphatidylinositol and PS, phosphatidylserine E. LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol and LPS, lyso-phosphatidylserine.

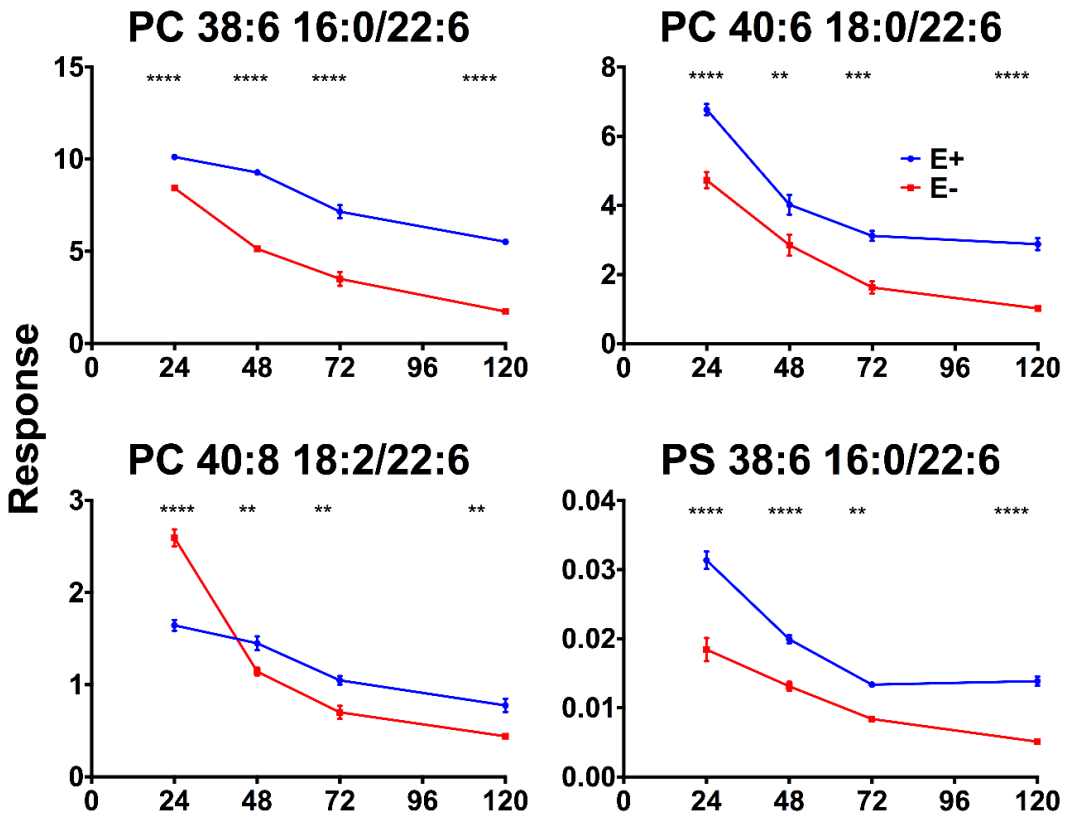


Figure 4. Four specific PLs containing DHA (22:6) are significantly lower in E- embryos. Lipidomic analysis of lipid extracts from E- and E+ embryo samples (*n*=15/sample; 4 samples/group) taken at 24, 48, 72, and 120 hours post-fertilization (hpf). Lipid species were confirmed by high-resolution MS, MS/MS fragmentation, and isotopic distribution, and then compared using the PeakView database. Peak intensities were used for relative quantification between E- and E+ conditions. The data shown are means ± SEM. Two-way ANOVA, Bonferroni's post-test for multiple comparisons (**p<0.01, ***p<0.001, ****p<0.0001).

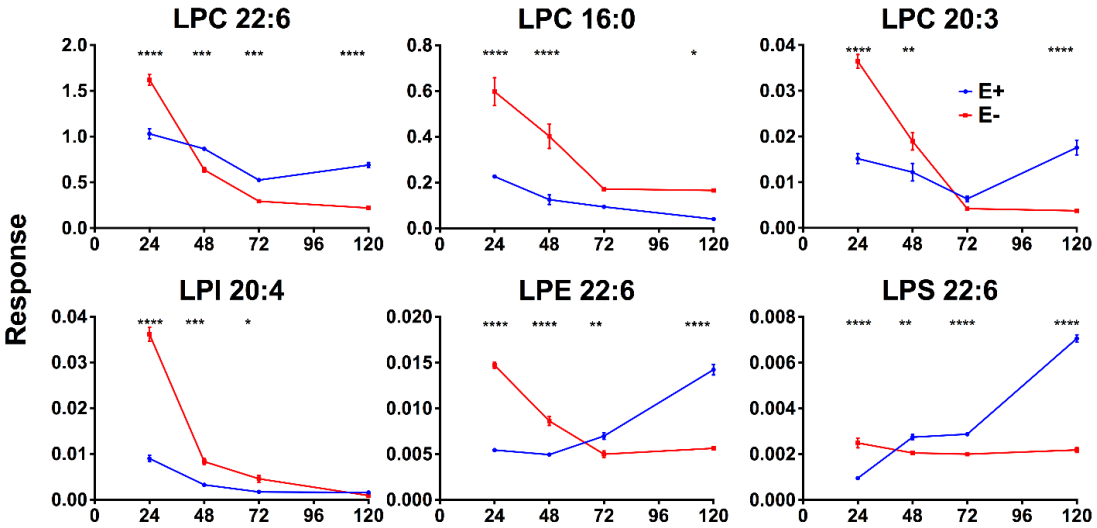


Figure 5. E- embryos show enhanced depletion of many lyso-PL species during development when compared with E+ embryos. Data were generated and analyzed as described in Figure 4. Notably, the lyso-PL species showing the greatest difference between the E- and E+ conditions all contained DHA (means ± SEM). Two-way ANOVA, Bonferroni's post-test for multiple comparisons (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). LPC 16:0 and LPC 20:3 levels at 72 hpf were significantly altered in E- vs. E+ embryos when analyzed using unpaired t-tests and Sidak-Bonferroni post-test (p<0.001, higher in E-; and p=0.0176, lower in E-, respectively), as were LPI 20:4 levels at 120 hpf (p<0.0001, lower in E-).

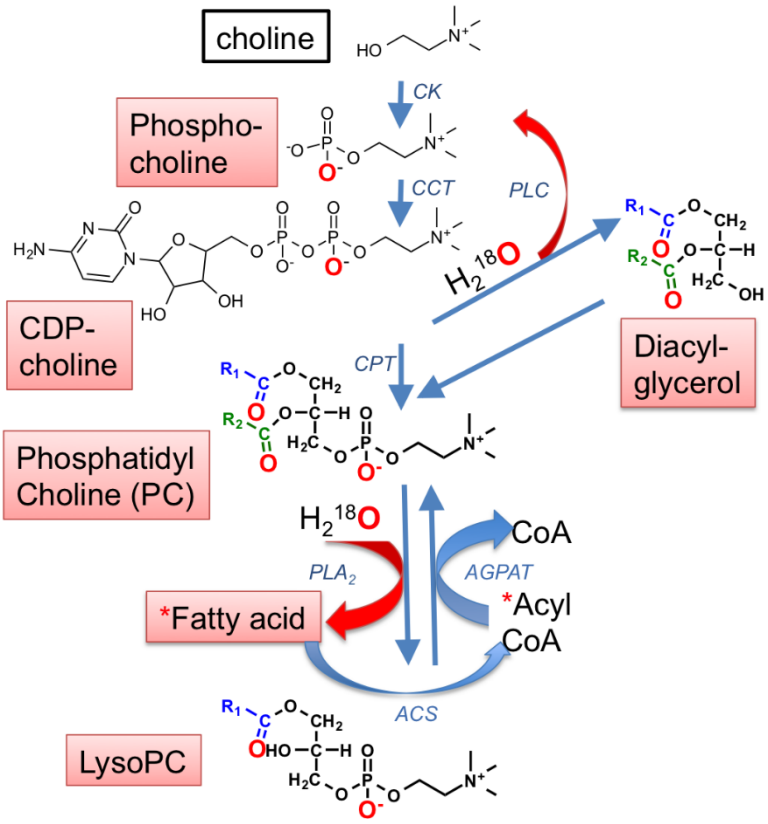


Figure 6. $H_2{}^{18}O$ incorporation into PC lipids. The CDP-choline pathway] is a representative metabolic pathway outlining reactions that allow for $H_2{}^{18}O$ incorporation into PCs and/or lyso-PCs. From the top, choline kinase (CK) phos¬phorylates choline to phosphocholine, which is converted to cytidine-diphosphocholine (CDP-choline) by CTP: phosphocholine cytidylyltransferase (CCT). Choline phosphotransferase (CPT) catalyzes PC synthesis. Fatty acyl chains are cleaved from the PC sn-2 position by phospholipase A2 (PLA2) to generate lyso-PC and free fatty acids. Free fatty acids are labeled (*), rapidly converted to acyl¬-CoA by acyl-CoA synthetase (ACS) and used for acylation of lyso-PL in a reaction catalyzed by acylglycerolphosphate acyltransferases (AGPAT). PC may be cleaved by PLC (phospholipase C) to yield diacyl¬glycerides (DAG) and phosphocholine. Hydrolysis reactions in which incorporation of the ^{18}O label may occur are shown in red, as are the indicated oxygens that may be labeled following PL remodeling.

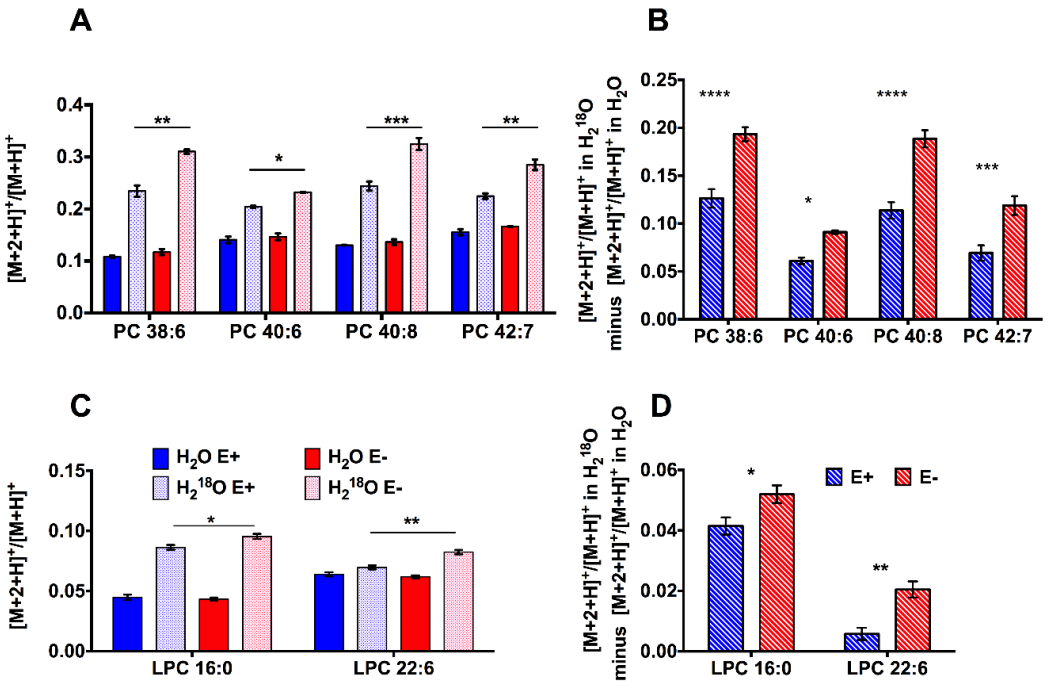
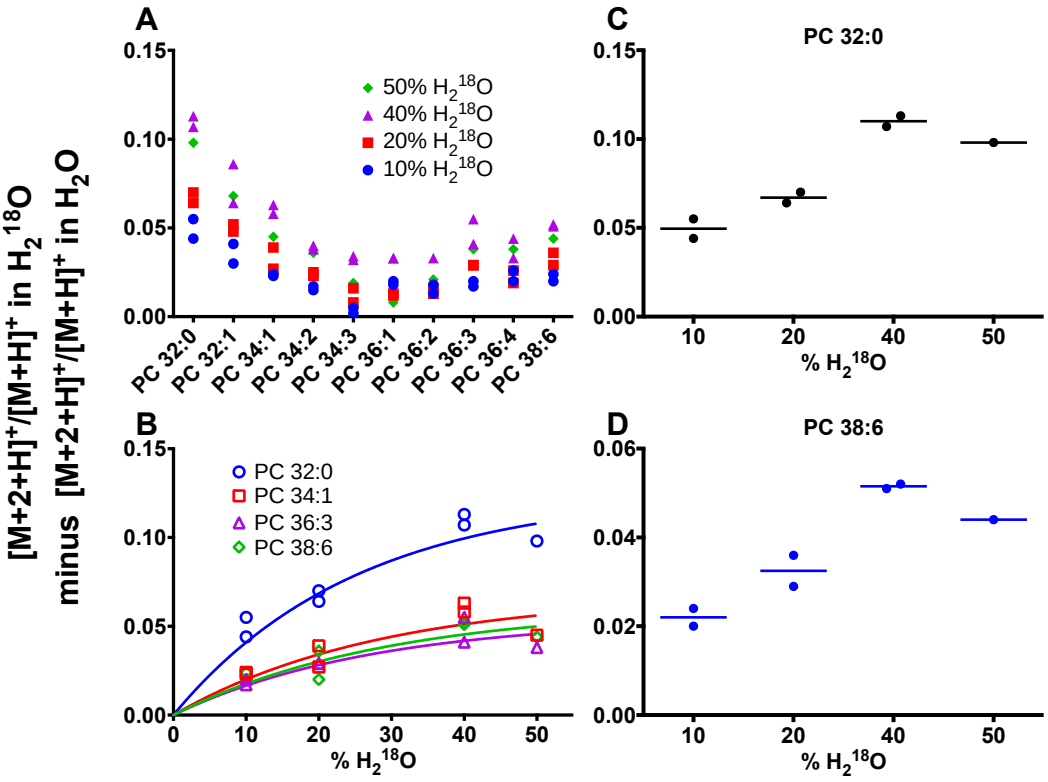


Figure 7. $H_2{}^{18}O$ incorporation into PCs and lyso-PCs is greater in E- than in E+ embryos. A and C. Ratios of [M+2+H]+/[M+H]+ from H2O-incubated E- vs. E+ embryos were similar (solid bars; no significant differences) in PCs (A) and lyso-PCs (C); ratios comparing label incorporation in $H_2{}^{18}O$ -incubated E- vs. E+ embryo (patterned bars) revealed enhanced labeling in E- embryos of DHA-containing PCs, as well as in LPC 16:0 and 22:6, suggesting increased PL remodeling. PC species (means ± SEM) were identified and confirmed as described in Figure 4; PC 38:6 (16:0/22:6); PC 40:6 (18:0/22:6); PC 40:8 (18:2/22:6); PC 42:7 (20:3/22:4). All PLs and lyso-PLs showed significant ($p<0.001$) label incorporation in H_2O- vs. $H_2{}^{18}O$ -incubated embryos within the same diet condition, with the exception of E+ embryos LPC 22:6 in H_2O- vs. $H_2{}^{18}O$ -incubated (no significant difference, indicating no significant remodeling in E+ embryos). Asterisks indicate statistical differences between $H_2{}^{18}O$ E- and E+ groups (red and blue patterned bars, respectively). B and D. [M+2+H]+/[M+H]+ in $H_2{}^{18}O$ minus [M+2+H]+/[M+H]+ in H_2O (mean± SEM) in E- vs. E+ embryos for PCs (B) and lyso-PCs (D). A-D: Two-way ANOVA with Tukey's (A, C) or Sidak's (B, D) post-test for multiple comparisons (*$p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$).



Supplementary Figure 1. $H_2{}^{18}O$ labeling optimization trial. A cohort of laboratory embryos (from adult 5D zebrafish not subjected to dietary manipulation) was incubated with increasing concentrations of $H_2{}^{18}O$ (0% to 50% v/v in EM, *n*=2 samples of 15 embryos each, except for 50%, *n*=1) for 24 h from 48 to 72 hpf to determine the % $H_2{}^{18}O$ that yielded the greatest label incorporation. Lipid species were identified and quantified as described for lipidomics analyses (See Methods) A. Incorporation of the label for representative phosphatidylcholine (PC) species are shown; incorporation was measured as the differences in the ratios of [M+2+H]+/[M+H]+ peak intensities in the $H_2{}^{18}O$ incubated minus the ratios of [M+2+H]+/[M+H]+ peak intensities in the H_2O incubated (PeakView software, SCIEX). Incorporation was found to be highest in the 40% v/v $H_2{}^{18}O$ incubation. B. Shown are four representative PC species fitted with non-linear regression curves showing a plateau in label incorporation at 40% v/v $H_2{}^{18}O$. C-D. Incorporation of the H218O label in PC with saturated (C) and highly unsaturated (D) acyl chains; indicating optimal labeling at 40% v/v $H_2{}^{18}O$ for both.

CHAPTER THREE:

Lethal Dysregulation of Energy Metabolism During Embryonic Vitamin E Deficiency

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2017 Mar; 104:324-332.
doi: 10.1016/j.freeradbiomed.2017.01.020

Abstract

Vitamin E (α-tocopherol, VitE) was discovered in 1922 for its role in preventing embryonic mortality. We investigated the underlying mechanisms causing lethality using targeted metabolomics analyses of zebrafish VitE-deficient embryos over five days of development, which coincided with their increased morbidity and mortality. VitE deficiency resulted in peroxidation of docosahexaenoic acid (DHA), depleting DHA-containing phospholipids, especially phosphatidylcholine, which also caused choline depletion. This increased lipid peroxidation also increased NADPH oxidation, which depleted glucose by shunting it to the pentose phosphate pathway. VitE deficiency was associated with mitochondrial dysfunction with concomitant impairment of energy homeostasis. The observed morbidity and mortality outcomes could be attenuated, but not fully reversed, by glucose injection into VitE-deficient embryos at developmental day one. Thus, embryonic VitE deficiency in vertebrates leads to a metabolic reprogramming that adversely affects methyl donor status and cellular energy homeostasis with lethal outcomes.

Introduction

Vitamin E (α-tocopherol, VitE) was discovered in 1922 to prevent embryonic mortality in rats [78], but the mechanisms involved remain unknown. VitE is a lipophilic antioxidant [49], protecting long-chain polyunsaturated fatty acids, especially docosahexaenoic acid (DHA) from lipid peroxidation [336, 337]. Is VitE's essentiality due to its antioxidant function? A leading theory argues VitE is an integral part of an "antioxidant network", where the one-electron VitE oxidation product generated by scavenging peroxyl radicals is reduced by ascorbic acid. Reduction of the ascorbyl radical utilizes other antioxidants, including glutathione (GSH); this process uses endogenous reducing equivalents, primarily NADPH, to serve as the final electron-acceptor [197]. The close association between GSH and VitE is emphasized further by studies of phospholipid hydroperoxide glutathione peroxidase (*GPX4*), a selenium-dependent peroxidase that reduces phospholipid hydroperoxides [338]. Could dysregulation of this antioxidant network cause embryonic mortality? Maternal VitE supplementation in mice prevents *GPX4* knockdown-induced lethality in offspring [339], suggesting that VitE is necessary to generate the lipid hydroperoxides and to prevent their subsequent oxidation to generate radicals and reinitiate lipid peroxidation.

In humans, VitE deficiency increases early miscarriage risk [80], which poses public health concerns since estimates of inadequate dietary VitE intakes exceed 80% of the global ≥14 year-old population [340]. Using our VitE-deficient zebrafish model as a tool, we undertook solving the mystery of why VitE is a necessary nutrient with an especially critical requirement during vertebrate embryonic development.

Materials and Methods

Study design

All experiments were performed in duplicate. All data trends and specific outcomes contained herein matched between the first and second experimental replicates. In order to maximize consistency, the results we report are all from the second set of experiments. Additional supplementary data and the complete data set is available in [307].

Materials and reagents

Reagents used for metabolomics analyses included: methanol and ultra-pure water (LC-MS grade, EMD Millipore, Gibbstown, NJ); formic acid, acetic acid (Optima LC/MS grade; Fisher Chemical, Pittsburgh, PA); and butylated hydroxytoluene (BHT, TCI America; Portland, OR), as well as zirconium oxide beads (Next Advance; Averill Park, NY). Deuterium (d) labeled internal standards DHA-d_5, ARA-d_8, EPA-d_5, LA-d_4, and 9(*S*)-HODE-d_4 (Cayman Chemical, Ann Arbor, MI) were used for quantification of total and free fatty acids, and oxidized DHA derivatives, respectively. Sterile d_6-α-tocopherol emulsion (Fresenius-Kabi, Graz, Austria) and D-(+)-glucose (Sigma Aldrich, St. Louis MO) were used for embryo microinjection rescue studies. Reagents used for bioenergetics profiling included: oligomycin (Cayman Chemicals; Ann Arbor, MI), carbonyl cyanide 4-(trifluoromethoxy) phenyl-hydrazone (FCCP), and sodium azide (Sigma-Aldrich; St. Louis, MO).

Zebrafish husbandry and diets

The Institutional Animal Care and Use Committee of Oregon State University approved this protocol (ACUP Number: 4344). Tropical 5D strain zebrafish were housed in the Sinnhuber Aquatic Research Laboratory. Adults were kept at standard laboratory conditions of 28°C on a 14-h light/10-h dark photoperiod in fish water consisting of reverse osmosis water supplemented with a commercially available salt (Instant Ocean®) to create a salinity of 600 microsiemens [252], adjusted to pH 7.4. At 55 days post-fertilization (dpf), adult zebrafish were randomly allocated to one of

two diet groups, α-tocopherol deficient (E–) or α-tocopherol sufficient (E+), for the duration of the study [296]. The defined diets, which contained only fatty acids with 18 or fewer carbons and no more than 3 double bonds, were prepared with the vitamin C source as StayC (500 mg/kg, Argent Chemical Laboratories Inc., Redmond, WA) and without (E–) or with added α-tocopherol (E+, 500 mg *RRR*-α-tocopheryl acetate/kg diet, ADM, Decatur, IL), as described previously [278, 296]. Diets were stored at -20°C until fed to the adult zebrafish.

E– and E+ embryos were obtained from adult fish fed either the E– or E+ diet, respectively, for a minimum of 80 days up to 9 months. Embryos were obtained through natural group spawning, collected, staged [252], and kept in standard embryo media (EM; as described [308]). Embryos used for biochemical analysis, described below, were euthanized prior to sampling by cold exposure (placed on ice for a minimum of 30 min.). Note that embryos are not fed. For all experiments, the E+ embryos are considered the control condition; lab embryos also were used as an additional control to monitor embryo quality (data not shown).

Vitamin E and ascorbic acid analyses

Using high-pressure liquid chromatography with electrochemical detection, diet and embryo α-tocopherol [286] and ascorbic acid [318] were determined. Measured α-tocopherol concentrations in the E– and E+ diets were 0.45 ± 0.01 and 369 ± 2 mg/kg, respectively; vitamin C was 143 ± 16 mg ascorbic acid/kg (n=3 replicate samples measured for each diet). This level of dietary vitamin C has been found to be adequate for the zebrafish [74].

Evaluation of phenotypic and developmental progress

At 24 hours post-fertilization (hpf), embryos were assessed for viability, developmental progression and spontaneous movements (earliest behavior in zebrafish), using the zebrafish acquisition and analysis program (ZAAP). ZAAP is a custom program designed to inventory, acquire, and manage zebrafish data, and was used to collect 18 developmental endpoints, as either present or absent (i.e. binary responses were recorded, described below [321]).

Developmental progression is considered perturbed if zebrafish are delayed more than 12 hours compared to control animals. Spontaneous movements are assessed over a 2-min. period and are considered perturbed if there is a lack of embryonic contractions and/or movement. At 96 and 120 hpf, larval morphology (body

axis, eye, snout, jaw, otic vesicle, notochord, heart, brain, somite, fin, yolk-sac, trunk, circulation, pigment, and swim bladder) was evaluated and recorded and behavioral endpoints (motility, tactile response) were thoroughly evaluated. If the embryo was dead at either 24 or by 96-120 hpf, the non-mortality endpoints were not included in the evaluations. All images were taken using a Keyence BZ-700X microscope with a 2X objective lens under standard bright-field conditions.

Behavioral assessments

Locomotor activity [258, 321] was measured in a total of *n*=128 embryos per VitE group using Viewpoint Zebrabox (software version 3.0, Viewpoint Life Sciences, Lyon, France). At 96 hpf, the plates containing the embryos were placed in a Viewpoint ZebraBox and embryo locomotor activity was assessed using the “tracking” setting during alternating periods of light and dark, a modification of [322]. Embryos subjected to this test typically move less during the light periods and more during dark periods, and behavioral differences can be determined by comparing distances moved during the light and/or dark periods. Locomotor activity in response to the light/dark transition was tracked during 3 min. periods of alternating light and dark for a total of 24 min. The integration time was set to 6 seconds to increase statistical power. A high definition camera (30 frames/second) tracked the total movement (swim distance, millimeters) in response to the multiple light-dark transitions.

Extraction and LC-MS/MS for metabolomic analysis

At 12 hpf, E– and E+ embryos were transferred one embryo per well into 96 well plates containing 100 µL EM per well. Following 24, 48, 72, and 120 hpf, embryos (*n*=15 per replicate, *n*=4 replicates per group) were transferred to 1.5 mL Eppendorf tubes, covered with EM, and kept on ice for 30 min. to euthanize the animals. EM was carefully removed to prevent loss of embryos and samples were stored at -80 °C overnight. To extract embryos for metabolomics analyses, solvent (300 µL 80:20 v/v methanol:water) was added, then sample extracts were homogenized with 0.5 mm zirconium oxide beads using a counter-top bullet blender for 6 min. Following 15 min. incubation on ice, the extracts were centrifuged at 4 °C at 15,000×*g* for 13 min. Aliquots (200 µL) of the upper layer were transferred individually to new tubes and stored at -80 °C until analysis via LC-MS/MS. To ensure the stability and repeatability of the LC–MS system, quality control (QC) samples (*n*=4), which were generated by pooling 10 µL aliquots from each embryo extract, were analyzed with the embryo samples.

Chromatography was performed with a Shimadzu Nexera system (Shimadzu; Columbia, MD, USA) coupled to a high-resolution hybrid quadrupole-time-of-flight mass spectrometer (TripleTOF® 5600; SCIEX; Framingham, MA, USA). Two different LC analyses using reverse phase and HILIC columns were used. In reverse phase LC, chromatographic separations were carried out using a 4.6 × 150 mm Inertsil phenyl-3 column (5 µm, GL Sciences Inc., Rolling Hills Estates, CA, USA) for positive and negative ion analyses, as we described [319]. The sample injection volume was 10 µL and the flow rate was 0.4 mL/min. The mobile phases consisted of water (A) and methanol (B), both with 0.1% formic acid. The gradient was as follows: an initial hold at 5% B for 1 min, followed by a gradient of 5–50% B in 11 min, to 100% B at 23 min, held until 35 min, then a shift to 5% B at 37 min until 50 min. The column temperature was held at 50 °C. In metabolomics HILIC LC analysis, separation was carried out using a 4.6 × 150 mm SeQuant ZIC-pHILIC (5 µm, EMD Millipore, Billerica, MA, USA). The flow rate was 0.4 mL/min and the injection volume was 10 µL. The two mobile phases consisted of 20 mM ammonium carbonate, pH 9.2 with ammonium hydroxide in water (A) and acetonitrile (B). The gradient was as follows: an initial hold at 80% B for 1 min, followed by a gradient of 80-20% B in 30 min, to 8% B at 31 min, held until 36 min, then a shift to 80% B at 37 min until 44 min. The column temperature was held at 50 °C.

Time-of-flight (TOF) mass spectrometry (MS) was operated with an acquisition time of 0.25 s and a scan range of 70–1000 Da. MS/MS acquisition was performed with collision energy set at 35 V and collision energy spread of 15 V. Each MS/MS scan had an accumulation time of 0.17 s and a range of 40–1000 Da using information-dependent acquisition (IDA). The source temperature was set at 500 °C and IonSpray voltage at 4.5 kV in positive ion mode and -4.0 kV negative ion mode, respectively.

Sample preparation, extraction and LC-MS/MS analyses of total or free DHA, EPA, ARA, and LA fatty acids and hydroxy-DHA

Analysis of total DHA, EPA, ARA, and LA were performed as described [286] with the following modifications: samples were obtained at 24, 48, 72, and 120 hpf (*n*=10-15 embryos per replicate, 3 replicates per group) and saponified in alcoholic KOH with 1% ascorbic acid; following cooling, the pH was adjusted to 2.5 with 12N HCl, then 2.0 mL heptane and 10 µL internal standard [DHA-d_5 (1.0 µg/mL), EPA-d_5

(1.0 µg/mL), ARA-d_8 (20.0 µg/mL) and LA-d_4 (20.0 µg/mL)] were added. Samples were mixed, the supernatant (organic layer) was removed and dried under nitrogen gas, the residue resuspended in 100 µL 80:20 v/v methanol:water with 0.5% acetic acid. Samples were stored at -80 °C until analysis by LC-MS/MS (see below).

Extracts for quantitative free fatty acid and hydroxy-DHA analyses were prepared as described for metabolomics samples, with the following modifications: extraction solvent (290 µL, 80:20 v/v methanol:water) included 50 µg/mL BHT and for quantification was combined with 10 µL per sample of internal standards containing DHA-d_5 (10.0 µg/mL), EPA-d_5 (5.0 µg/mL), ARA-d_8 (20.0 µg/mL), LA-d_4 (20.0 µg/mL), and 9(*S*)-HODE-d_4 (2.0 µg/mL). Embryo numbers ranged from *n*=15-30 embryos per replicate, with *n*=4 replicates per group. QC samples were prepared as described for metabolomic analysis. Amounts were quantitated by relative comparison to internal standards, see below.

Chromatographic separations were carried out on 4.6 × 250 mm J'sphere ODS-H80 (4 µm, YMC Co, Kyoto, Japan) for negative ion analysis. TOF-MS and TOF-MS/MS were operated with same parameters as for metabolomics, described above. The sample injection volume was 10 µL and the flow rate was 1 mL/min. The mobile phases consisted of water (A) and acetonitrile (B), both with 0.1% acetic acid. The gradient was as follows: an initial hold at 30% B for 4 min, followed by a gradient of 30–60% B in 1 min, to 65% B at 13 min, to 80% B at 25 min, to 100% at 26 min held until 32 min, then a shift to 30% B at 33 min until 38 min. The column temperature was held at 35 °C.

Extracellular flux analyzer assay for bioenergetic profiling

Oxygen consumption rate (OCR) and proton production rate (PPR) measurements were performed using the XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA) with methods based on [341]. Dual-analyte sensor cartridges were soaked in 500 µl XF Calibrant Solution (Seahorse Bioscience, Billerica, MA) in 24-well cell-culture microplates (Seahorse Bioscience, Billerica, MA) overnight at 30°C to hydrate. Embryos were staged and placed into 20 of 24 wells on an islet microplate; the remaining four wells served as temperature control wells. Islet plate capture screens were placed over the top of the measurement area to keep the embryos in place. Preliminary experiments were performed to determine the number of embryos needed to achieve OCRs that fell within the recommended specifications

of the XF24 instrument for each developmental age: four embryos per well at 24 hpf, and two embryos per well at 48 hpf. At 24 hpf, embryos (4 embryos per well; *n*=7 wells per VitE condition) were rinsed and placed in unbuffered EM in the XF24 islet capture plate. Briefly, we used oligomycin A to inhibit ADP phosphorylation by the mitochondrial ATP synthase, thereby initiating a decrease in OCR that approximates the fraction of total basal respiration coupled to ATP turnover. Treatment with the uncoupling/protonophore agent, FCCP, causes translocation of protons from the mitochondrial intermembrane space to the matrix, leading to an increase in the OCR that approximates “maximal respiratory capacity”. Sodium azide (NaN_3) blocks the respiratory chain by inhibiting cytochrome c oxidase, leaving “non-mitochondrial respiration”. The difference in minimum OCRs measured after oligomycin A and sodium azide treatments is a metric of respiration attributed to proton leak (*i.e.* ion movements requiring proton motive force) across the mitochondrial inner membrane [342, 343]. Titrations of each reagent were performed at 24 and 48 hpf, to determine the concentrations that produced the maximum change in respiration without inducing death within the experimental time frame. Concentrated stocks of oligomycin and FCCP were prepared in DMSO at 10 mM and 20 mM, respectively. A concentrated stock of sodium azide (5 M) was prepared in phosphate-buffered saline.

OCR and PPR were measured before and after the addition of 50 µL of 63.2 µM oligomycin A (ATP synthase inhibitor), 55.6 µL 2.5 µM FCCP (mitochondrial uncoupler), and 61.6 µL sodium azide (mitochondrial complex IV inhibitor). The same protocol was used for 48 hpf embryos (2 embryos per well; *n*=7 wells per VitE group) with addition of 50 µL 9.2 µM oligomycin A; then 56.0 µL 2.8 µM FCCP; then 62 µL 1.25 mM NaN_3. The Seahorse protocol consisted of calibration, equilibration, 8 measurements of baseline, 8 measurements after injection of oligomycin A and FCCP, and 12 measurements after injection of NaN_3. OCRs and PPRs were calculated using a modified AKOS algorithm, available with the Seahorse Bioscience software, that takes into account oxygen diffusion through the plate and atmospheric leak, in addition to the oxygen consumed by the organism [341]. Buffer capacity was calculated by monitoring pH of the media, and PPR values derived from calculated buffer capacity and chamber volume of each well.

Microinjection rescue studies

Embryos were microinjected using a nanoliter 2000 injector (World Precision Instruments, Sarasota, FL, USA) linked to a stereoscopic microscope. Needles for microinjection were made in a puller (Sutter Instruments, model P-97, Novato, CA, USA) using glass capillary with an internal filament (1.14 o.d.; 0.5 mm i.d.). For pilot α-tocopherol injections, embryos were injected at the 1-2 cell stage (approximately 0.5 hpf according to [252]) into the yolk-sac with a sterile vitamin E preparation made for intravenous use in humans containing d_6-α-tocopherol (d_6-α-T, 5.4 g/L) in an oil-water emulsion made with soybean oil and phospholipids. Embryos were injected with 4.0 nL of the d_6-α-T emulsion, a quantity (20 ng, or 46 pmol) that provides an amount of α-tocopherol to the E– embryos equal to that in 0 hpf E+ embryos [286]. Glucose injections were performed as described [344]. Briefly, at 24 hpf embryos were injected (into the yolk-sac) with 4.0 nL of 2.0 M glucose solution prepared with *D*-glucose (Sigma-Aldrich, St Louis, MO, USA) dissolved in sterile saline solution. This dose was based on the maximum solubility of glucose in water. Control-injection conditions for both experiments consisted of saline injection with sterile saline solution (NaCl, 58 mmol/L; KCl, 0.7 mmol/L; $MgSO_4$, 0.4 mmol/L; $Ca(NO_3)_2$, 0.6 mmol/L; Hepes, 5 mmol/L; pH 7.3). Criteria used to assess supplementation tolerance of zebrafish embryos using ZAAP were embryonic development, growth, and mortality, assessed at 24, 48, and 120 hpf.

Data processing and statistical analyses

Targeted metabolomics data processing was performed using PeakView software (SCIEX). Sample peaks for each targeted metabolite of interest were annotated using the extracted ion chromatograms (XIC) lists based on high resolution MS, MS/MS fragmentation, isotopic distribution, and retention time compared with an in-house library of 635 metabolite standards (IROA Technologies, Bolton, MA, USA). In addition to the IROA database, metabolite identities were confirmed further using the METLIN web-based metabolomics database (http://www.metlin.scripps.edu). Peak intensities for each individual metabolic feature were normalized using the corresponding mean QC sample (n=4) intensity for that feature, as described [345], to balance their differences in intensities that may have arisen due to discrepancies in the sample homogenization (sample preparation). Student's *t*-tests (Excel, Microsoft) to compare the two VitE groups at each developmental time-point (24, 48, 72, and 120

hpf) were performed with statistical significance set at $p < 0.05$. To control for false discovery rates for these metabolomics data during follow-up analyses, study outcome significance was set at $Q < 0.05$, using an adaptive linear step-up procedure [346]. Subsequent statistical analyses (*e.g.* 2-way ANOVA with Tukey's or Sidak's multiple comparison tests, as recommended by the software) were performed using GraphPad Prism 6.0 software (GraphPad, La Jolla, CA).

Quantification of total and free fatty acids, and hydroxy-DHA was performed using MutliQuant Software version 3.0.2 (SCIEX). Lipid peaks were identified based on the accurate masses and retention times of each individual lipid, then quantified by integrating peak area using MultiQuant Software. Raw area counts for each lipid were normalized using area counts for the corresponding internal standard, then corrected for internal standard concentration. Statistical analyses (*e.g.* 2-way ANOVA with Tukey's multiple comparison test) were performed using GraphPad Prism 6.0 software (GraphPad, La Jolla, CA).

For OCR analyses, the extracellular flux analyzer assay data was collected and processed using the XF Reader software (Seahorse Biosciences, Agilent Technologies, Santa Clara, CA) and exported using Excel 2013 (Microsoft). The baseline OCR and PPR were calculated, in addition to the ATP-linked OCR, the maximum OCR, proton leak, and mitochondrial reserve capacity, as described by [341, 342]. Additional statistical analyses (*e.g.* 2-way ANOVA with Tukey's multiple comparison tests) were performed using GraphPad Prism 6.0 software.

Statistical analyses for morphological and behavioral endpoints were performed using code developed in R (R Developmental Core Team 2014, http://www.R-project.org). For morphological assessments at 24 and 96 hpf, binary responses were recorded as either absent (0) or present (1) for each of the 18 endpoints. For statistical analyses of behavior, raw data files were processed using custom R scripts with methodologies based on [258]. In brief, the distance traveled by each individual animal, over each integration period, was measured and then the total area under the curve (AUC) was computed for each animal. The overall AUCs for the E– compared to the E+ embryos then were compared using a combination of percent change (minimum 30% difference from E+ embryos) and a Kolmogorov-Smirnov test ($p<0.01$) to determine statistical significance.

Results and Discussion

We obtained E– and E+ embryos by spawning adult 5D zebrafish fed either E– or E+ diets. E+ embryos grew normally, while E– embryos suffered >80% morbidity and mortality by 120 hpf (**Figure 8A-B**). Measured α-tocopherol concentrations in *living* E– and E+ embryos were 1.09 ± 0.01 and 22.41 ± 0.04 pmol/embryo, respectively, at 120 hpf.

At 48 hpf E– embryos contained decreased concentrations of DHA-containing phospholipids (DHA-PL), particularly phosphatidylcholine (PC) and phosphatidylethanolamine (PE), as well as DHA-lyso-PC [305], a major DHA transporter to the brain [316, 347]. Since DHA is critical for normal neurodevelopment [347], we hypothesized that VitE deficiency causes embryonic death by depleting DHA-PC, thereby depriving the embryo of adequate DHA. We tested this hypothesis by assessing the impact of VitE status on relative quantities of total, free (unesterified) and oxidized DHA in E– vs. E+ embryos. Initially, total and free DHA were elevated in E– embryos, but by 48 hpf both were significantly lower than E+ embryos (**Figure 8C,** see also Supplementary Figure 2 and Tables 1 and 2 in [307]); further, the lipid peroxidation products, 7- and 10-hydroxy-DHA [348], as well as 9-HODE, were elevated in E– (**Figure 8C**), ranging from 3-7% of the free DHA at 120 hpf, confirming increased DHA peroxidation.

Craniofacial malformations are observed at 48 hpf in E– embryos (**Figure 8B)** and in zebrafish embryos genetically manipulated to *completely* lack VitE [297] more severe defects are seen as early as 15-17 hpf, an embryonic stage that coincides with neurulation [252]. Since choline is a nutrient essential for neurulation in vertebrate embryos [349] and PC is a major source of choline [108], we hypothesized that the increased DHA-PC replenishment in E– embryos [305] induces a secondary choline deficiency. We found that as development progressed both choline and various choline-containing compounds in the cytidine-5-diphosphocholine (CDP-choline) pathway for PC synthesis decreased in the E– embryos (**Figure 9A;** see also Supplementary Figure 3, Tables 1 and 2 in [307]), consistent with our previous lipidomics-based investigations [72].

PC can also be generated by the serial methylation of PE via the phosphatidylethanolamine N-methyltransferase (PEMT) pathway using the requisite methyl-donor, S-adenosylmethionine (SAM). Unlike the CDP-choline pathway that

generates PC containing mono- and di-unsaturated fatty acids, the PEMT pathway generates PC containing polyunsaturated fatty acids [350] and enhances DHA transfer from mother to fetus in humans [351]. Evidence that the PEMT pathway in E– embryos caused depletion of methyl-donors is shown by the decreases at 120 hpf of SAM, methionine, and betaine, while the SAM oxidation product, S-adenosyl-homocysteine increased (**Figure 9A;** see also Supplementary Figure 3, Tables 1 and 2 in [307]). Thus, PC synthesis via the PEMT pathway in E– embryos became compromised due to choline and methyl-donor depletion.

VitE deficiency not only induced oxidative damage by increasing lipid peroxidation, but depleted other antioxidants as well (see also Supplementary Figure 4, Tables 1 and 2 in [307]). Specifically measured ascorbic acid concentrations in E– embryos were halved at 120 hpf (22.2 ± 0.3 vs. 59.8 ± 1.0 pmol/embryo in E– and E+, respectively, see also Supplementary Figure 4, Tables 1 and 2 in [307]). We, therefore, hypothesized that the E– embryos have an increased need for reducing equivalents (NADPH) and that glucose would be shunted to the pentose phosphate pathway to generate NADPH [352]. In E– embryos, glucose was depleted with increases in 6-phosphogluconate and ribose-5-phosphate intermediates (**Figure 9B**), while glycolytic and TCA cycle intermediates in E– embryos (although increased at 24 hpf) decreased at subsequent time-points (**Figure 9B;** see also Supplementary Figure 5, Tables 1 and 2 in [307]). Moreover, NADPH was increasingly oxidized in E– embryos and NADP+/NADPH ratios increased over time (**Figure 9B;** see also Supplementary Figure 4, Tables 1 and 2 in [307]). This metabolic shift – aimed at ameliorating the effects of oxidative stress – occurred at the expense of energy-generating pathways, such as glycolysis and flow through the respiratory chain of tricarboxylic acid (TCA) cycle-generated reducing equivalents. Evidence for the uncoupling of the TCA cycle in E– embryos (*i.e.* citrate transported to cytosol for fatty acid synthesis and cytosolic reduction of pyruvate to lactate) is provided by their 1) increased saturated fatty acids (palmitic and stearic, **Figure 9B,** see also Supplementary Figure 6, Tables 1 and 2 in [307]), 2) decreased glutamine and glutamate (see also Tables 1 and 2 in [307]), and 3) increased lactate (**Figure 9B,** see also Supplementary Figure 5, Tables 1 and 2 in [307]). Overall, this metabolic profile in E– embryos resembles the Warburg effect seen in cancer cells [353]. Thus, E– embryos experience dysregulation of energy metabolism, a phenomenon that is a major driver of cellular dysfunction and death as

an ultimate consequence of VitE deficiency. Potentially, ferroptosis, a mechanism of programmed cell death due to increased lipid peroxidation [354, 355], which is dependent on *GPX4* and GSH as well as VitE to prevent cell death [339], is responsible for the embryonic lethality. Notably, it has been suggested that VitE functions to inhibit lipoxygenase enzyme activity and, thus, the oxidation of arachidonic and adrenic PE, thereby preventing the generation of these oxidized PE signaling molecules for ferroptosis [42]. These latter molecules were doubly and triply oxygenated, and therefore beyond the technical capabilities of the approaches used for the present study. Further research is needed to assess the role of ferroptosis during VitE deficiency and embryogenesis.

Bioenergetic profiles of E– and E+ embryos were assessed in real-time by measuring mitochondrial and non-mitochondrial respiration (**Figure 10A**). At 24 hpf, E– embryos had higher basal oxygen consumption rates (OCR), but at 48 hpf their basal OCRs were decreased (**Figure 10B**). At 24 and 48 hpf E– embryos had reduced levels of “maximal respiration” (**Figure 10B, 11A**), demonstrating a lack of mitochondrial reserve capacity [342], and had an elevated proton production rates (PPR, **Figure 10C, 11B**), concomitant with their increased lactate concentrations (**Figure 9B**). Thus, E– embryos experienced a “metabolic switch” from a hyper- to a hypo-metabolic state consistent with non-energy generating glucose metabolism (*i.e.* pentose phosphate pathway) and TCA cycle uncoupling (*i.e.* citrate transport into cytosol) by 48 hpf (**Figure 9B**, **11C**).

We hypothesized that impairment of aerobic, mitochondrial glucose metabolism in E– embryos caused their neurobehavioral abnormalities. Morphologically normal 96 hpf E– embryos were 84% less responsive to light-dark stimuli in a locomotor assay (**Figure 11D**; see also Supplementary Figure 7 in [307]). To determine if increased glucose status would ameliorate the neurobehavioral defects, 24 hpf E– and E+ embryos were microinjected into the yolk sac with D-(+)-glucose in saline or with saline alone. Glucose rescued the neurological deficits in 50%, and morbidity and mortality in 31%, of E– embryos (**Figure 11D**; see also Supplementary Figure 8 in [307]), but they still sustained craniofacial deformities (not shown). Thus, it may be surmised that other nutrients, *e.g.* DHA and choline, are required by E– embryos to obtain a complete phenotypic rescue, as observed in a pilot trial employing micro-injection of a VitE-PL emulsion at 0 hpf (see Supplementary

Figure 6 in [307]). Deuterated polyunsaturated fatty acids, which are less susceptible to lipid peroxidation [343] and protect mitochondria from metabolic dysregulation caused by lipid peroxidation [356], could be used as a potential rescue for E– embryos to evaluate the role of the initial lipid peroxidation in embryonic lethality. Similarly, ferroptosis inhibitors, such as ferrostatin and liproxstatin [357], could be protective during VitE deficiency, and would be a means to test directly the role of ferroptosis in E– embryo lethality.

The mechanisms underlying VitE's essentiality during neurodevelopment include its antioxidant protection of DHA and DHA-PLs, which prevents lipid peroxidation, additional antioxidant depletion, and metabolic dysregulation with secondary decreases in both choline and glucose. Additionally, specific effects on lipoxygenase enzymes may play critical roles in embryonic lethality. Such pleiotropic outcomes cause death by effectively starving the embryonic brain not only of vital nutrients, namely DHA and choline, but also of the energy needed for development. These findings acquire even greater significance upon consideration that – nearly 100 years after its discovery [78] – they provide mechanistic insights into *why* the vertebrate fetus requires VitE, and, as a corollary, expand the role VitE deficiency plays in human miscarriage.

Acknowledgements

The authors thank Carrie Barton, Greg Gonnerman, Andrea Knecht, Jane La Du, Scott Leonard, and Lisa Truong for providing outstanding technical assistance. Tory Hagen, Siva Kolluri and Andrew Karplus are gratefully acknowledged for reading the manuscript and providing critical advice. NIH S10RR027878 (MGT and JFS) and NIEHS P30 ES000201 (RT) supported this work. MM was supported in part by NSF DGE 0965820. H-KK sabbatical support provided by The Catholic University of Korea. MGT supported in part by Helen P Rumbel endowment to the LPI.

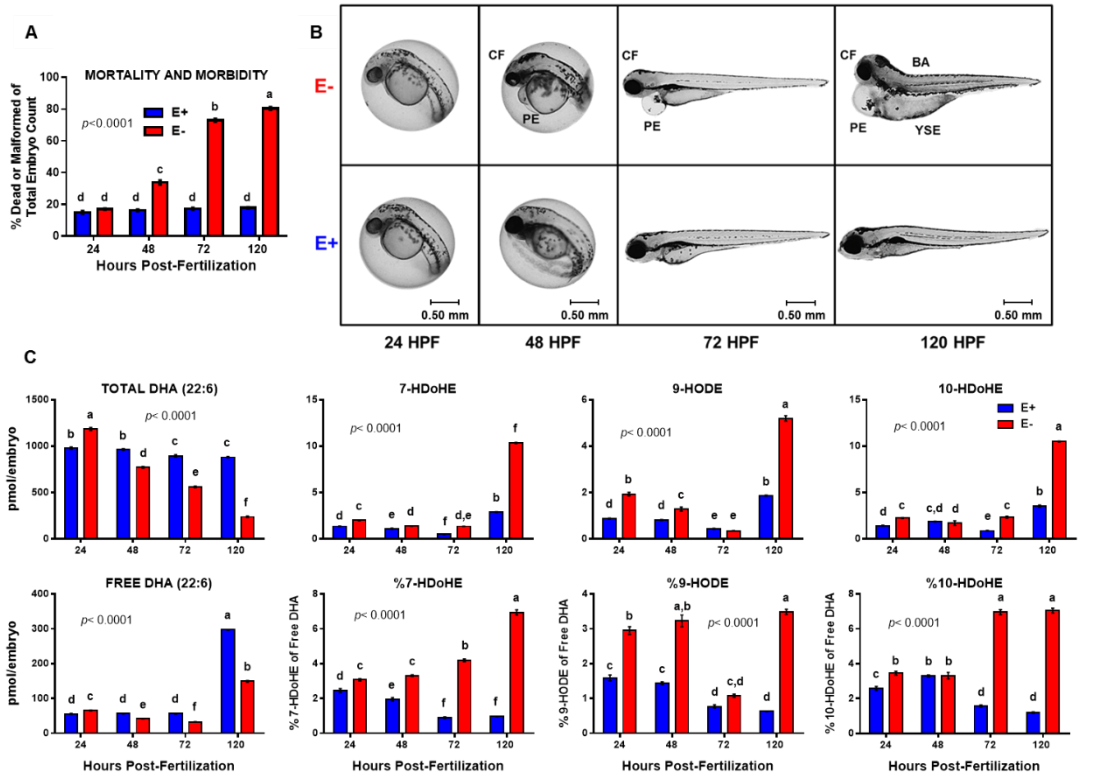


Figure 8. VitE deficiency-induced morbidity and mortality coincides with decreased DHA and increased hydroxy-DHAs. A. Increasing morbidity and mortality in E– embryos. B. Phenotypic deformities in E– embryos: CF=cranial-facial malformation, PE= pericardial edema, YSE= yolk-sac edema, BA= skewed body-axis. C. Relative-quantitation of free DHA and hydroxy-DHAs in E– vs. E+ embryos; area counts normalized using internal standards (*n*= 4 samples/group, *n*=15-30 embryos/sample). Percentages of each hydroxy-DHA = (hydroxy-DHA/free DHA)*100. Shown are means ± SEM; *p*-values from 2-way ANOVA with unique letters indicating differences (Tukey's post-test, $p<0.05$).

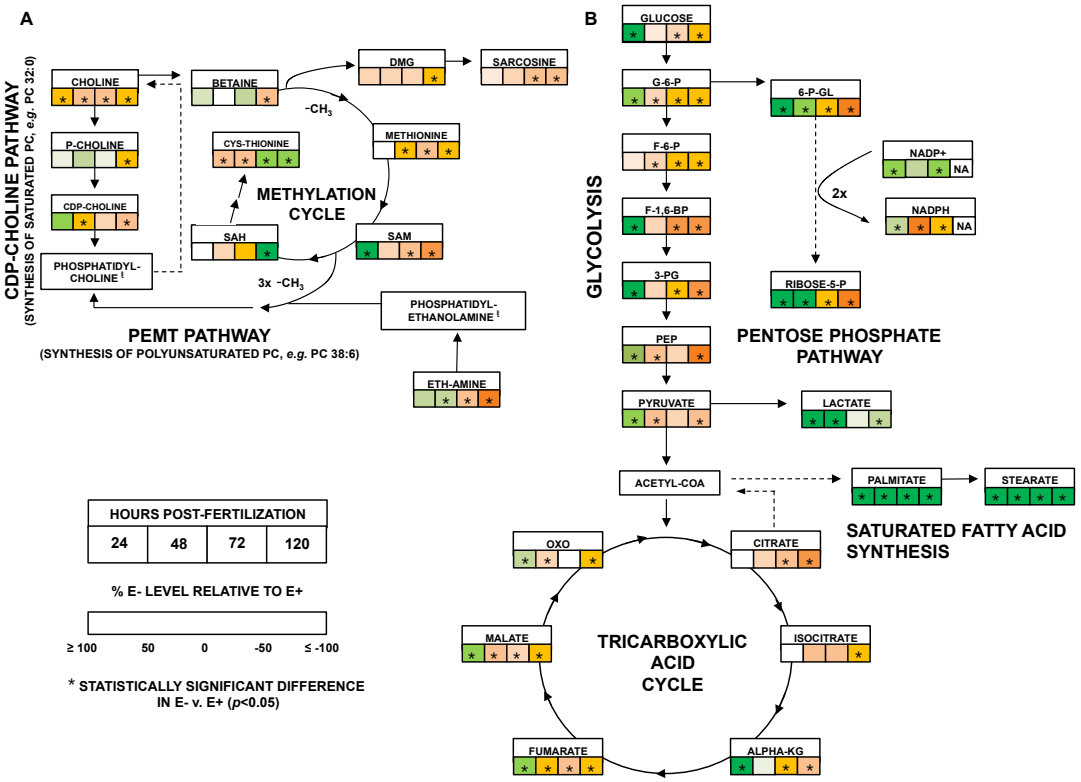


Figure 9. Time course in E– embryos shows disrupted metabolism. A. Choline regeneration. B. Glucose metabolism and TCA cycle. Shown are targeted metabolites (boxes (□) indicate comparisons at each time-point) with significance calculated using 2-way ANOVA (*p*-value = 0.05 to <0.0001, Table S3) and Sidak's post-test ($p<0.05$) of normalized, log-transformed intensity values ($n=4$ samples/group, 15 embryos/sample, see Methods). NA = no data available. Abbreviations: A. P-Choline (phosphocholine), DMG (dimethylglycine), Eth-amine (ethanolamine), Cys-thionine (cystathionine), SAM (S-adenosyl-methionine), SAH (S-adenosyl-homocysteine), -CH_3 (methyl group). B. G-6-P (glucose-6-phosphate), F-6-P (fructose-6-phosphate), F-1,6-BP (fructose-1,6-bisphosphate), 3-PG (3-phosphoglycerate), PEP (phosphoenolpyruvate), Alpha-KG (alpha-ketoglutarate), OXO (oxaloacetate), 6-P-GL (6-phosphogluconate), Ribose 5-P (ribose-5-phosphate).

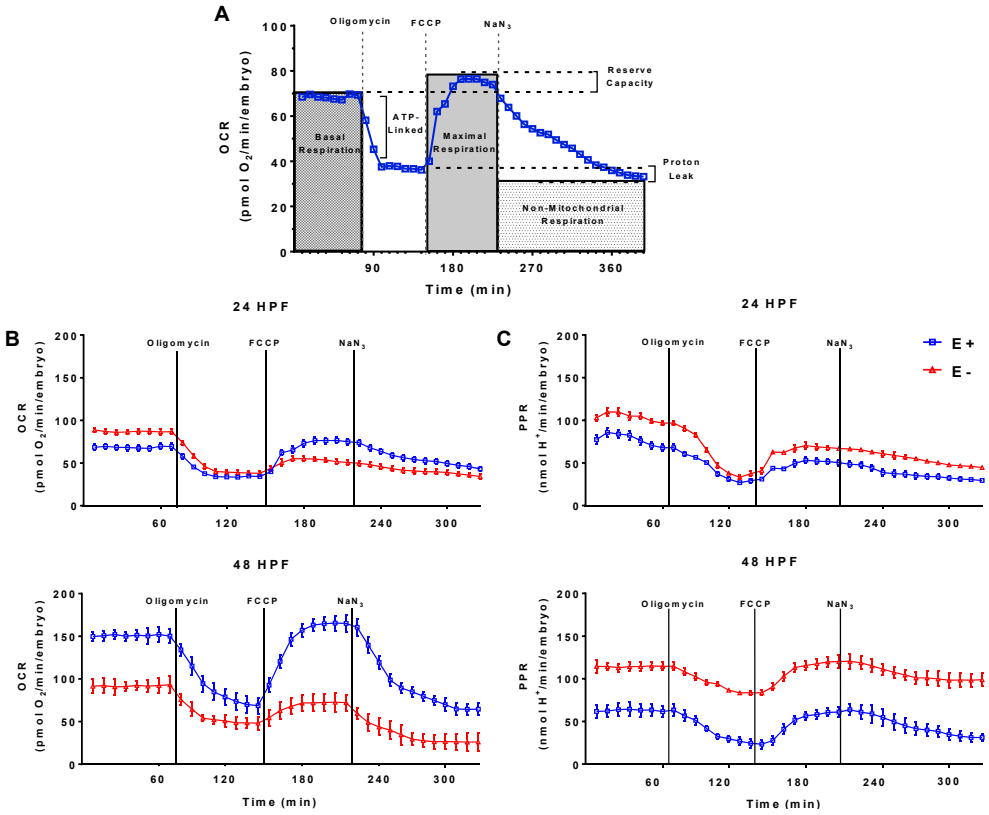


Figure 10. Bioenergetic profiling in E– embryos. A. Representative oxygen consumption rates (OCR) in 24 hpf E+ embryos (*n*=7); indicated are responses after sequential exposure to oligomycin, cyanide 4-(trifluoromethoxy) phenyl-hydrazine (FCCP) and sodium azide (NaN_3). B. OCR and C. Proton production rates (PPR) in E– vs. E+ embryos, showing means ± SEM, *n*=7 samples/group; for 24 hpf: 4 embryos/sample for 36/44 trials; for 48 hpf: 2 embryos/sample, 36/36 trials.

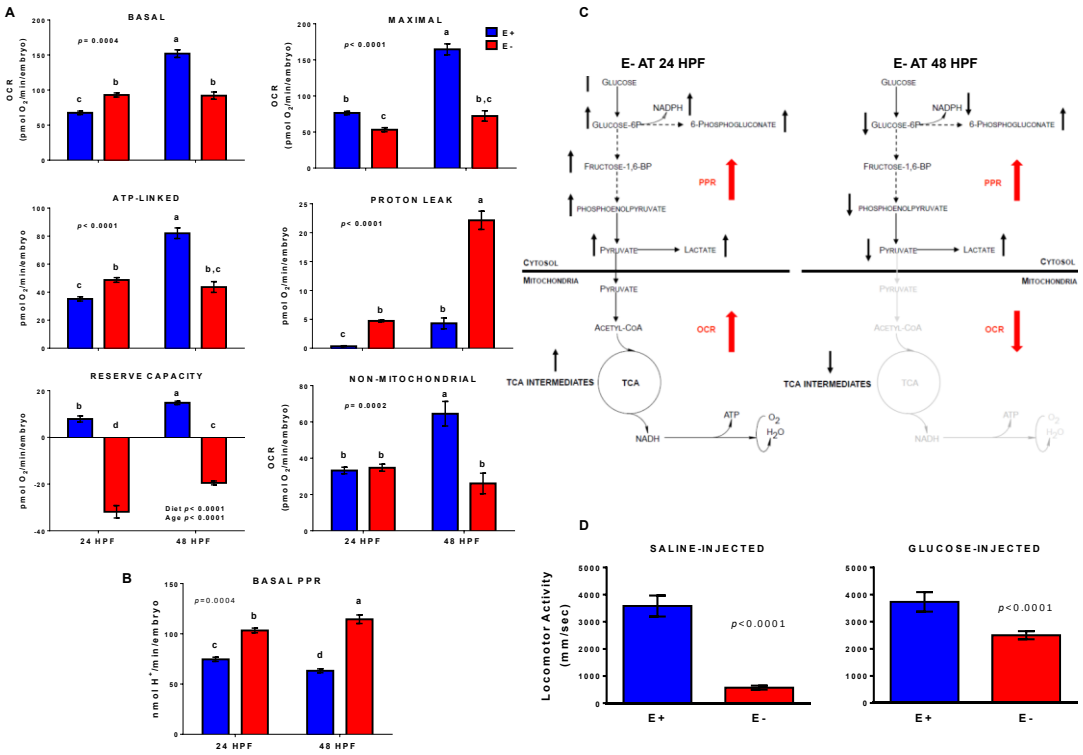
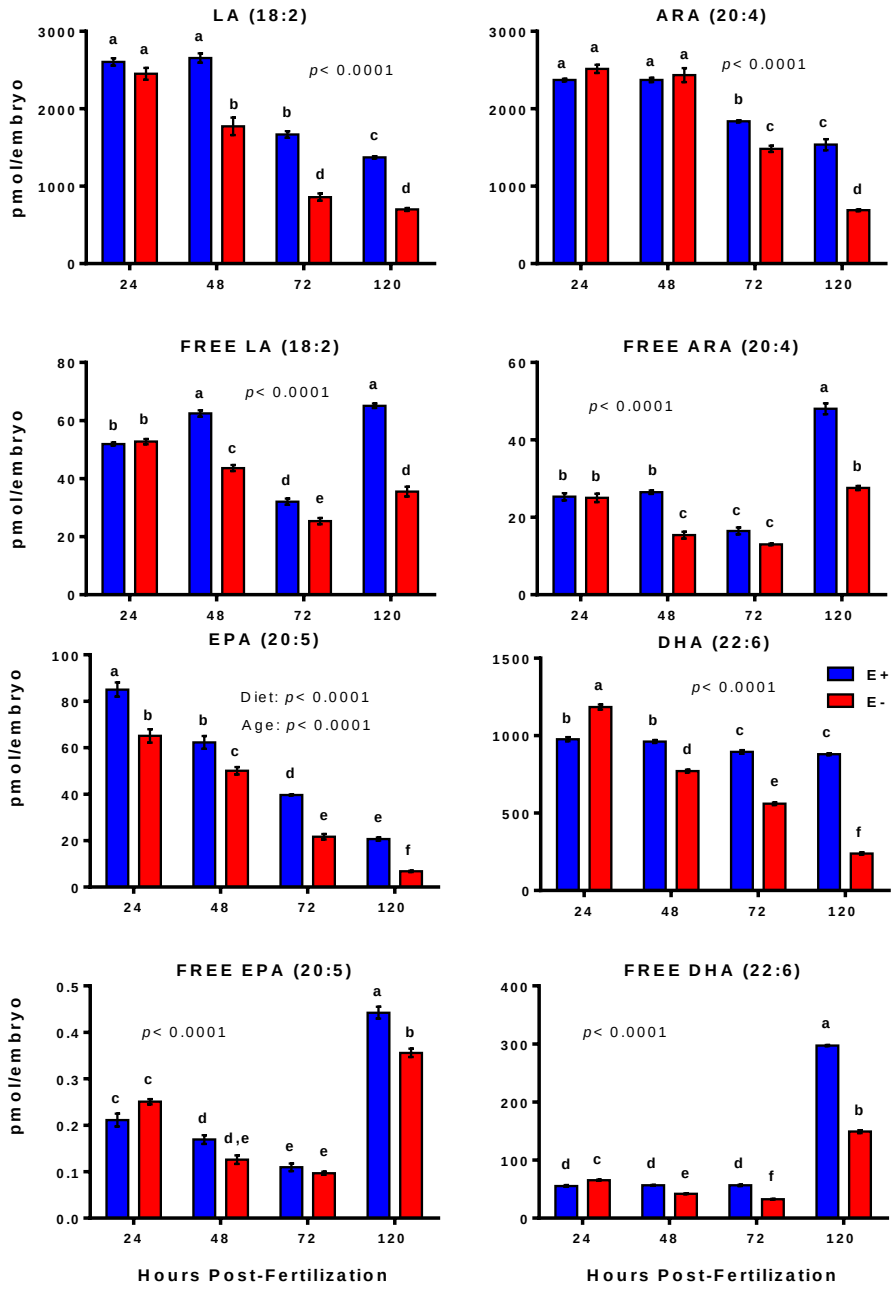
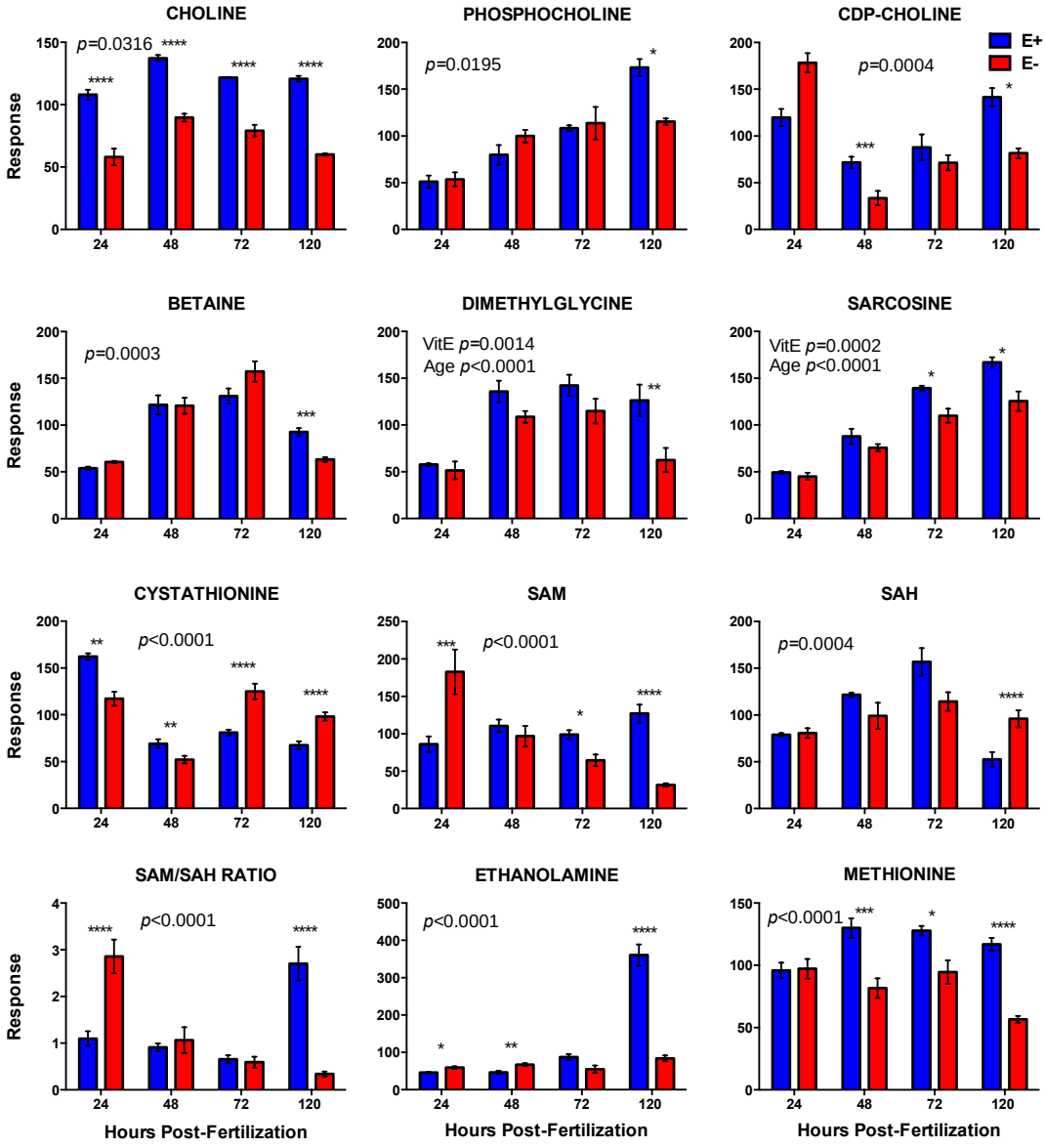


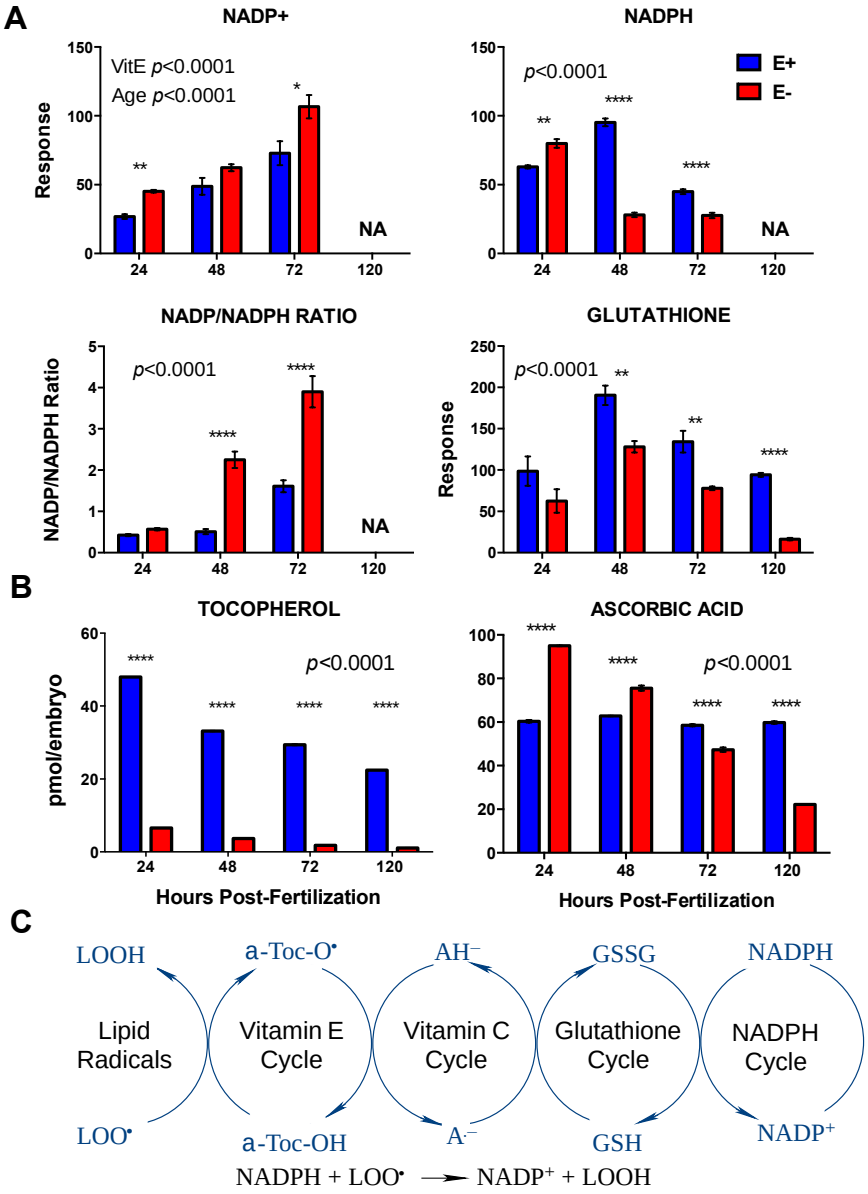
Figure 11. Quantitation of bioenergetic profiling in E– embryos and glucose rescue. A. OCR and B. PPR show means ± SEM (data from Figure 10); *p*-value = 2-way ANOVA, Tukey's post-test, unique letters indicate differences ($p<0.05$). C. Disrupted energy metabolism in E– embryos. Activity of gray pathways is reduced; red arrows denote bioenergetic rates showing metabolic switch between 24 and 48 hpf. Black arrows indicate differences in select metabolites between E– and E+ embryos (data from Figure 9). D. E– and E+ embryos (n=128/group) injected into the yolk sac at 24 hpf with either saline or glucose were analyzed for locomotor activity at 96 hpf (mm/sec; see Methods). Glucose injection attenuated neurobehavioral abnormalities in E– embryos by ~50% (Kolmogorov–Smirnov test; $p<0.0001$).



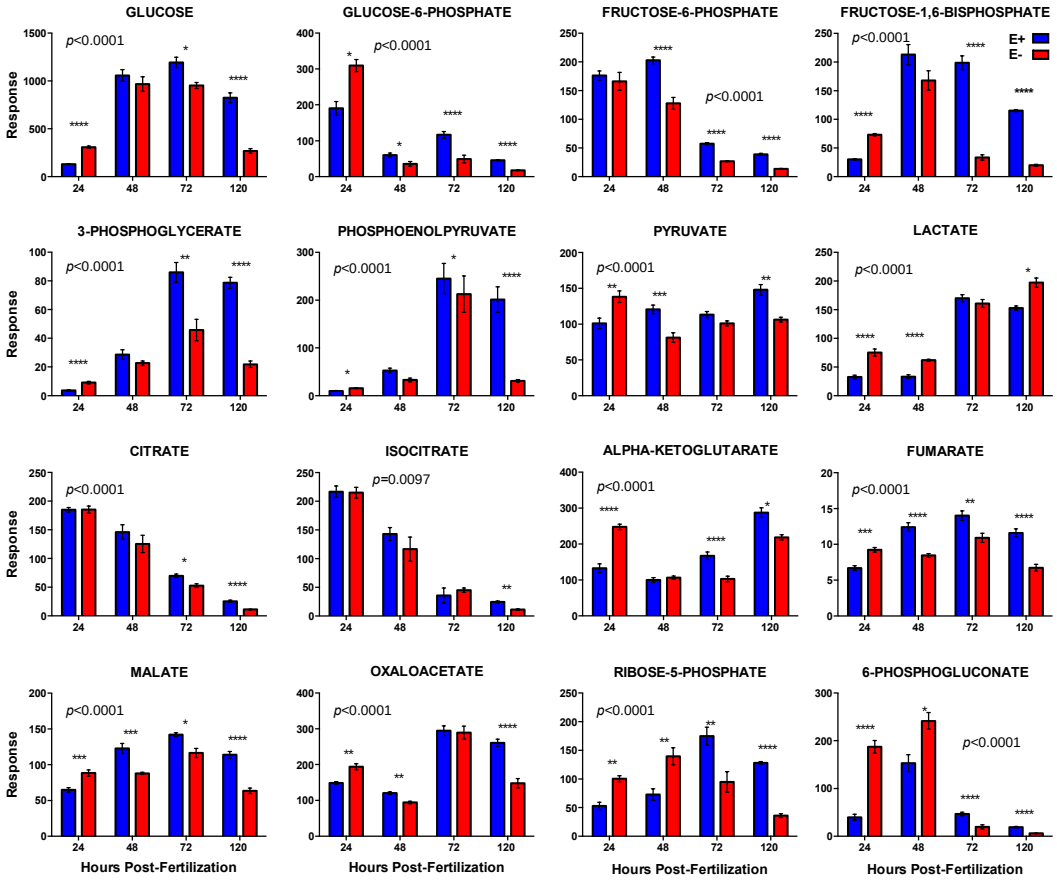
Supplementary Figure 2. Quantified levels of total and free (unesterified) fatty acids in E– vs. E+ embryos. Area counts normalized using internal standards (*n*= 3 samples/group, with *n*=10-15 embryos/sample for total lipids; *n*=4 samples/group with *n*=15-30 embryo/sample for free fatty acids). Shown are saponified (upper row) or extracted only (lower row) samples, means ± SEM; p-values are for VitE x Age interactions, unless indicated otherwise (Tukey's post-test, p<0.05 for bars bearing different letters). Abbreviations: LA (linoleic acid); ARA (arachidonic acid); EPA (eicosapentaenoic acid); DHA (docosahexaenoic acid).



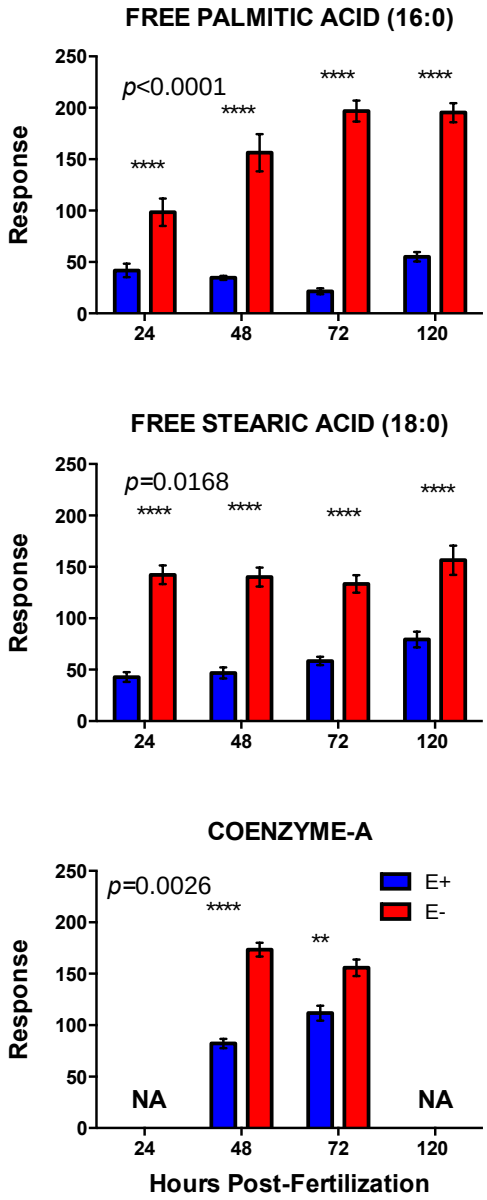
Supplementary Figure 3. Relative response intensities of choline and methylation pathway intermediates. E– and E+ embryo (*n*=15/sample; 4 samples/group) data were normalized against QC sample intensities (*n*=4) for each individual metabolite. Statistical significance ($p<0.05$) was calculated using 2-way ANOVA with Sidak's post-test for multiple comparisons of normalized and natural log-transformed intensity values. Shown are means ± SEM; *p*-values are for VitE x Age interactions, unless indicated otherwise. Paired comparison, *p*-values are indicated as *<0.05, **<0.005, ***<0.001, **** <0.0001.



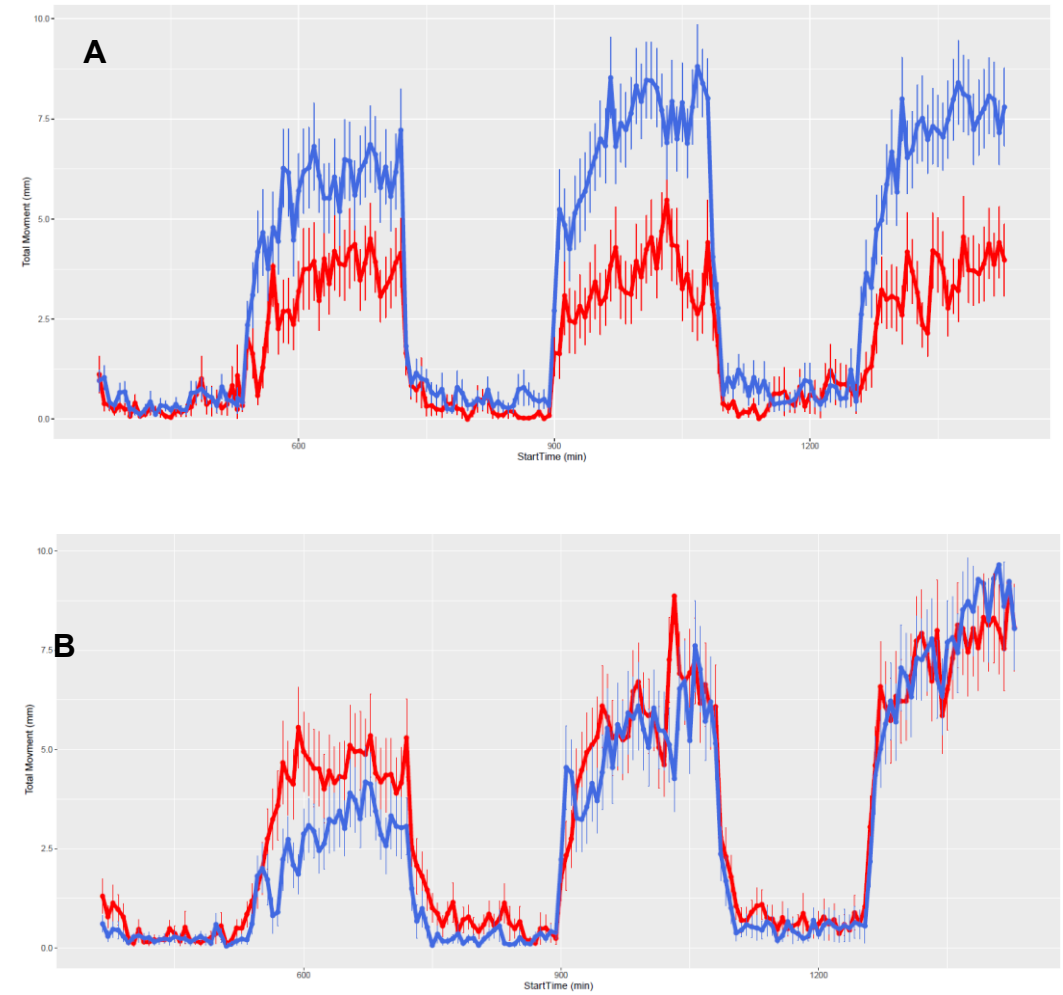
Supplementary Figure 4. Relative response intensities of antioxidant network components from metabolomics and quantification of α-tocopherol and ascorbic acid. A. E– and E+ embryo (*n*=15/sample; 4 samples/group) relative response data was normalized against QC sample intensities (*n*=4) for each individual metabolite. B. Quantified levels of α-tocopherol and ascorbic acid, respectively. Statistical significance (p<0.05) was calculated using 2-way ANOVA with Sidak's post-test for multiple comparisons of normalized and natural log-transformed intensity values. Shown are means ± SEM; p-values are for VitE x Age interactions, unless indicated otherwise. Paired comparisons p-values are indicated as *<0.05, **<0.005, ***<0.001, **** <0.0001. C. Antioxidant network scheme showing interaction of antioxidants with lipid radicals and consumption or NADPH.

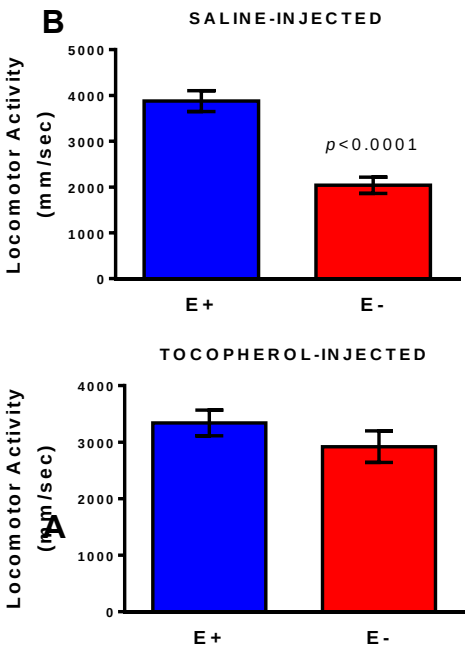


Supplementary Figure 5. Relative response intensities of glycolytic and tricarboxylic acid cycle intermediates. E– and E+ embryo (*n*=15/sample; 4 samples/group) data were normalized against QC sample intensities (*n*=4) for each individual metabolite. Statistical significance ($p<0.05$) was calculated using 2-way ANOVA with Sidak's post-test for multiple comparisons of normalized and natural log-transformed intensity values. Shown are means ± SEM; *p*-values are for VitE x Age interactions. Paired comparisons *p*-values are indicated as *<0.05, **<0.005, ***<0.001, **** <0.0001.

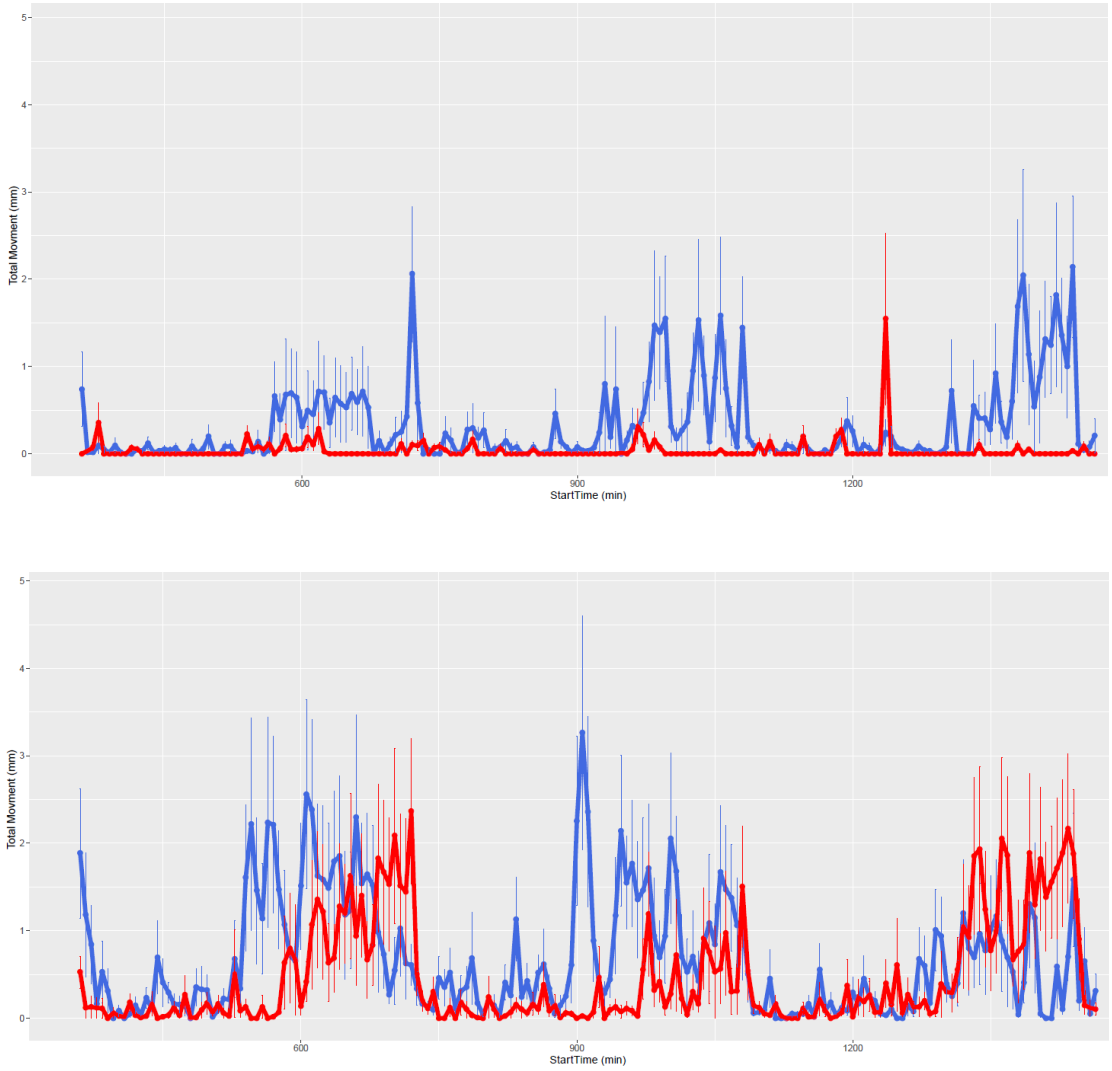


Supplementary Figure 6. Relative response intensities of free saturated fatty acids and coenzyme A from metabolomic analyses. E– and E+ embryo (*n*=15/sample; 4 samples/group) data were normalized against QC sample intensities (*n*=4) for each individual metabolite. Statistical significance ($p<0.05$) was calculated using 2-way ANOVA with Sidak's post-test for multiple comparisons of normalized and natural log-transformed intensity values. Shown are means ± SEM; *p*-values are for VitE x Age interactions. Paired comparison *p*-values are indicated as *<0.05, **<0.005, ***<0.001, **** <0.0001.





Supplementary Figure 7. E– compared with E+ embryos have impaired behavior when injected with saline (upper panel), but restored responses when injected with VitE (lower panel). A. Embryos were analyzed in 96-well plates (128 embryos per group). Locomotor activities following a series of light stimuli (a stimulus every 6 for 24 min) were measured as distance moved (mm) over time (seconds). At 96 hpf, E- (red) embryos treated with saline (upper panel) were 47% less responsive to light than were E+ embryos (E– area-under-curve, AUC: 2040±178; E+ AUC: 3877±228; $p<0.0001$). Embryos with morphological defects were not included in data analysis. E- behavior was restored using VitE injection into the yolk at the 1 cell stage (lower panel E– AUC: 2970±280; E+ AUC: 3340±226). Statistical significance was determined using a Kolmogorov–Smirnov test ($p<0.01$). B. Bar chart comparisons of respective time-course data. VitE (tocopherol)-injected E– and E+ embryo locomotor activities were not significantly different.



Supplementary Figure 8. Locomotor response assay activity data showing neuro-behavioral impairment. E– and E+ embryos (96 hpf) were analyzed in 96-well plates (128 embryos per group). Locomotor activities following a series of light stimuli (every 6 for 24 min) were measured as distance moved (mm) over time (seconds). E– (red) embryos treated with saline (A, upper panel) were 84% less responsive to light than were E+ (blue) embryos (E– area-under-curve, AUC: 572±72 E+ AUC: 3580±387; p<0.0001). Embryos with morphological defects
were not included in data analysis. E– behavior was partially restored by approximately 50% following glucose injection into the yolk at 24 hpf (B, lower panel; E– AUC: 2502±150; E+ AUC: 3734±359; p<0.0001). Statistical significance was determined using a Kolmogorov–Smirnov test (p<0.01).

Supplementary Table 1

Supplementary Table 2

Click here to download **Supplementary Tables 1 and 2**

CHAPTER FOUR:

Chronic vitamin E deficiency impairs cognitive function in adult zebrafish via dysregulation of brain metabolism due to redox-mediated mechanisms

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Unpublished Manuscript

Abstract:

Zebrafish (*Danio rerio*) are a recognized model for studying the pathogenesis of cognitive deficits and the mechanisms underlying behavioral impairments, including the consequences of increased oxidative stress within the brain. The lipophilic antioxidant vitamin E (α-tocopherol; VitE) has an established role in neurological health and cognitive function, but the biological rationale for this action remain unknown. In the present study, we investigated behavioral perturbations due to isolated, chronic VitE deficiency in adult zebrafish fed diets that were either VitE-deficient (E- group) or sufficient (E+ group) for up to 18-months of age. We hypothesized that E- adult zebrafish would display significant cognitive impairments associated with elevated lipid peroxidation and additional metabolic disruptions in the brain. Quantified VitE levels in brains from E- adults (5.7 ± 0.1 pmol/mg) were approximately 22-times lower than in E+ adult brains (122.8 ± 1.1 pmol/mg; *n*= 10/group) when measured at 18-months. Using assays of both associative (avoidance conditioning) and non-associative (habituation) learning, we found E- adults were learning impaired compared with E+ fish, and that these functional deficits occurred concomitantly with the following observations in adult E- brains: decreased concentrations and increased peroxidation of polyunsaturated fatty acids (especially of docosahexaenoic acid, DHA), altered brain phospholipid and lysophospholipid composition, dysregulation of the cellular antioxidant network, and perturbed energy (glucose/ketone), phosphatidylcholine, and choline/methyl-donor metabolism. Collectively, these data suggest that chronic VitE deficiency could lead to cognitive dysfunction through multiple potential mechanisms, including decreases in DHA, antioxidants, glucose, and choline, as well as corresponding dysfunction in related metabolic pathways (*e.g.* energy/NAD(P)H and methyl-donor metabolism) within the brain.

Introduction

Cognitive impairment, or *cognitive decline*, a noticeable and measurable decline in cognitive abilities (*e.g.* memory and learning) that exceeds those attributed to normal aging, represents an early symptom of neurodegeneration and increased risk for progression to more severe dementias, such as Alzheimer's disease (AD) [3]. Cognitive impairment and ensuing dementia are increasingly pressing public health concerns as the global population ages [358]. While the complex etiology of these

conditions remains an area of active investigation, oxidative stress has been implicated as a primary factor in neurodegenerative disease pathogenesis [359]. The vertebrate brain is especially enriched in long-chain polyunsaturated lipids, such as *ω-3* docosahexaenoic acid (DHA; 22:6 ω-3) [18]; therefore, lipid peroxidation is a likely contributor to neuropathology [360]. Oxidative stress can be mediated by lifestyle factors, including diet and nutrition [361].

The lipophilic antioxidant vitamin E (α-tocopherol; VitE) has an established role in neurological health and mitigation of oxidative stress. Its biological half-life in the brain is distinctively slow, compared to other tissues, suggesting that there are tissue-specific mechanisms that actively sequester VitE [45, 62]. Expression of the α-tocopherol transfer protein (α-TTP; required for VitE trafficking to and within the brain [58]) is markedly elevated in brain samples from human patients afflicted with oxidative stress-related neurodegenerative diseases, such as ataxia with vitamin E deficiency (AVED) and AD [63]. Selective α-TTP expression in the cerebellum is regulated both by oxidative stress and VitE status [64, 65]. Taken together, these data demonstrate that VitE's action as a lipophilic antioxidant is critical for optimal brain function.

The hallmark clinical outcome of prolonged VitE deficiency is cerebellar dysfunction, which manifests as neurodegeneration and lack of fine motor control (*ataxia*) [362]. In addition, studies focused on dementia show that inadequate VitE causes enhanced cognitive decline due to increased lipid peroxidation within the brain [211, 363]. However, results from human trials examining the efficacy of VitE supplementation for the treatment of dementia are inconclusive [210, 295]. These findings and others [60, 215] collectively suggest VitE is necessary for preserving cognitive health, but the specific mechanism(s) underlying such a role – whether uniquely dependent on VitE's antioxidant capacity or of a more multifaceted nature – remain unknown. Therefore, to elucidate VitE's neurological function, we sought to answer the question: how does chronic VitE deficiency contribute to cognitive decline?

Previously, we reported that 12-month-old adult VitE-deficient (E-) zebrafish exhibit reduced swimming behaviors compared with VitE-sufficient (E+) zebrafish, indicating neuropathy and/or myopathy in the E- group [278]. In subsequent studies, we showed that, with concomitant VitE and vitamin C (ascorbic acid) deficiencies, E- fish suffer degenerative myopathy, which decreased their responsiveness to behavioral (startle response) assays [74]. These combined nutritional deficiencies

precluded assessment of neurological and cognitive consequences of VitE deficiency alone; therefore, in the present study, we investigated the behavioral perturbations of *isolated* VitE deficiency in adult E- and E+ zebrafish fed a diet with adequate ascorbic acid [72, 74]. Further, given our past findings demonstrating increased lipid peroxidation and perturbed PL composition – specifically, depletion of PLs containing DHA – in adult E- zebrafish brains [72], we hypothesized that E- adult zebrafish would display significant cognitive impairments associated with elevated brain lipid peroxidation and, potentially, additional metabolic disruptions resulting from compromised brain antioxidant status, as found in recent studies using VitE-deficient zebrafish embryos [305, 306].

Materials and Methods

Materials

Reagents used for lipidomic analyses included: methanol and ultra-pure water (LC-MS grade, EMD Millipore, Gibbstown, NJ), formic acid, acetic acid (Optima LC/MS grade; Fisher Chemical, Pittsburgh, PA), and butylated hydroxytoluene (BHT, TCI America; Portland, OR), as well as zirconium oxide beads (Next Advance; Averill Park, NY). 1,2-ditridecanoyl-sn-glycero-3-phosphocholine (DT-PC) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and used without further purification. Deuterium (d) labeled internal standards docosahexaenoic acid (DHA)-d_5, arachidonic acid (ARA)-d_8, eicosapentaenoic acid (EPA)-d_5, linoleic acid (LA)-d_4, α-linolenic acid (ALA)-d_{14}, 5-hydroxyeicosatetraenoic acid (5-HETE)-d_8 and 9-hydroxy-docosahexaenoic acid (9(S)-HODE)-d_4 (Cayman Chemical, Ann Arbor, MI) were used for quantification of total and free fatty acids and oxidized DHA derivatives, respectively.

Zebrafish Husbandry

The Institutional Animal Care and Use Committee (IACUC) of Oregon State University

approved this protocol (ACUP Numbers: 4344, 4706). Tropical 5D strain zebrafish (Danio rerio) were housed in the Sinnhuber Aquatic Research Laboratory. Adults 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 (Instant Ocean®) to create a salinity of 600 microsiemens. Sodium bicarbonate was added as needed to adjust the

pH to 7.4. At two-months of age, adult zebrafish were randomly allocated to one of two diet groups, α-tocopherol (VitE) deficient (E-) or sufficient (E+), and fed one of the defined diets [296] for the duration of the study. The defined diets, which contained only fatty acids with 18 or fewer carbons and 2 or 3 double bonds [278, 305], were prepared with the vitamin C source as StayC (500 mg/kg, Argent Chemical Laboratories Inc., Redmond, WA) and without (E-) or with added α-tocopherol (E+, 500 mg *RRR*-α-tocopheryl acetate/kg diet, ADM, Decatur, IL), as described previously [74]. Every effort was made to minimize suffering. Prior to lipidomics sampling at 18-months of age, all fish were euthanized by cold exposure, then snap-frozen whole in liquid nitrogen and stored at -80°C until analyses.

Tocopherol and ascorbic acid analyses

Using high pressure liquid chromatography with electrochemical detection (HPLC-ECD),

α-tocopherol was measured both in diet samples and adult brain tissue, as described previously [317]. Ascorbic acid content in diet and muscle tissue was measured using HPLC-ECD as previously described [318]. Measured α-tocopherol concentrations in the E- and E+ diets were 0.45 ± 0.01 and 369 ± 2 mg/kg (*n*= 3 replicate samples measured for each diet), respectively. Vitamin C concentration was 148 ±10 mg ascorbic acid/kg diet. This level of dietary vitamin C has been found to be adequate for the zebrafish [74]. The α-tocopherol concentrations in the E- and E+ muscle (*n*= 10/group) were 1.3 ± 0.1 and 114.8 ± 0.9 pmol/mg tissue, respectively. Muscle tissue vitamin C concentrations were similar between groups (*n*= 4/group; E-: 84.9 ± 0.3 pmol/mg tissue; E+: 84.7 ± 0.3 pmol/mg tissue).

Shuttle-box testing

Associative learning and memory in adult zebrafish, was performed using custom-build shuttle-boxes, as described [259], with modifications as in [364]. Briefly, the protocol conditioned zebrafish to leave the dark side (“reject side”) of the shuttle-box and swim into the compartment with blue light (“accept side”). Following a 10-minute acclimation period (in white light) prior to beginning each trial, a single adult zebrafish was allotted 8 seconds to “seek” the accept side to avoid a moderate shock; if it did not move from the reject side, a 16 second shock period was initiated in which a moderate pulse of ~0.7V/cm was delivered for a duration of 500 milliseconds. There was a total of 30 trials. If a fish failed to swim to the lighted chamber and received a

shock for eight consecutive trials, then it was removed from the study for ethical reasons and considered a “fault-out”. The number of fault-out fish was not different between E- and E+ groups, thus the data was not included in subsequent analysis, summarized below.

Startle response testing

Assessment of non-associative learning (habituation) was performed using an updated modification of the startle response assay outlined in [74]. In brief, the experimental set-up consisted of 8 side-by-side tanks with an observation window facing an LED screen. Three sides of each tank were masked opaque white to prevent interactions between fish. All tanks were filled with 750 mL of water. An IP surveillance camera (Q-SeeHD) with infrared sensitivity was placed above the tanks to allow for top view video recording. The setup was placed over a custom LED lightbox with solenoid tap units directly below each tank. A total of 64 naïve zebrafish (32/diet group) were gently netted into individual tanks. The assay consisted of a 10-minute acclimation period, followed by three startle stimuli (one every 5 minutes). Swim motion was recorded throughout using Media Recorder software and analyzed using Noldus Ethovision 11.5. For each video, a tracking arena was defined by size and position. The fish were tracked at 25 frames per second and total distance swam (in centimeters) and velocity for one minute; 10 second bins were reported for acclimation and startle phases, respectively.

Extraction, LC-MS/MS, and data analysis for lipidomics

The brains from E- and E+ zebrafish (*n*= 10 brains/group) were dissected and weighed. Brain weights were not significantly different between groups (E- 2.3 g ± 0.2 g compared with E+ 2.4 ± 0.2 g). Brains were extracted individually in solvent (300 µL, 25:10:65 v/v/v methylene chloride: isopropanol: methanol, with 50 µg/mL butylated hydroxytoluene [BHT]) and internal standard (0.5 µg/µL, 1,2-ditridecanoyl-*sn*glycero-3-phosphocholine [DT-PC, PC 26:0] in methanol) for LC-MS/MS analyses as in [305, 306]

Extraction and LC-MS/MS, analysis for metabolomics

E– and E+ brains (*n*= 2 brains/sample; four replicate samples per group) were extracted for metabolomics analyses as described [306]. Chromatography was performed with a Shimadzu Nexera system (Shimadzu; Columbia, MD, USA) coupled to a high-resolution hybrid quadrupole-time-of-flight mass spectrometer (TripleTOF®

5600; SCIEX; Framingham, MA, USA). Two different LC analyses using reverse phase and HILIC columns were used, as previously described [306]

Sample preparation, extraction, and LC-MS/MS analyses of total or free DHA, EPA, ARA, and LA fatty acids and oxidized lipid derivatives

Analysis of total DHA, EPA, ARA, ALA, and LA of brain tissue samples obtained from E- and E+ adult zebrafish (*n*= 10/diet) were performed as described previously [306], with the use of 10 µL internal standard mixture [DHA-d_5 (1.0 µg/mL), EPA-d_5 (1.0 µg/mL), ARA-d_8 (2.0 µg/mL), ALA-d_{14} (1.0 µg/mL) and LA-d_4 (2.0 µg/mL)]. Extracts for free fatty acid and oxidized lipid analyses were prepared as described for lipidomics samples [306], with the following adjustments: individual E- and E+ brains were homogenized with extraction solvent (290 µL, 80:20 v/v methanol:water with 50 µg/mL BHT) combined for quantification with 10 µL per sample of internal standard mixture containing DHA-d_5 (1.0 µg/mL), EPA-d_5 (1.0 µg/mL), ARA-d_8 (2.0 µg/mL), ALA-d_{14} (1.0 µg/mL), LA-d_4 (2.0 µg/mL), 5-HETE-d_8 (1.0 µg/mL) and 9(*S*)-HODE-d_4 (1.0 µg/mL).

Statistical analyses

For shuttle-box assays, numerous parameters previously described in Truong et al. [259] were collected, including “Time to a Side (seconds)”, the time required by the fish to make the initial crossing from the reject to the accept side and the number of times a fish returned to the dark (the reject chamber) after having escaped to the lighted, accept chamber (“Returns to Dark”). The statistical methods remained the same as described previously [259] using custom R scripts. Briefly, data was acquired for individual fish in each diet group (*n*= 37 E- and *n*= 34 E+), with responses fit to a linear regression to determine if a fish learned. To calculate an overall learning rate (slope) for each diet group, the average response of all E- and E+ fish, respectively, for each trial was taken and used to model a group linear regression. A student’s *t*-test ($p<0.05$) was used to calculate statistical significance between E- and E+ learning rates.

To analyze startle response data, all tracked data files were imported into R software (R Developmental Core Team 2014, http://www.R-project.org) and analyzed using custom-made scripts to generate mean ± SEM outputs for tracked movement over each trial interval (both Acclimation and Tap response intervals, respectively). Statistical significance was set at $p< 0.05$. Additional statistical analyses (*e.g.* 2-way

ANOVA with Tukey's or Sidak's multiple comparison tests, as recommended by the software) were performed using GraphPad Prism 6.0 software (GraphPad, La Jolla, CA).

Lipidomics data processing was performed as described [72, 305]. Student's *t*-tests (Excel, Microsoft) to compare the two VitE groups were performed with statistical significance set at $p < 0.05$. Targeted metabolomics data processing was performed as described previously [306]. The Holm-Sidak method for multiple comparisons was used to compare normalized metabolite intensity values (responses) between the two diet groups for metabolites ($n \leq 12$ metabolites per pathway/category analyses) involved in separate metabolic pathways/categories (**Supplementary Table 3**) with statistical significance set at $p< 0.05$. All statistical analyses for lipidomics and metabolomics were performed using GraphPad Prism 6.0 software (GraphPad, La Jolla, CA). Quantification of total and free fatty acids, and oxidized lipids was performed using MutliQuant Software version 3.0.2 (SCIEX), as performed previously [306]. Statistical analyses were performed as for lipidomics/metabolomics (above).

Results

Adult behavior and cognitive function

We first investigated the effects of chronic VitE deficiency on cognitive outcomes in adult E- and E+ zebrafish. To assess learning, we utilized the shuttle-box active avoidance assay, a modification of the protocol described [259, 364], in which 12-month-old adult E- and E+ zebrafish were trained to actively "shuttle" from a dark, "unsafe" chamber into a lighted, "safe" chamber to avoid an aversive stimulus (mild electrical shock). Grouped linear regressions of the "Time to a Side" parameter, (the cumulative time per trial that a fish required to shuttle to the lighted chamber, in seconds) revealed that over the 30-trial testing phase, E- adults took significantly longer to swim to safety than E+ adults (**Figure 12**), suggesting an impaired ability to associate presentation of the discriminative/operant light stimulus with safety (*i.e.* avoidance of the shock stimulus). Similar analyses using the "Returns to Dark" parameter (the number of times within a trial that a fish returns to the dark chamber and receives a shock *after* having shuttled to the lighted chamber) as another metric of learning ability showed that E- adults returned to the unsafe chamber significantly more than did E+ fish (p =0.0048; mean ± SEM Returns over 30 trials E-: 2.87 ± 0.35; E+: 1.21 ± 0.29, data not shown), which again suggests impaired cognitive function.

To determine whether impaired swimming ability in E- adults, as observed previously [74], contributed to the shuttle-box results, we performed non-associative learning (habituation) evaluations in which a startle response (increased swimming following a loud noise) was measured following a series of tap-stimuli. In this assay, the startle response was expected to decrease with successive taps as the animal habituates to the stimulus; that is, learns that the noise is not harmful. Baseline swimming activity was the same in both E- and E+ adults, as was the respective groups' startle response to the first tap (**Figure 13**). However, while the E+ adults mounted sequentially lower startle responses to later tap stimuli, indicating a successful ability to habituate/learn, E- adults did not: they mounted an equally robust startle response to all three taps (**Figure 13**). This data indicates that the E- fish did not suffer compromised locomotion; therefore, outcomes from both shuttle-box and habituation assays suggest reduced learning ability and cognitive impairment associated with chronic VitE deficiency.

VitE status, lipid peroxidation, and lipidomics assessments

We hypothesized that the E- adults' behavioral perturbations were likely related to altered VitE status within the brain. Quantified VitE levels in brains from E- adults (5.7 ± 0.1 pmol/mg) were approximately 22-times lower than in E+ adult brains (122.8 ± 1.1 pmol/mg; *n*= 10/group) when measured at 18-months of age. Muscle VitE levels were significantly lower in E– adults (E-: 1.3 ± 0.1 vs E+: 114.8 ± 0.9 pmol/mg tissue). Given (a) VitE's role as a lipophilic antioxidant [49], and (b) the brain's high concentrations of long-chain polyunsaturated fatty acids (PUFAs) are prone to peroxidation [365], especially DHA [337], we further posited that such extreme differences in brain VitE content would cause significant lipid peroxidation of PUFAs within E- compared to E+ brains. Indeed, quantified total and unesterified (free) levels of ω-3 and ω-6 PUFAs were markedly lower in E- vs. E+ brains (**Figure 14**); the most drastic decreases were found for total DHA and arachidonic acid (ARA; 20:4 ω-6), which both were reduced by approximately two-fold in the E- fish, as compared to the E+ (**Figure 14A**). Additional measurements of oxidized lipids revealed that, concomitant with the observed decreases in PUFA levels, E- brains contained significantly higher amounts of several ω-*3* and ω-6 oxidized species than did E+ brains (**Figure 15**). Affected lipids included F_4-neuroprostanes, which are autoxidized DHA-derivatives known to be elevated with neurodegenerative disease and/or

neurological oxidative injury [135]. These data demonstrate that long-term VitE deficiency led to enhanced lipid peroxidation and, potentially, consequent depletion of select PUFAs, particularly DHA and ARA, in brains from the E- group.

The present data showing increased peroxidation of brain DHA in 18-month-old adult zebrafish E- brains resembles our previous measurements of oxidized DHA-containing PL species found in brains from 12-month-old E- zebrafish [72]. This latter (younger) cohort of fish also sustained significant perturbations in the overall brain PL composition [72]; thus, we performed lipidomics analyses in brains from our 18-month-old cohort of E- and E+ fish to investigate whether an extended duration of VitE deficiency caused greater disruption of brain PLs in older fish. Whereas our prior report found only four PLs to be decreased in 12-month-old E- relative to E+ brains [72], brains from 18-month-old E- fish were lower in 13 different PLs (**Figures 16** and **17**); notably, ten contained DHA (**Figure 16**) and the remaining three also contained highly-unsaturated lipids (*e.g.* eicosapentaenoic acid [EPA 20:5 ω-3]; **Figure 17**). Moreover, we show herein for the first time that E- brains contained more highly-*saturated* PL species than did E+ brains (**Figure 18**), potentially indicating replacement of damaged (oxidized) unsaturated fatty-acyl chains with saturated species to generate molecules that are less vulnerable to lipid peroxidation. Lower levels of 12 lyso-phospholipids (lyso-PLs), including lysophosphatidylcholine containing DHA (LPC 22:6), the preferred form of DHA uptake into the brain [315, 316, 366], also were evident in E- compared to E+ brains (**Figure 19**).

Metabolomic assessment of antioxidant and energy metabolism

We postulated that the severe decrease in VitE in E- vs. E+ brains not only resulted in increased lipid peroxidation (**Figure 15**), but also likely disrupted the cellular antioxidant network by reducing levels of additional antioxidants in the brain. Using targeted metabolomic analyses, we found relative amounts of ascorbic acid, GSH, and NADH (**Figure 20**), all were significantly lower in E- compared to E+ brains, while corresponding oxidation products were elevated in the E- group (**Figure 20**).

Additional targeted metabolomics analyses revealed considerable decreases in brain glucose and glycolytic intermediates (**Figure 21**), as well as reduced levels of pentose-phosphate pathway intermediates and NADPH (**Figure 21**) in E- vs. E+ brains, suggesting VitE deficiency-induced dysregulation of glucose metabolism both for energy production and for generation of reducing equivalents (*e.g.* NADH and

NADPH) to replenish and maintain the cellular antioxidant network. Note that NADH was significantly lower in E- adult brains as well (**Figure 20)**. Interestingly, E- brains contained higher levels of the ketone body β-hydroxy-butyrate than did E+ brains; ketogenic amino acids (including strictly-ketogenic lysine and leucine) and palmitic acid (18:0), both of which may be utilized for ketone synthesis from acetyl-CoA, also were elevated in E- samples (**Figure 21**).

Metabolomics assessments of phospholipid and choline metabolism

Given the significant disruption of PL/LPL composition (**Figures 16-19**) and of cellular energy metabolism (**Figures 21** and **22**), we investigated whether VitE deficiency also perturbed other metabolic pathways; particularly those related to phosphatidylcholine (PC) synthesis, since this PL/lyso-PL variety was most affected by VitE deficiency (as reported previously [72, 305]). Not only were metabolites of the CDP-choline pathway decreased in E- vs. E+ brains (*e.g.* choline), but ethanolamine, a requisite substrate for serial methylation of phosphatidylethanolamine (PE) via the phosphatidylethanolamine methyltransferase (*PEMT*) pathway, which generates PC species enriched with DHA [367], also was significantly lower in the E- group (**Figure 23**). This indicates possible disruption of metabolic pathways specifically associated with synthesis of DHA-containing PC. Accordingly, altered PC metabolism (presumably due to upregulated remodeling and turnover of damaged membrane PLs, as discovered in VitE-deficient embryos [305]), and decreased choline levels in E- brains (**Figure 23**) were associated with observed decreases in choline-derived methyl-donor metabolites such as betaine and *S*-adenosylmethionine (SAM; **Figure 24**). We also found parallel increases in oxidized methylation cycle metabolites *S*-adenosylhomocysteine (SAH) and homocysteine (**Figure 24**). Lower levels of methyl-donors, like SAM, correspond with dysregulation of the *PEMT* pathway, as well as suggest that choline inadequacy induced by VitE deficiency may disturb more universal methyl-donor status and, hence, other methylation reactions (*e.g.* epigenetic processes like DNA methylation).

Discussion

Zebrafish (*Danio rerio*) are a recognized model for studying the pathogenesis of cognitive deficits [260] and the mechanisms underlying behavioral impairments, including the consequences of increased oxidative stress within the brain [368]. Herein, we demonstrated that chronic VitE deficiency impaired both associative

(avoidance conditioning) and non-associative (habituation) learning in adult zebrafish, and that these functional deficits occurred concomitantly with the following observations in adult E- brains: decreased concentration and increased peroxidation of PUFAs (especially of DHA), altered brain PL and lyso-PL composition, dysregulation of the cellular antioxidant network; and perturbed energy (glucose/ketone), phospholipid (PC), and choline/methyl-donor metabolism.

In the present study, we utilized complementary behavioral assays to show that VitE deficiency specifically compromised *cognition*, rather than perception and/or locomotion [74, 278]. The shuttle-box assay, while widely employed as a paradigm to evaluate learning in zebrafish via avoidance conditioning [259], has several limitations; namely, the data from this assay alone do not necessarily indicate impaired cognition because the trained avoidance response requires functional visual and motor systems – the fish must perceive and swim towards the light to escape receiving a shock. Chronic VitE deficiency can lead to blindness [369], severe motor dysfunction [266], and, in zebrafish, impaired swimming due to degenerative myopathy [74]. While follow-up analyses to measure visual acuity [370] in E- vs. E+ adults are warranted, the fact that the E- fish *did* readily respond to the light stimulus by directly (rather than aimlessly) swimming into the illuminated chamber – albeit less promptly than E+ fish – suggests the E- group perceived the light without difficulty, and that their visual system remained sufficiently functional.

To address the possibility of compromised locomotion, we next performed a habituation (startle-response) assay to determine if the E- adult's swimming ability was reduced. Our present findings demonstrated that E- adults did not have impaired locomotion, and, in fact, had *elevated* swimming activity in response to the startle stimulus. These results contrast with previous findings [74]; however, this discrepancy may be attributed to the combined VitE and vitamin C deficiency in our past report, which caused significant myopathy in the E- adults. Such differential findings are analogous to other studies showing that compounded antioxidant-nutrient deficiencies lead to significantly more severe physiological (myopathic) consequences than do isolated deficiencies [371]. E- fish in the present study were neither vitamin C-deficient (muscle ascorbic acid content was 84.9 ± 0.3 pmol/mg tissue in E- and 84.7 ± 0.3 pmol/mg tissue in E+ adults) nor motor-impaired relative to E+ fish; rather, their enhanced startle response suggests a neurological failure to habituate to the tap

sound (*i.e.* learn to ignore a repeated, non-harmful stimulus). This could be due to an underlying disruption of inhibitory signaling within the brain, as levels of γ-amino-butyric acid (GABA), a primary inhibitory neurotransmitter in vertebrates [372], were significantly lower in E- compared to E+ brains ($p < 0.0001$; **Supplementary Table 3**). Whether, and to what extent, disruption of neurotransmitter signaling contributed to the learning impairments associated with VitE deficiency requires additional research beyond the scope of the present work (for example, using transgenic zebrafish lines [373] or neuron recordings [374]); though, intriguingly, recent studies suggest a role for VitE in the delay of both cognitive and motor declines in aged rodents due to increased monoamine neurotransmitter synthesis [375]. Overall, what our behavior data *does* show is that isolated, chronic VitE deficiency resulted in strictly cognitive abnormalities, as evidenced by significant learning deficits in the E- adults.

Behavioral impairments in the adult E- fish are perhaps not surprising considering the extreme lack of brain VitE within this group. VitE deficiency has well-established neurological consequences [362], including compromised cognition [211, 363]. These detrimental effects are mostly attributed to increased neurological oxidative stress, resulting from inadequate antioxidant protection from VitE within the brain [215]. Such an explanation, however, does not provide mechanistic insight as to how VitE deficiency contributed to the etiology of neurodegenerative disease, nor how VitE functions to preserve cognitive health. Our lipid and metabolomics analyses help fill these gaps in knowledge, as we show that VitE deficiency-induced oxidative stress caused multiple secondary nutrient deficiencies, each of which perturbed potentially inter-related metabolic pathways that may underlie neuronal dysfunction and, ultimately, impaired cognition.

First, we demonstrate VitE deficiency led to decreases in brain PUFAs (**Figure 14**), likely due to elevated lipid peroxidation, especially of DHA (**Figure 15**). Numerous animal and human studies emphasize the importance of sufficient DHA for optimal brain health and prevention of cognitive decline (as reviewed [85]); for example, lower levels of plasma DHA are associated with age-related cognitive impairment [376], and many animal [118], epidemiological [219], and clinical [377] reports show that high DHA consumption is associated with reduced dementia risk. Thus, DHA peroxidation and reduced levels in the E- zebrafish potentially constitute major factors explaining their behavioral defects. While enhanced peroxidation and oxidized lipid-derivative

biological activity of other PUFAs, such as ARA [378], cannot be ignored, we focus here on DHA since its status and metabolism appeared most affected in E- brains.

Mechanism(s) via which VitE deficiency-induced peroxidation and decreased DHA could contribute to cognitive dysfunction are myriad, but likely include resulting alterations to the cellular membrane PL profile (**Figures 16-19**). We found significant disruption of membrane PLs in E- adults; specifically, lower levels of 10 DHA-containing PLs (**Figure 16**), several of which are biomarkers for increased dementia risk in humans [277], as well as two lyso-PLs with DHA, including LPC 22:6 (**Figure 19**), the primary uptake form of DHA into the vertebrate [315, 366] (and more specifically, zebrafish [316]) brain. Such drastic changes may compromise learning, as PL DHA-content modulates membrane fluidity and function [118]; within the brain, such effects influence neuronal signaling [133]. For example, membrane DHA is suggested to facilitate N-methyl-D aspartate (NMDA) receptor responses [379], which result in long-term potentiation, a crucial process for synaptic modifications underlying long-term memory and learning in vertebrates [380], including zebrafish [381]. DHA supplementation also has been shown to increase the levels of hippocampal brain-derived neurotrophic factor (BDNF) [382], as well as promote the activation of protein kinase B (PKB; known also as Akt), a crucial signaling protein for neuronal survival, via alterations to the membrane PL profile [300]. DHA-influenced changes in gene expression could contribute to perturbed brain function in the E- group as well: DHA modulates gene transcription by activating peroxisome proliferator-activated receptor (PPAR) family members [133]; hence, DHA alters expression of genes involved in energy metabolism within the brain [383] and the mRNA stability of several enzymes associated with glucose and lipid metabolism [384]. In summary, the numerous and varied functions of DHA, as an integral part of brain PLs, constitute several, probably cumulative, processes through which VitE deficiency-induced decreases in brain DHA and DHA-containing PLs adversely impacted E- adult behavior.

Additionally, our data showing more general increased lipid peroxidation in E- brains (**Figure 15**) also is highly significant because it provides a mechanistic link between observed perturbations in the cellular antioxidant network (**Figure 20**) and compromised energy metabolism (**Figures 21** and **22**): hypothetically, reduced lipophilic antioxidant protection from VitE and the ensuing peroxidation of brain PUFAs disrupts glucose utilization by enhancing requirements for the endogenous reducing

equivalents NADH and NADPH [385], leading to the eventual depletion of brain glucose in an attempt to mitigate oxidative injury. This process is proposed to underlie dementia pathogenesis, as brain glucose uptake [386] and utilization [385] decline well before the clinical symptoms of cognitive decline present [387]. Our findings that β-hydroxy-butyrate and ketogenic amino acids were elevated in E- brains (**Figure 21**), while glycolytic and citric acid cycle intermediates were decreased (**Figures 21** and **22**) provide additional, provocative evidence to support the notion that VitE-induced oxidative stress perturbs brain energy metabolism by causing a shift in substrate preference that is meant to counter the production of oxidizing free-radicals. In fact, recent interventions have found ketogenic diets provide therapeutic benefit in animal models of dementia [33] by reducing mitochondria-generated oxidative species. While the redox control of metabolism – and associated disruptions of this relationship in neurodegenerative conditions – is not a new phenomenon [139], our combined lipid and metabolomics data signify mitochondrial dysfunction in the brain may be a primary mechanism that contributes to VitE deficiency-related cognitive deficits. Further research to unravel molecular and signaling details of these findings, particularly enzyme- and gene-focused studies related also to targets of DHA signaling [384], is necessary to substantiate this supposition and better link the results reported herein.

Finally, while disruption of the membrane PL fatty acid content in E- adult brains is noteworthy, so too is evidence of disrupted membrane PL synthesis (**Figure 23**), especially for PC and the related decrease in choline. Exhaustion of both CDP-choline and PEMT pathway metabolites suggests enhanced PC turnover and remodeling following oxidative damage, particularly of PC-species with DHA, as previously reported in VitE-deficient zebrafish embryos [305, 306]. Associated decreases in choline within E- brains deserves individual attention, given the role choline has in both neurodevelopment [30] and preservation of cognitive function throughout adulthood [217]. These neuroprotective effects may be mediated through choline's role as a methyl-donor nutrient [40]. Therefore, our findings that choline and choline-derived methyl-donor metabolites, such as betaine and SAM, are decreased in E- brains (**Figure 24**) suggests disruption of methylation reactions that could subsequently affect cognition, since methyl-donor deficiency has been found to compromise learning by disturbing epigenetic DNA and/or histone methylation in the brain [238]. The impact of such epigenetic consequences on cognitive function and

dementia risk remains an active area of research (as reviewed [224]), and recent studies link redox-mediated processes to epigenetic modifications that specifically influence brain mitochondrial energy metabolism [388] as well as more general cognitive health [228]. That VitE deficiency may have downstream epigenetic effects by inducing choline inadequacy within the brain constitutes an intriguing and novel focus for future studies.

In conclusion, we demonstrate VitE's critical role in maintaining neurological health may be attributed to several pleiotropic functions that likely converge and interrelate to explain how chronic VitE deficiency contributes to dementia pathogenesis. Addressing and resolving the possibility of pervasive, sub-clinical VitE deficiency in adult humans [79], therefore, warrants urgent attention to help prevent and/or delay progressive cognitive decline in the globally aging population.

Acknowledgements:

The authors thank Carrie Barton, Scott Leonard, and Dr. Michael Simonich for their outstanding technical assistance. NIH S10RR027878 (MGT), NIEHS P30ES000210 (RT), and OSU Center for Healthy Aging Research LIFE (MM) grants supported this work.

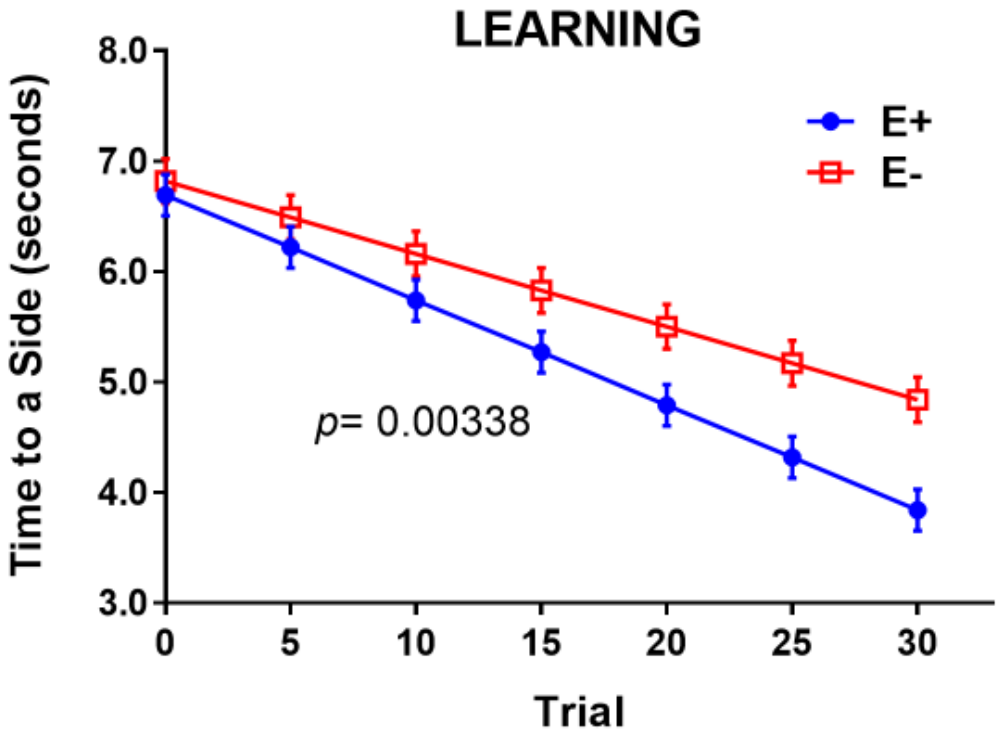

Figure 12. E- adults were learning impaired when compared to E+ adults Grouped linear regressions of the time required ("Time to a Side", in seconds) for an individual fish to decide to swim to the lighted, "safe" chamber in the E- (*n*= 37) vs. E+ (*n*= 34) diet conditions. By the 30th trial, E- adults took significantly longer to associate the light stimulus with safety, indicating impaired learning. For each diet condition, the data were fit using group linear regression models, which allowed observance of variance and reduced the sensitivity to outliers. E- vs. E+ learning rates were compared using a student's *t* test to calculate statistical significance ($p< 0.05$).

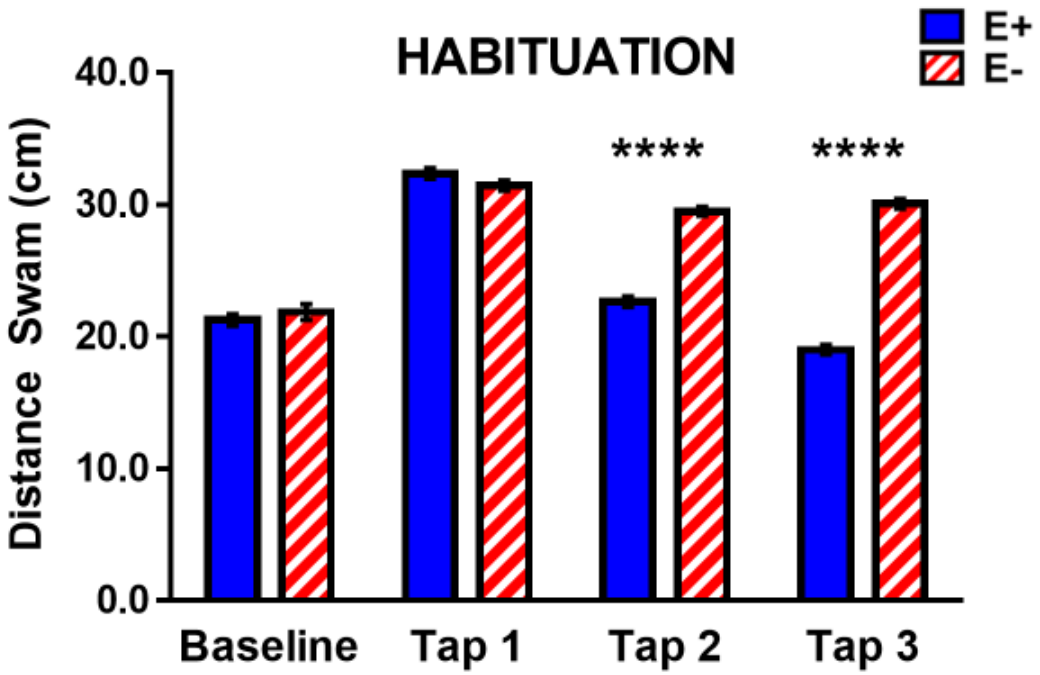

Figure 13. E- adults had a compromised habituation (startle) response compared to E+ adults A total of 64 naïve E- and E+ zebrafish (*n*= 32/diet condition) were tracked at 25 frames/sec for total distance swam (cm) and velocity over one-minute intervals during acclimation (Baseline) and following each tap stimulus, shown above as mean distances ± SEM. During the 10-minute acclimation period prior to the first startle tap, E- and E+ fish exhibited the same amount of swimming activity. In response to the initial tap (TAP 1), both E+ and E- zebrafish mounted a similar swim response, but E- zebrafish mounted nearly the same magnitude of response to all three startles (taps), indicating failure to habituate to the stimulus (Two-way ANOVA with Tukey's post-test for multiple comparisons; overall p <0.0001 for Diet x Tap interaction; ****p <0.0001 at indicated Taps).

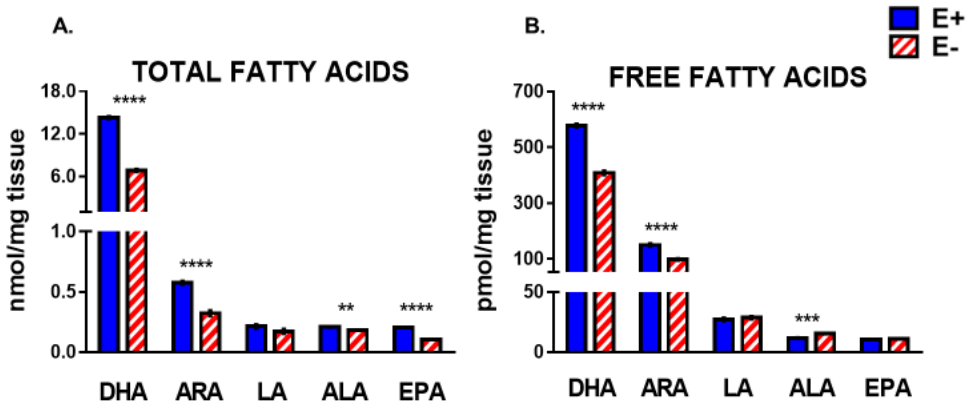

Figure 14. Quantified levels of total and unesterified (free) fatty acids in E– vs. E+ adult brains Shown are area counts for individual fatty acids normalized using internal standards (*n*= 10 samples/diet). A. Saponified and B. extracted only samples; means ± SEM; *p*-values are indicated as *<0.05, **<0.005, ***<0.001, **** <0.0001. from unpaired student's *t*-tests. Abbreviations: ALA (alpha-linolenic acid); ARA (arachidonic acid); DHA (docosahexaenoic acid); EPA (eicosapentaenoic acid); LA (linoleic acid).

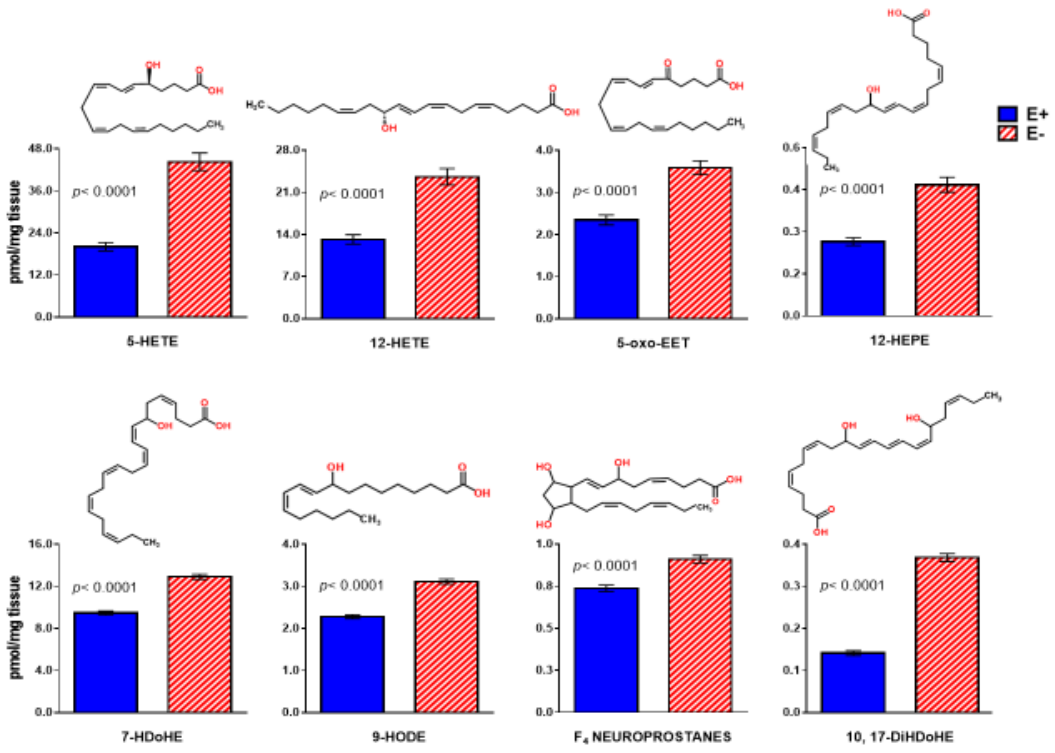

Figure 15. Oxidized fatty acids were elevated in E- compared to E+ brains Individual fatty acids (means ± SEM, area counts normalized using internal standards, obtained and analyzed as for Figure 3B) are shown from adult E- and E+ zebrafish brains (*n*= 10/diet). Significant differences (p <0.05) were determined for each lipid using logarithmically transformed data and an unpaired student's *t*-test. Abbreviations: 5-HETE (5-hydroxyeicosatetraenoic acid); 12-HETE (12-hydroxyeicosatetraenoic acid); 5-oxo-EET (5-oxo-eicosatetraenoic acid); 12-HEPE (12-hydroxy-eicosapentaenoic acid); 7-HDoHE (7-hydroxy-docosahexaenoic acid); 9-HODE (9-hydroxy-docosahexaenoic acid); F4-neuroprostanes (shown as 7-series; 9-3,5-Dihydroxy-2-2,5-octadien-1-yl cyclopentyl-7-hydroxy-4,8-nonadienoic acid); 10, 17-DiHDoHE (10, 17-dihydroxy-docosahexaenoic acid).

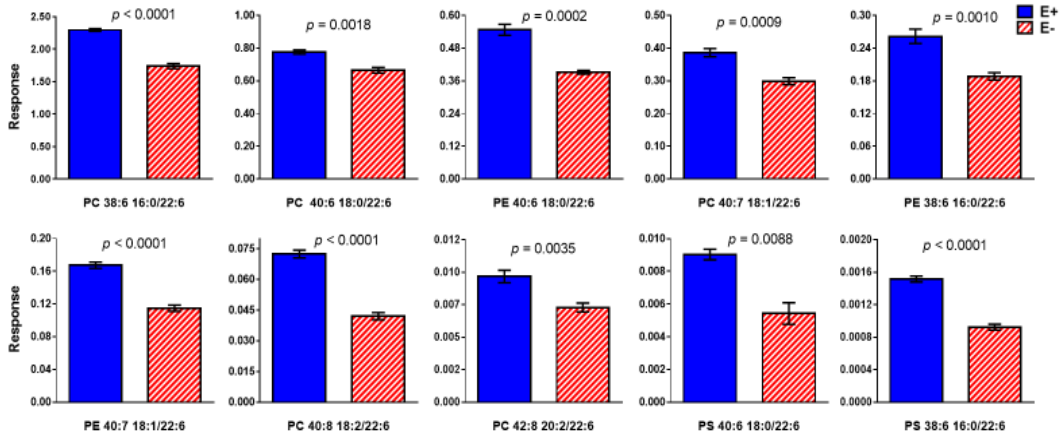

Figure 16. Ten specific DHA-PLs were lower in E- compared with E+ brains Differences between each identified DHA-PL from brains of 18-month-old E- and E+ (mean ± SEM, peak MS responses were used for relative quantification, *n*= 10/diet group) were determined using logarithmically transformed data and an unpaired student's *t*-test ($p < 0.05$).

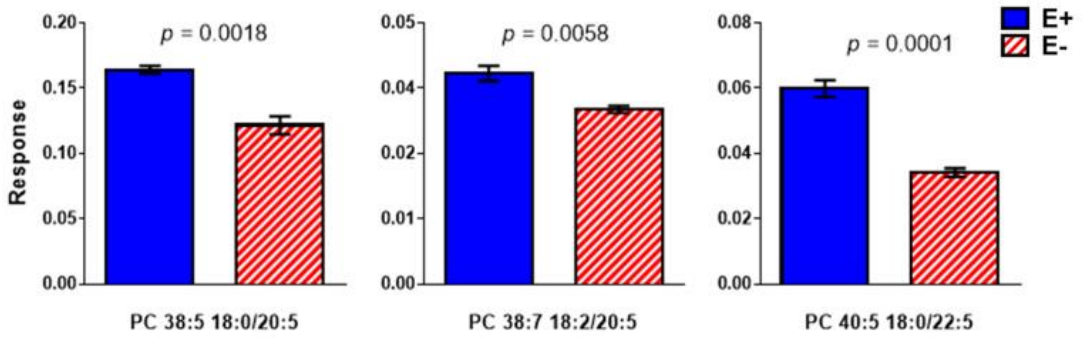

Figure 17. E- zebrafish brains had significantly lower levels of unsaturated PL species compared to E+ brains Highly unsaturated PLs (including DHA-PLs shown in Figure 16) were decreased in E- compared to E+ brains, indicating the targeted depletion of unsaturated brain PLs due to chronic VitE deficiency. The data shown was obtained and analyzed as for Figure 16.

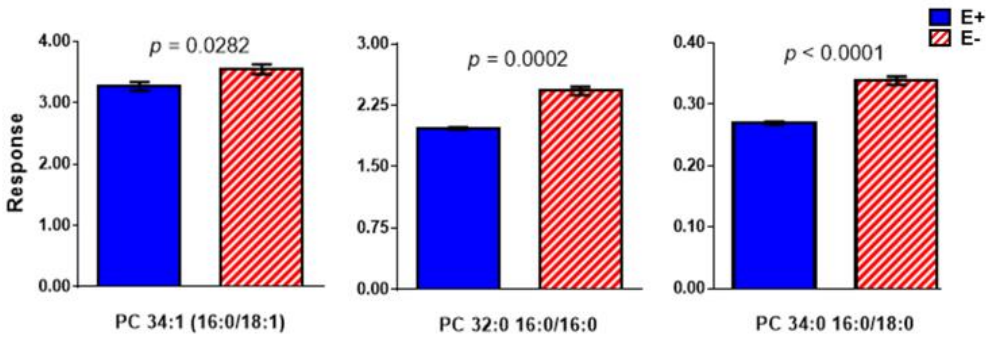

Figure 18. E- zebrafish brains had significantly higher levels of saturated PL species compared to E+ brains Highly saturated PL species were increased in E- brains. The data shown was obtained and analyzed as for Figure 16.

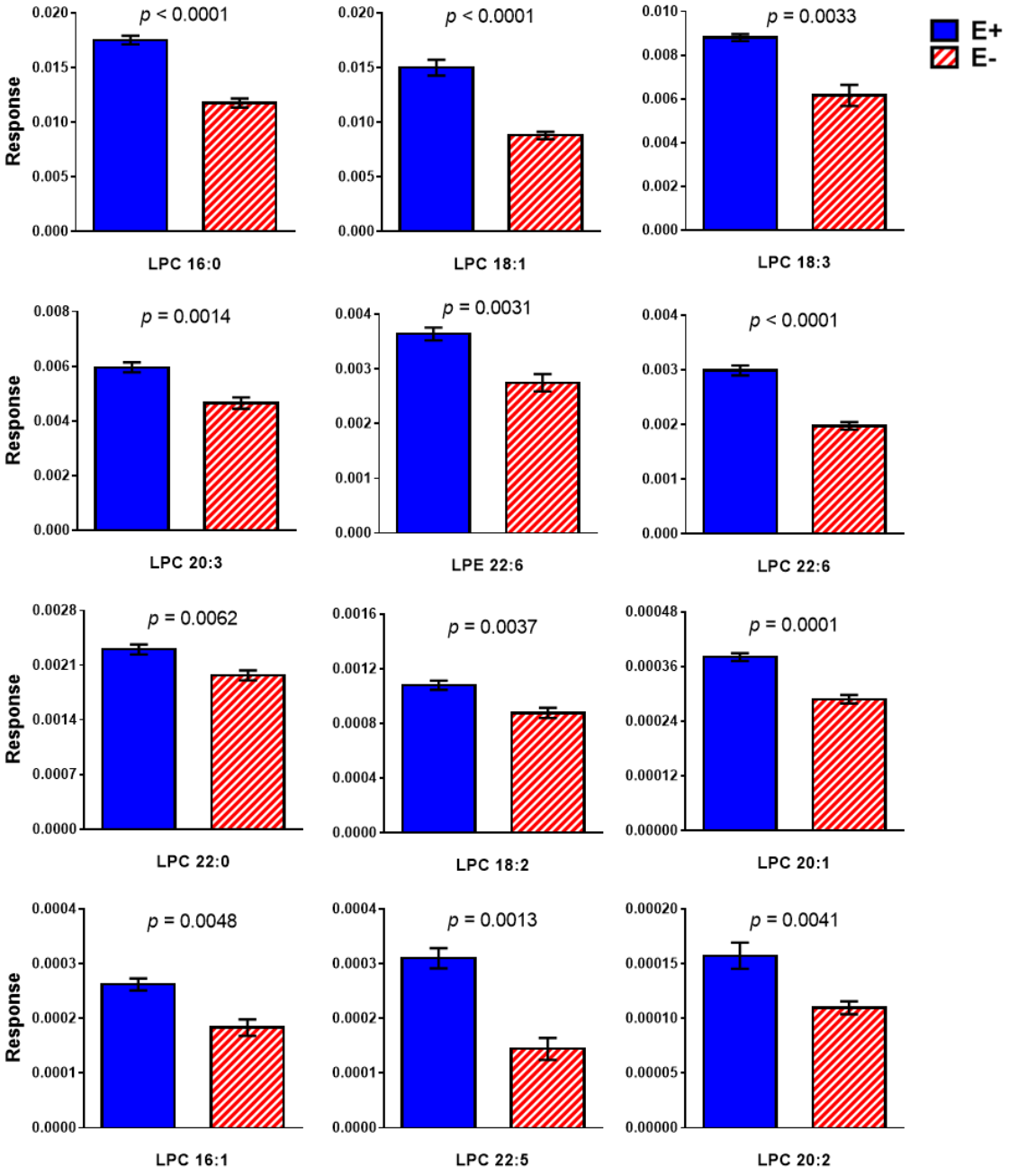

Figure 19. E- zebrafish brains were significantly lower in 12 lyso-PLs compared to E+ brains The data shown was obtained and analyzed as for Figure 16.

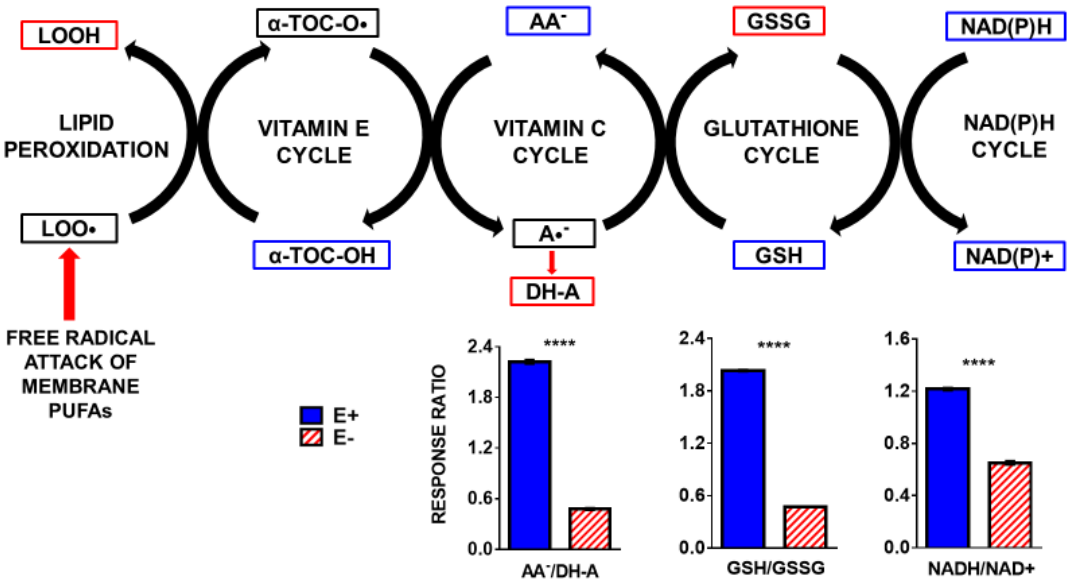

Figure 20. The cellular antioxidant network was disrupted in E- adult brains Antioxidant network scheme showing interaction of antioxidants with lipid radicals and consumption or NAD(P)H. Bar graphs show relative responses between brains from E- and E+ adults ((*n*= 2 brains/sample; four replicates per group); data was normalized against QC sample intensities (*n*= 4) for each individual metabolite. Boxes shown in Red (increased in E– or Blue (increased in E+) represent metabolites that were higher in E– or E+ adults, respectively. Black boxes indicate relative levels of a given metabolite not shown in the figure. Statistical significance ($p<0.05$) was determined using the Holm-Sidak method for multiple comparisons of normalized intensity values. Shown are means ± SEM, *p*-values are indicated as **** <0.0001. Abbreviations: LOO, lipid radical; LOOH, oxidized lipid; α-TOC-OH, α-tocopherol; α-TOC-O, α-tocopheroxyl radical; AA^-, ascorbic acid; A^- ascorbate radical; DH-A, dehydroascorbate; GSH, glutathione; GSSG, glutathione disulfide; PUFAs, polyunsaturated fatty acids.

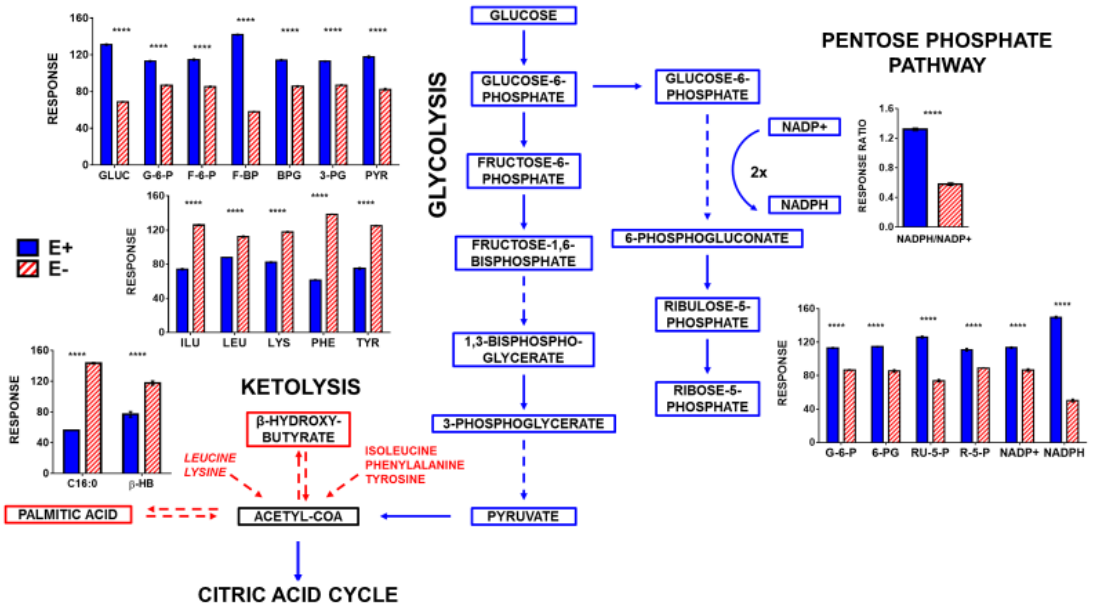

Figure 21. Cytosolic energy metabolism pathways in the brain were perturbed in E- adults The data shown in bar charts compare levels of individual metabolites included in the outlined metabolic pathway diagram (left). Boxes shown in Red (increased in E– or Blue (increased in E+) represent metabolites that were higher in E– or E+ adults, respectively. Black boxes indicate relative levels of a given metabolite not shown in the figure. Solid lines indicate direct reactions and dashed lines denote several reaction steps between metabolites. Data were analyzed as described in Figure 20. Abbreviations: GLUC, glucose; G-6-P glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-BP, fructose-1,6,-bisphosphate; 1,3-BPG, 1,3-bisphosphoglycerate; 3-PG, 3-phosphoglycerate; PYR, pyruvate; 6-PG, 6-phosphogluconate; RU-5-P, ribulose-5-phosphate; R-5-P, ribose-5-phosphate; β-HB, β-hydroxy-butyrate; C16:0, palmitic acid.

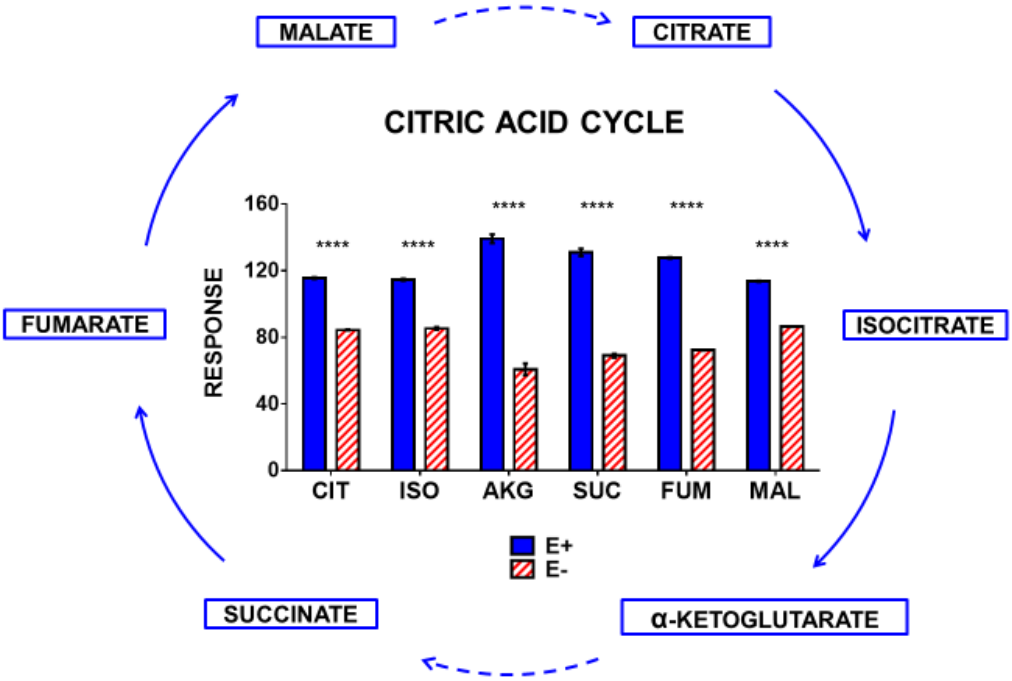

Figure 22. Mitochondrial energy metabolism was decreased in E- compared to E+ adult brains The data shown in bar charts compare levels of individual metabolites included in the outlined metabolic pathway diagram (left). Boxes shown in Blue represent metabolites that were higher in E+ compared to E- adults. Solid lines indicate direct reactions and dashed lines denote several reaction steps between metabolites. Data were analyzed as in Figure 20. Abbreviations: CIT, citrate; ISO, isocitrate; AKG, α-ketoglutarate; SUC, succinate, FUM, fumarate; MAL, malate.

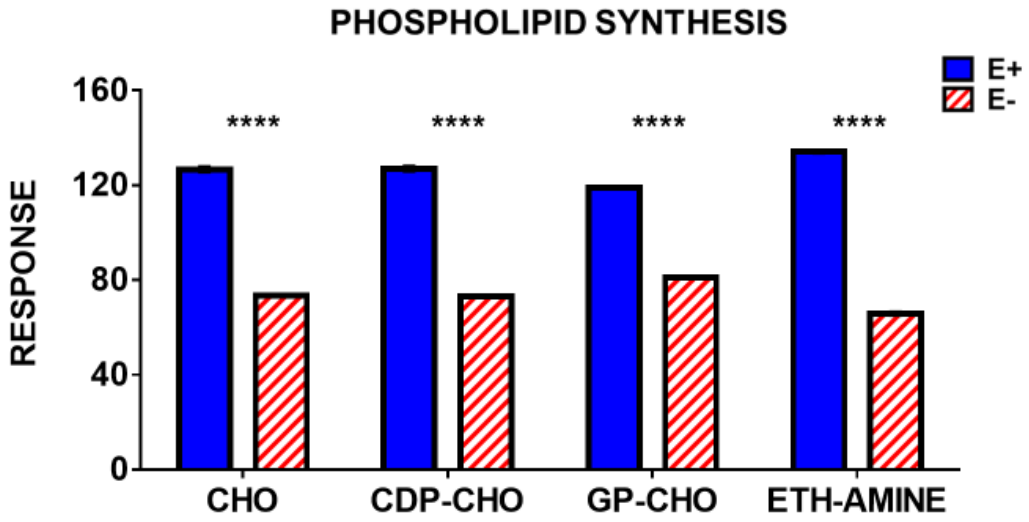

Figure 23. Phospholipid synthesis in the brain was altered in E- compared to E+ adults Metabolomics results for phosphatidylcholine metabolism pathway intermediates in E- vs. E+ adult zebrafish brains (*n*= 2 brains/sample; 4 replicates per group). Data were analyzed as in Figure 20. Abbreviations: CHO, choline; CDP-CHO, cytidine 5'-diphosphocholine; GP-CHO, glycero-phosphocholine; ETH-AMINE, ethanolamine.

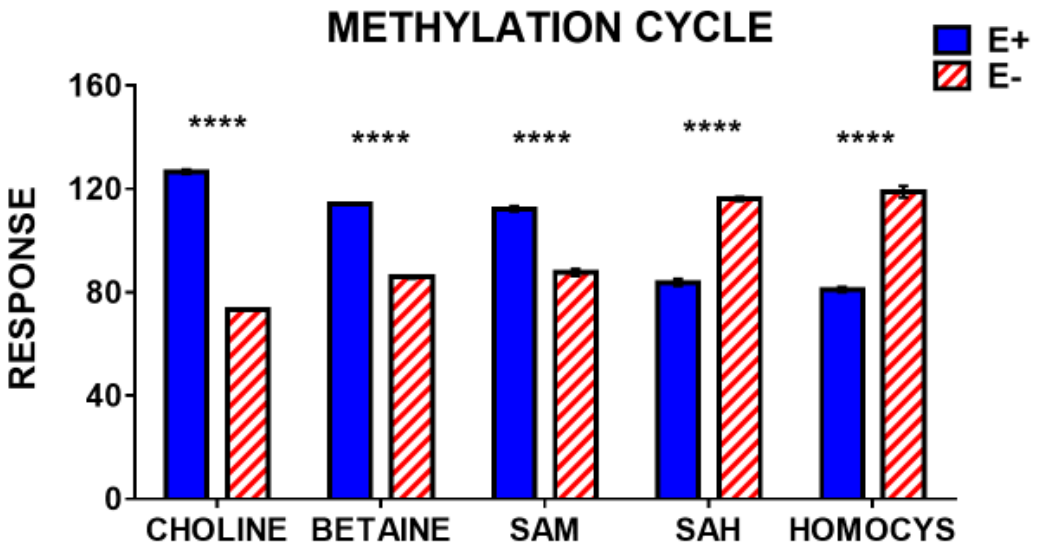

Figure 24. Choline-derived methyl-donors were decreased in the brains of E- adults Metabolomics results for choline-derived methylation-cycle intermediates in E- vs. E+ adult zebrafish brains (*n*= 2 brains/sample; 4 replicates per group). Data were analyzed as in Figure 20. Abbreviations: SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; HOMOCYS, homocysteine.

Supplementary Table 3

Click here to download **Supplementary Table 3**

CHAPTER FIVE:

Vitamin E deficiency during embryogenesis in zebrafish causes lasting metabolic and cognitive impairments despite refeeding adequate diets

Melissa McDougall, Jaewoo Choi, Lisa Truong, Robert Tanguay, Maret G. Traber

Unpublished Manuscript

Abstract

Vitamin E (α-tocopherol; VitE) is a lipophilic antioxidant required for normal embryonic development in vertebrates, but the long-term effects of embryonic VitE deficiency, and whether they are ameliorated by feeding VitE–adequate diets, remain unknown. We addressed these questions using a zebrafish (*Danio rerio*) model of developmental VitE deficiency followed by dietary remediation. Adult zebrafish maintained on VitE–deficient (E–) or sufficient (E+) diets were spawned to obtained E– and E+ embryos, respectively, which we evaluated up to 12 days post-fertilization (dpf). The E– group suffered significantly increased morbidity and mortality as well as altered DNA methylation status through 5 dpf when compared to E+ larvae, but upon feeding with a VitE-adequate diet from 5-12 dpf both the E– and E+ groups survived and grew normally; the DNA methylation profile also was similar between groups by 12 dpf. However, 12 dpf E– larvae still had behavioral defects. These observations coincided with sustained VitE deficiency in the E– vs. E+ larvae ($p< 0.0001$), despite adequate dietary supplementation. We also found in E– vs. E+ larvae continued docosahexaenoic acid (DHA) depletion ($p< 0.0001$) and significantly increased lipid peroxidation. Further, targeted metabolomics analyses revealed persistent dysregulation of the cellular antioxidant network, the CDP-choline pathway, and glucose metabolism. While anaerobic processes were increased, aerobic metabolism was decreased in the E– vs. E+ larvae, indicating mitochondrial damage. Taken together, these outcomes suggest embryonic VitE deficiency causes lasting behavioral impairments due to persistent lipid peroxidation and metabolic perturbations that are not resolved via later dietary VitE supplementation.

Introduction

Vitamin E (α-tocopherol; VitE) is required for healthy fetal development, as its deficiency during pregnancy causes fetal resorption in rodents [78], and human studies associate maternal VitE inadequacy with early miscarriage [80]. Further, our recent work using a zebrafish model of embryonic VitE deficiency indicates VitE is essential for normal *neuro*development: VitE-deficient (E–) compared to VitE-sufficient (E+) zebrafish embryos have increased mortality, severe morphological abnormalities including cranio-facial deformities [305, 306], and also exhibit marked neurobehavioral perturbations [305-307]. Such outcomes likely are mediated via multiple, pleiotropic mechanisms that all arise from VitE's role as a potent lipophilic antioxidant [49].

Initially, increased lipid peroxidation in E– embryos depletes them of docosahexaenoic acid (DHA, 22:6*n*–3) [286, 306], resulting in the increased recycling/turnover and depletion of DHA-containing phospholipids and lysophospholipids [305], particularly those containing choline (phosphatidylcholine; PCs and lyso-PCs, respectively), including lyso-PC 22:6, the form of DHA preferentially taken up by the brain [366] through the specific transporter, MFSD2A [315, 316]. This secondary depletion of choline coincides with cranio-facial deformities analogous to the neural tube defects evident in animal [349] and human [186] reports of maternal choline deficiency that, notably, also occur during neurulation [252] with *complete* VitE deficiency in genetically-manipulated zebrafish embryos at 15-17 hours post-fertilization (hpf) [297]. Given choline's established role in cognitive development [183], VitE likely is essential for the fetal brain not only because it protects membrane DHA from excessive peroxidation, but also because it facilitates adequate perinatal choline status.

Furthermore, the increased lipid peroxidation in E– embryos perturbs their cellular antioxidant network, which ultimately disrupts aerobic energy metabolism, causing a significant decrease in whole-body (and, presumably, brain) glucose levels, and thus adversely impacts neurobehavioral outcomes by depriving the embryonic brain of sufficient energy to grow and function [306, 307]. These consequences are avoided with proactive VitE repletion because an α-tocopherol emulsion administered into the yolk of 0 hpf E– embryos entirely prevents mortality and morbidity outcomes [307]. However, remediation of VitE deficiency-induced (*i.e.* secondary) nutrient deficiencies only partially rescues E– embryos, as observed following glucose supplementation into the yolk at one day of age (24 hpf; after established VitE deficiency but prior to glucose depletion) [306]. Together, this data suggests the effects of developmental VitE deficiency may be *prevented*, but not necessarily *reversed*. We hypothesized, therefore, that deleterious outcomes of embryonic VitE deficiency cannot be ameliorated fully though later supplementation with VitE and other depleted nutrients (*e.g.* DHA and choline), and that long-term cognitive defects will persist in E– compared with E+ embryos despite dietary intervention. To test this hypothesis, we selected normal appearing E– or E+ embryos, then fed them a complete diet for 7 days and analyzed them for behavioral, biochemical, and morphological changes.

Materials and Methods

Materials and reagents

The following reagents were used for metabolomics analyses: methanol and ultra-pure water (LC-MS grade, EMD Millipore, Gibbstown, NJ); zirconium oxide beads (Next Advance; Averill Park, NY); formic acid, acetic acid (Optima LC/MS grade; Fisher Chemical, Pittsburgh, PA); Deuterium (d) labeled internal standards DHA-d_5, ARA-d_8, EPA-d_5, LA-d_4, ALA-d_{14}, 9(*S*)-HODE-d_4, and 5-HETE-d_8 (Cayman Chemical, Ann Arbor, MI) and butylated hydroxytoluene (BHT, TCI America; Portland, OR) were used for quantification of total and free fatty acids, and oxidized lipid derivatives, respectively.

Zebrafish husbandry and diets

The Institutional Animal Care and Use Committee of Oregon State University approved this protocol (ACUP Number: 4706). Tropical 5D strain (5D) zebrafish (*Danio rerio*) were housed in the Sinnhuber Aquatic Research Laboratory. Adults 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 (Instant Ocean®) to create a salinity of 600 microsiemens [252]. Sodium bicarbonate was added as needed to adjust the pH to 7.4. At 55 days post-fertilization (dpf), zebrafish were randomly allocated to one of two diet groups, α-tocopherol deficient (E–) or α-tocopherol sufficient (E+), and fed one of the defined diets for the duration of the study [296]. The defined diets, which contained only fatty acids with 18 or fewer carbons and two or three double bonds, were prepared with the vitamin C source as StayC (500 mg/kg, Argent Chemical Laboratories Inc., Redmond, WA) and without (E–) or with added α-tocopherol (E+, 500 mg *RRR*-α-tocopheryl acetate/kg diet, ADM, Decatur, IL), as described previously [296, 297]. Diets were stored at -20°C until fed to the adult zebrafish.

E– and E+ embryos were obtained from adult fish fed either the E– or E+ diet, respectively, for a minimum of 80 days up to 9 months. Larvae were harvested through natural group spawning, collected, staged [252], and kept in standard embryo media (EM; as described [308] using 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 4.2 mM $NaHCO_3$; pH 7.2-7.4) for up to 12 days. Media was changed and freshly replenished once daily. Note that embryos are not fed prior to 5 days post-fertilization (dpf), as each is a complete unit containing

only those nutrients present in the yolk when eggs are laid, which the embryo fully utilizes by approximately 5-6 dpf (larval stage). Beginning at 5 dpf, larvae were hand-fed once daily for 7 days a commercial, nutritionally complete zebrafish food (GEMMA Micro 75 ZF, Skreeting; Fontaine les Vervins, France). The food is manufactured with fish meal and fish oils (sources of long-chain polyunsaturated ω-3 fatty acids), α-tocopheryl acetate (minimum 400 IU/kg diet) and vitamin C (minimum 1000 mg/kg diet). Proximate analysis of the diet composition provided by the company was Proteins (59%), Oil (14%), Ash (14%), Fiber (0.2%), and Phosphorous (1.3%). For all experiments described below, the E+ larvae are considered the control condition; lab 5D larvae from adult zebrafish maintained on GEMMA Micro 300 ZF (Skreeting; Fontaine les Vervins, France) also were used as an additional control to monitor larval quality (data not shown). Larvae used for biochemical analysis, described below, were euthanized by cold exposure (placed on ice for ≥30 minutes) then snap-frozen in liquid nitrogen prior to sampling. Levels of α-tocopherol in embryos and larvae were quantified as described [3].

Diet fatty acid composition and quantification

The total fatty acid compositions of the defined adult E– and E+ diets as well as the commercial GEMMA Micro 75 ZF diet were analyzed and quantified via saponification followed by GC-MS using established protocols [389].

Evaluation of phenotypic and developmental progress

Morbidity and mortality outcomes were assessed as described previously [306], using the zebrafish acquisition and analysis program (ZAAP). ZAAP is a custom program designed to inventory, acquire, and manage zebrafish data, and was used to collect 22 developmental endpoints, as either present or absent (i.e. binary responses were recorded, described below [321]). At 4 and 12 dpf, larval morphology (body axis, eye, snout, jaw, otic vesicle, notochord, heart, brain, somite, fin, yolk-sac, trunk, circulation, pigment, and swim bladder) was evaluated and recorded and behavioral endpoints (motility, tactile response) were thoroughly evaluated. If the embryo was dead at either 4 or 12 dpf, the non-mortality endpoints were not included in the evaluations. All images were taken using a Keyence BZ-700X microscope with a 2X objective lens under standard bright-field conditions.

Locomotor response assay

Locomotor activity was measured in a total of *n*=80 morphologically normal larvae per diet group (3 replicate trials) using Viewpoint Zebrabox [321]. Briefly, at 12 dpf, six-well plates containing the larvae (5 larvae per well) were placed in a Viewpoint ZebraBox (software version 3.0, Viewpoint Life Sciences, Lyon, France). Embryo locomotor activity was assessed using the “tracking” setting during alternating periods of light and dark, a modification of [322]. Locomotor activity in response to the light/dark transition was tracked during 3-minute periods of alternating light and dark for a total of 24 min. The integration time was set to 6 seconds to increase statistical power. A high definition camera (30 frames/second) tracked the total movement (swim distance, millimeters) in response to the multiple light-dark transitions.

Larval avoidance assay

The larvae were imaged with a custom-built imaging system made as described previously by [262]. In brief, the system includes a 15-megapixel Canon EOS Rebel T1i digital camera and an Acer Aspire 5517 laptop with a 15.6-inch screen to provide visual stimuli to the larvae. Larval behavior was examined in ‘five-lane’ plates, with 5 larvae per lane (25 larvae per plate: 10 E–, 10 E+, and 5 lab control; 4 plates per trial for 3 separate trials). The five-lane plate is made using a Nunc 1-well rectangular plate (Fisher 12-565-493), 50 ml of 0.8% agarose in water, and a CNC-milled plastic mold. Each lane is 18 mm wide, 70 mm long and 3.5 mm deep and has 60° sloping edges to avoid shadows and blind spots along the perimeter of the swimming area. The lanes have ample space to examine larval avoidance of aversive visual stimuli. Larvae were first imaged for 15 minutes without visual stimuli and then for 15 minutes in the presence of a moving red bar, which is 1.3 cm wide and moves up and down at a speed of 17 mm/second in the upper half of the lanes. Images were acquired every 6 seconds for a 30-minute period and were analyzed in ImageJ. ImageJ macro (version 25k), downloaded by request from [390], was used to automatically separate the color channels, subtract the background, apply a threshold, identify larvae based on particle size, and repeats these steps for subsequent images in a series. The macro generates a list of X,Y coordinates indicating the location and orientation of the larvae over time, which are used to calculate (Excel, Microsoft): a) the percentage of time that the larvae are located in the lower half of the lane, away from the visual stimuli, b) the swim speed, c) the percentage of time that the larvae

rest, which is defined as the percentage of time the larvae move less than 1 mm in a 6 second interval, d) the average distance between larvae, e) the percentage of time that larvae are together, which is defined as less than 5 mm apart from the nearest neighbor, and f) the percentage of time that larvae are located along the edge of the lane, which is defined as the outer 3 mm perimeter of the swimming area. Parameters a, b, c, and f were used for behavioral analyses in the present study.

Extraction and LC-MS/MS for metabolomic analysis

At 12 dpf, E– and E+ morphologically normal larvae (*n*= 10 per replicate, *n*= 4 replicates per group) were transferred to 1.5 mL Eppendorf tubes, covered with EM, and euthanized via cold-exposure (see above). EM was carefully removed to prevent loss of larvae and samples were stored at -80 °C overnight. To extract larvae for metabolomics analyses, solvent (300 µL 80:20 v/v methanol:water) was added, then sample extracts were homogenized with 0.5 mm zirconium oxide beads using a counter-top bullet blender for 6 min. Following 15 min. incubation on ice, the extracts were centrifuged at 4 °C at 15,000×*g* for 13 min. Aliquots (200 µL) of the upper layer were transferred individually to new tubes and stored at -80 °C until analysis via LC-MS/MS. To ensure the stability and repeatability of the LC–MS system, quality control (QC) samples (*n*= 4), generated by pooling 10 µL aliquots from each larval extract, were analyzed with the larval samples. Chromatography was performed with a Shimadzu Nexera system (Shimadzu; Columbia, MD, USA) coupled to a high-resolution hybrid quadrupole-time-of-flight mass spectrometer (TripleTOF® 5600; SCIEX; Framingham, MA, USA), as described previously [306].

Sample preparation, extraction and LC-MS/MS analyses of total or free DHA, EPA, ARA, ALA, and LA fatty acids and oxidized lipids

Extraction for total docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), arachidonic acid (ARA), alpha linolenic acid (ALA), and linoleic acid (LA) were performed as described [286, 306] with the following modifications: samples were obtained at 12 dpf (*n*=5 larvae per replicate, 5 replicates per group) homogenized in a solution of 1.0 mL water and 2.0 mL 1% ascorbic acid in ethanol (w/v), then saponified with the addition of 300 µL saturated KOH; following cooling, the pH was adjusted to 2.5 with 12N HCl, then 2.0 mL heptane and 10 µL internal [DHA-d_5 (1.0 µg/mL), EPA-d_5 (1.0 µg/mL), ARA-d_8 (2.0 µg/mL), ALA-d_{14} (1.0 µg/mL) and LA-d_4 (2.0 µg/mL)] were added. Samples were mixed, the supernatant (organic layer) was

removed and dried under nitrogen gas, resuspended in 100 µL 80:20 v/v methanol:water with 50 µg/mL BHT and 0.5% v/v acetic acid. Samples were stored at -80 °C until analysis by LC-MS/MS (see below).

Extracts for quantitative free fatty acid and hydroxy-DHA analyses were prepared as described for metabolomics samples, with the following modifications: extraction solvent (290 µL, 80:20 v/v methanol:water) included 50 µg/mL BHT and for quantification was combined with 10 µL per sample of internal standards of DHA-d_5 (1.0 µg/mL), EPA-d_5 (1.0 µg/mL), ARA-d_8 (2.0 µg/mL), ALA-d_{14} (1.0 µg/mL), LA-d_4 (2.0 µg/mL), 5-HETE-d_8 (1.0 µg/mL) and 9(*S*)-HODE-d_4 (1.0 µg/mL). QC samples were prepared as described for metabolomic analysis. Amounts were quantitated by relative comparison to internal standards, see below. Chromatographic separations were carried out on 4.6 × 250 mm J'sphere ODS-H80 (4 µm, YMC Co, Kyoto, Japan) for negative ion analysis. TOF-MS and TOF-MS/MS were operated with same parameters as for metabolomics, described previously [306].

Global DNA methylation and hydroxy-methylation quantification

DNA isolation was performed using a Zymo ZF-Duet DNA/RNA MiniPrep Kit (Zymo Research; Irvine, CA). Embryo (1-5 dpf) and larval (12 dpf) samples (*n*=8-16 per sample depending on age; 4 replicate samples per group) were collected into 1.5 mL lock-top tubes; excess EM was removed and 425 uL lysate buffer (from kit) was added. Samples were homogenized using a bullet blender, as described for metabolomics sample preparation above. Global DNA methylation (5-methylcystosine quantification, 5-mC) and hydroxy-methylation (5-hmC) were assessed using MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) and MethylFlash Global DNA Hydroxymethylation (5-hmC) ELISA Easy Kit (Colorimetric), respectively, from EpiGentek USA (Farmingdale, NY) Experiments were repeated in duplicate.

Statistical analyses

Statistical analyses for morphological and locomotor endpoints were performed using code developed in R (R Developmental Core Team 2014, http://www.R-project.org), as reported [306]. Avoidance assay data were exported to Microsoft Excel, individual parameters averaged (means with SEM) for baseline and stimulus periods for each diet condition, and then transferred to GraphPad Prism 6.0 software (GraphPad, La Jolla, CA) for subsequent analyses using 2-way ANOVA with

Tukey's post-test for multiple comparisons ($p< 0.05$). Targeted metabolomics data processing was performed as described previously [306] using PeakView software (SCIEX). The Holm-Sidak method for multiple comparisons was used to compare normalized metabolite intensity values (responses) between the two diet groups for metabolites (n ≤ 12 metabolites per pathway/category analyses) involved in separate metabolic pathways/categories (**Supplementary Table 4**) with significance set at $p< 0.05$. All statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad, La Jolla, CA). Quantification of total and free fatty acids, and oxidized lipids was performed using MutliQuant Software version 3.0.2 (SCIEX), as performed previously [306]. Statistical analyses were performed as for metabolomics data (above). Quantification of global DNA methylation and hydroxymethylation was determined per respective kit instructions. Additional statistical analyses (*e.g.* 2-way ANOVA with Sidak's post-test for multiple comparisons with significance set at $p< 0.05$) were performed using GraphPad Prism 6.0 software (GraphPad, La Jolla, CA).

Results

Larvae development and behavior

E– embryos suffer increased morbidity and mortality over the first 5 days post-fertilization (dpf) relative to E+ embryos [306]; however, a small subset remains morphologically normal. We selected 5 dpf (larval stage) E– and E+ larvae without evident physical deformities to be hand-fed once daily for 7 days a commercial, nutritionally complete zebrafish food. Larvae in both groups appeared to develop normally and grew similarly (**Figure 25**). They displayed similar daily swimming and feeding activity; however, high-throughput behavioral screening at 12 dpf showed that the E– larvae sustained neurodevelopmental perturbations, as they were 85% less responsive to light-dark transition stimuli in a locomotor response assay (**Figure 26**).

While the locomotor response assay is a robust screening tool, it does not differentiate between neurological impairments in *cognitive* function (*i.e.* inability to interpret and respond appropriately to a stimulus) from physiological defects in *perception* (*i.e.* inability to sense or observe a stimulus). To investigate cognitive endpoints, we used an active avoidance assay [262] to evaluate characteristics of larval behavior in response to an aversive stimulus (visual presentation of a moving red bar). Swimming parameters (*e.g.* speed, location, overall activity/movement) were similar between E– and E+ larvae during baseline conditions, indicating no motor

impairments in the E– group (**Figure 27A**). By contrast, during the stimulus only the E+ larvae demonstrated obvious aversive behavior by swimming faster (**Figure 27A**), increasing overall swimming (**Figure 27B**), and moving away from the red bar (**Figure 27C**), as well as demonstrating *thigmotaxis* (preference for lane edges; **Figure 27D**), an expected behavior and validated index of anxiety in zebrafish larvae [391]. The E– larvae failed to demonstrate a typical avoidance response and instead showed *decreased* overall movement in response to the moving bar with a greater percentage of the larvae remaining "at rest" compared to baseline conditions (**Figure 27B**), although, apparently, the E– larvae could adequately perceive the moving bar because they increased avoidance swimming significantly compared to baseline (**Figure 27C**).

Evaluation of VitE repletion

The behavioral abnormalities in the E– larvae were unexpected given their apparent normal phenotype and their week-long adequate dietary intake (413 ± 4 mg α-tocopherol per kg diet). Although measured VitE concentrations in E– larvae at 12 dpf were increased significantly from those measured at 5 dpf, they were approximately 2.5 times lower in E– compared to E+ larvae. VitE levels were unchanged from 5 to 12 dpf in the E+ group, indicating the commercial food contained adequate VitE to maintain sufficiency in E+ larvae (**Figure 28**).

We hypothesized that this sustained VitE deficiency in the E– larvae were due to the abundant amounts of long-chain polyunsaturated fatty acids present in the commercial diet (DHA: 1169 ± 2; EPA: 592 ± 2; ARA: 73 ± 0.4 μg/g food). In E– compared with E+ larvae measured concentrations of total (saponified) and free (unesterified) DHA, as well as other ω-3 and ω-6 LC-PUFAs, were significantly lower (**Figure 29**). These respective decreases likely resulted from increased lipid peroxidation in the E– larvae, based on their increases in oxidized ω-3 (HDoHE, HODE) and ω-6 (HETE, oxo-ETE) fatty acid concentrations (**Figure 30**). Notably, the F_4-series neuroprostanes (F_4-NPs, **Figure 30A**), which are non-enzymatic DHA peroxidation products specific to nervous tissue [392], were increased more than 3-fold (Note that though the approach used did not permit unequivocal identification of the specific F_4-NP isomer(s), we did confirm that the metabolite mass, retention time, and ms/ms fragmentation pattern matched those of the 4-series F_4-NPs, *e.g.* high-intensity [M+H] precursor ion m/z at 343.227). Additionally, non-enzymatic ω-6 ARA-derived F_2- and F_3-isoprostanes [378] were also higher in E– vs E+ larvae (**Figure**

30B). Together, the increases in F_4-NPs as well as F_2- and F_3-isoprostanes show increased free radical-induced lipid peroxidation in 12 dpf E– larvae. Previously, oxidized and unoxidized lipids were reported in Medaka and higher F_2-isoprostanes were found relative to F_3-isoprostanes and F_4-neuroprostanes; similar (within a factor of 10) values are reported for muscle as compared with our whole larvae [468].

Metabolic consequences of continued VitE inadequacy

Increased lipid peroxidation in the E– larvae resulted in significant oxidation of the cellular antioxidant network: targeted metabolomics analyses showed E– compared with E+ larvae had lower levels of ascorbic acid and glutathione concomitant with increased levels of each metabolite's respective oxidized form (**Figure 31A-B**). Interestingly, both NADPH and NADP+ were elevated in the E– larvae (**Figure 31B**), but the NADPH/NADP+ ratio was significantly decreased (**Figure 32A**), indicating enhanced NADPH oxidation (consumption) and a reduced NADPH reserve. We hypothesized that these perturbations in E– larvae would prompt greater metabolic activity via the pentose phosphate pathway (PPP) – a primary endogenous source of NADPH known to be active in the brain [393]. Our metabolomics data revealed that, indeed, glucose and PPP intermediates were significantly increased in the E– larvae (**Figure 32B**), indicating enhanced metabolic flux through this pathway. Similar elevations in glycolytic intermediates also were observed (**Figure 33**), mimicking the metabolomic profile of E– embryos at 1 dpf [306], a time at which the embryo still retains ample nutrient-stores in the yolk. These parallels suggest that VitE-deficiency promotes a hypermetabolic response, aimed at ameliorating insults to (and preserving function of) the cellular antioxidant network, when sufficient substrate (glucose) is available.

Interestingly, in contrast to the above, levels of citric acid cycle intermediates were *decreased* in the E- embryos (**Figure 34**), indicating the possibility of impaired aerobic metabolism and compromised mitochondrial function, as found during more prolonged developmental VitE deficiency in 2 dpf (but not 1 dpf) E– embryos [306].

VitE deficiency-induced lipid peroxidation (and ensuing DHA depletion) also causes increased DHA-containing phospholipid (DHA-PL) and lysophospholipid (lyso-PL) recycling/turnover, which leads to the depletion of choline-containing PLs (PCs) [305] and results in a secondary choline deficiency in E– embryos [306]. Thus, we hypothesized that choline levels also would be significantly lower in E– compared to

E+ larvae. Targeted metabolomics analyses of choline and choline-containing compounds involved in PC synthesis revealed these metabolites were significantly lower in the E– larvae (**Figure 35**). Analyses of choline-derived methyl-donor metabolites (*e.g.* betaine, *S*-adenosylmethionine), however, showed no differences between E– and E+ groups (**Supplementary Table 4**); similarly, global DNA methylation status, while reduced over 1-4 dpf in E- vs. E+ embryos, was the same between groups when assessed at 12 dpf (**Figure 36**). Thus, the dietary choline supply was presumably adequate to restore and maintain normal methyl-donor status and essential methylation reactions – notably, DNA methylation (**Figure 36**) – in the 12 dpf E– larvae.

Discussion

Developmental VitE deficiency causes neurobehavioral impairments that are resolved with preventative VitE supplementation initiated early during the embryonic period [307]; however, these perturbations are not readily *reversed* via later dietary remediation. Results from our behavioral assessments in 12 dpf E– and E+ larvae show rescue was not possible using a nutritionally complete diet as an intervention strategy, despite the apparent normal phenotype of the animals (**Figure 25**). We found that E– larvae sustain marked locomotor deficits in response to light/dark stimuli (**Figure 26**). An established symptom of overt VitE deficiency in humans and rodent is spinocerebellar ataxia [43, 61], therefore outcomes of our locomotor assay alone were insufficient to indicate specifically *cognitive* impairments in the E- larvae because lasting motor-related neuropathologies could contribute to the reduced locomotor response observed in the E- group. To deduce specifically whether this outcome was due to cognitive, motor, or perceptual (*e.g*. vision) defects in E– larvae, we next performed an active avoidance assay using a visual threat (moving red bar). Although E– larvae behaved similarly to E+ larvae during baseline conditions and showed no signs of motor (swimming) impairments (**Figure 27**), the E– group did not exert a typical avoidance response (**Figure 27A-B**) [262], or evident measures of anxiety (**Figure 27D**) [391] as observed in E+ larvae. Instead, most E– larvae “froze” (*i.e.* stopped moving appreciably; **Figure 27B**). Such freezing may constitute a maladaptive avoidance response suggestive of cognitive dysfunction, as reported in studies of neurologically impaired adult zebrafish [394]. A small subset of E– larvae appeared to perform avoidance swimming by moving away from the red bar (**Figure**

27C), but this response was far less pronounced than that of the E+ larvae, and E– swimming speeds between baseline and stimulus conditions remained unchanged (**Figure 27A**). Overall, since the E– larva responded to, and therefore perceived, stimuli presentation, they presumably suffer cognitive (not perceptual) defects, which adversely impacted their ability to quickly and appropriately execute behavioral tasks. Additional tests of visual acuity in E– vs. E+ larvae [256] are necessary to verify this interpretation.

The continued neurocognitive impairments may be attributed to sustained VitE deficiency at 12 dpf in the E– compared with E+ larvae (**Figure 28**). We hypothesize that concomitant dietary supplementation with copious amounts of LC-PUFAs led to substantially increased antioxidant requirements and utilization resulting from the enhanced lipid peroxidation in the E– state (especially of highly-unsaturated DHA, [337]), as found in previous reports [395, 396]. Our observations that (1) VitE status was maintained from 5 to 12 dpf in the E+ larvae, (2) E– larval VitE levels increased significantly from 5 to 12 dpf (**Figure 28**), and (3) differences over time in quantified total, unesterified (free), and oxidized lipid levels previously measured in 5 dpf larvae [306, 307] compared to levels in 12 dpf larvae (**Figures 29** and **30**) showed parallel patterns of change in both E– and E+ groups, provide evidence that although the diet contained adequate VitE to support normal antioxidant requirements in growing zebrafish, the E– group, given their initial state of extreme VitE deficiency, may require greater amounts of VitE to achieve repletion (*i.e*. VitE levels equal to the E+ group). Future experiments utilizing different feeding regimens are needed to better address the requirements and/or potential for complete resolution of VitE deficiency in the E– larvae, and to determine if, once deficiency is fully ameliorated, the E– group remains cognitively impaired.

Decreases in DHA and ARA (**Figure 29**) resulting from elevated lipid peroxidation in E– compared to E+ larvae (**Figure 30**) also potentially underlie the former group's cognitive dysfunction, since both DHA [397] and ARA [147] are essential for healthy fetal neurodevelopment. Of relevance to brain function are the F_4-NPs, which were ~3.5 times higher in E– compared to E+ larvae (**Figure 30A**). Both animal [398] and human [136] studies demonstrate that brain F_4-NPs are elevated during enhanced oxidative stress and pathological cognitive decline. Higher levels of additional autoxidized DHA products such as 10-HDoHE [348] (**Figure 30A**) further

suggest free radical-induced lipid peroxidation of brain DHA due to inadequate antioxidant (VitE) protection in E– larvae, though the possibility of aberrant enzymatic activity (*e.g.* of lipoxygenases) warrants attention as well.

Our metabolomics data showing consequent depletion of antioxidants (**Figure 31**), a decrease in the NADPH/NADP+ ratio (**Figure 32A**), and elevated glucose metabolism through the PPP (**Figure 32**) in E– larvae suggest that continued lipid peroxidation resulted in the diversion of glucose into the PPP in the E– group, as the animal attempted to generate adequate NADPH to replenish and preserve antioxidant network function under conditions of VitE deficiency. This resembles outcomes reported in models of increased cerebral oxidative stress [385]. Interestingly, glucose utilization via cytosolic, anaerobic metabolic pathways (specifically, the PPP (**Figure 32**) and glycolytic pathway (**Figure 33**) were higher in E– compared with E+ larvae, as observed in 1 dpf E– embryos [306]. However, aerobic metabolism via the citric acid cycle was decreased in E– vs. E+ larvae (**Figure 34**), which matches the metabolic profile of 2 dpf E– embryos, which also exhibit deranged mitochondrial function in bioenergetic profiling assays [306]. Thus, it may be that once VitE deficiency devastates mitochondrial function during the embryonic period, the damage is irreparable, and normal aerobic metabolism cannot be restored regardless of substrate (glucose) availability. Such would be especially detrimental to the brain, since neurons are highly dependent on aerobic metabolism/oxygen utilization [33] and die rapidly if deprived of energy substrate (*e.g.* glucose), as in cerebral ischemia [399].

Targeted metabolomics analyses also indicated continued relative choline deficiency in the E– vs. E+ larvae, potentially due to upregulated PC synthesis (**Figure 35**), especially via the phosphatidylethanolamine N-methyltransferase (PEMT) pathway, which utilizes PLs with an ethanolamine headgroup (PEs; notably, ethanolamine was decreased in E– vs. E+ larvae) to synthesize PC species enriched in DHA [112]. These data match our previous reports [305, 306] and provide evidence for continued perturbations in membrane PL remodeling. Lasting effects of reduced choline availability may partially underlie compromised cognition in the E– larvae, as demonstrated in mouse models of prenatal choline deficiency [239]. These consequences possibly are mediated by methyl donor-dependent epigenetic mechanisms [238] related to DNA methylation status, established either during the embryonic and/or larval periods.

Numerous studies focused on the redox-control of energy metabolism [400] show that DNA methylation (epigenetic) events mediate cellular bioenergetics [401]. We found decreased global DNA methylation (5-methylcytosine content; 5-mC) in E– embryos from 1-4 dpf (**Figure 36**) during periods of more severe choline deficiency [306, 307]; however, levels measured at 12 dpf were not different. Presumably, once provided with an adequate dietary source, the E- larvae utilized available choline to restore vital methyl-donor dependent reactions such as DNA methylation at the expense of maintaining PC synthesis (**Figure 35**), indicating a potential hierarchy in the endogenous functions of choline. Our findings showing elevated levels of global 5-hydroxymethylcytosine (5-hmC) by 5 dpf as well as an increased 5-hmC/5-mC in E- vs. E+ larvae by 2 dpf (**Figure 36**), both indicative of oxidative stress-induced chemical changes to DNA [401], also suggest that developmental perturbations – though no longer present at 12 dpf – may partially underlie the continued behavioral disruptions in E- larvae, as evidenced in models of autism [402]. Both DNA methylation and hydroxymethylation are known to regulate neural crest formation [403] and other neurodevelopmental processes [404]; thus, future studies focused on brain and gene-specific methylation status rather than global DNA methylation alone are warranted to investigate the possibility of altered methylation and hydroxymethylation profiles that specifically, and potentially permanently, affects cognitive development.

A unifying mechanism potentially linking developmental VitE deficiency with many of the metabolic perturbations reported herein – all of which may perturb brain function – is *ferroptosis,* a process of non-apoptotic programmed cell death [405] that involves several key features observed in our E– larvae, most critically: 1) enhanced enzymatic lipid peroxidation [406], especially due to excessive ARA metabolism via 5- [407] and 15-lipooxygenase [408] activities (**Figure 30A-B**; including 5- and 15-HETE; 5-oxo-ETE [409]); 2) glutathione depletion [410] (**Figure 31**); and 3) mitochondrial dysfunction [411] (**Figure 34**). We also found E– larvae had increased levels of glutamate ($p< 0.001$; **Supplementary Table 1**) relative to E+ larvae, which could signify glutamate toxicity, another metabolic perturbation associated with ferroptosis [407] via inhibition of the cellular cysteine/glutamate antiporter system X_c- [405]. Excessive glutamate is neurotoxic [412] and may be a consequence of compromised mitochondria [413]; additional experiments regarding the regulation of neuronal intracellular glutamate levels are required to determine whether these mechanisms

also apply to our VitE deficiency model. Importantly, VitE supplementation successfully prevents ferroptosis [339] and associated neurological impairments [414], while VitE deficiency exacerbates the latter [415], indicating that ferroptosis may constitute a biological rationale for the consequences of embryonic VitE deficiency we observed in E– larvae.

Taken together, our data reveals embryonic VitE deficiency causes lasting perturbations in lipid, antioxidant, and energy metabolism, despite consumption of an adequate diet at the larval stage. These combined outcomes may underlie the sustained neurocognitive abnormalities evident in the E– larvae, suggesting that embryonic VitE inadequacy has long-term adverse effects on the brain, as observed with prenatal DHA [416] and choline [417] deficiencies, respectively, during critical periods of brain development. Such findings substantiate the provocative contention that VitE also is a nutrient of similar status and significance, both for the fetal brain and for long-term cognitive function, and provide evidence to encourage future reevaluation of recommended VitE intake levels to promote optimal neurodevelopment and cognitive health in humans.

Acknowledgements:

The authors thank Carrie Barton, Jane La Du, Greg Gonnerman, Dr. Michael Simonich, and Dr. Donald Jump for their superior technical assistance. NIH grant S10RR027878 (MGT) and NIEHS P30ES000210 (RT) supported this work.

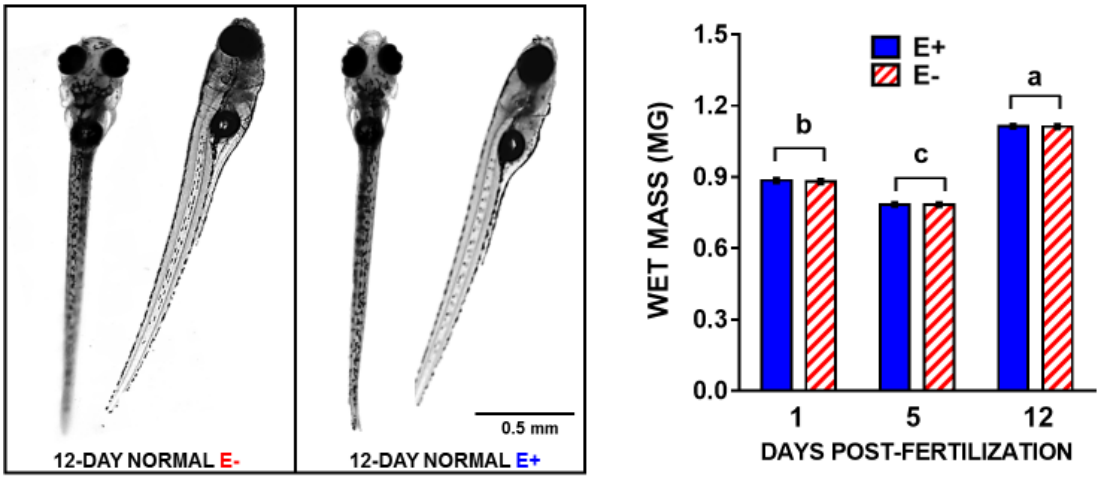

Figure 25. E– and E+ larvae maintained on standard zebrafish diets from 5-12 days post-fertilization grow and develop normally. Morphologically normal 12-day-old E– and E+ larvae used in the present study (left). Wet weight of morphologically normal E– and E+ larvae (*n*= 10 per group; right). Shown are means ± SEM; 2-way ANOVA with unique letters indicating significantly different values ($p< 0.05$) with Tukey's post-test for multiple comparisons.

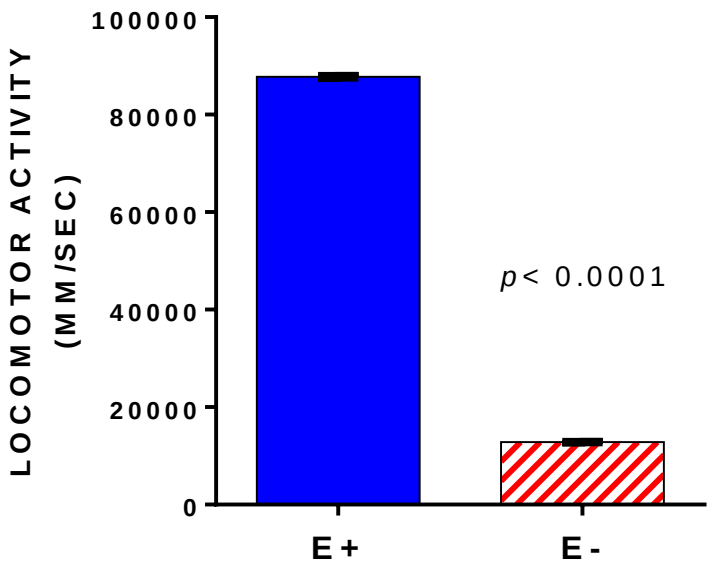

Figure 26. Locomotor behavior is impaired in E– compared with E+ larvae despite consuming an adequate diet. The 12-day-old larvae were analyzed in 6-well plates (*n*= 80 per group; 3 replicate trials). Embryos with morphological defects were not included in data analysis. Locomotor activities following a series of light stimuli (a stimulus every 6 for 24 min) were measured as distance moved (mm) over time (seconds). Bar chart comparisons of respective time-course data (area under the curve ± SEM); E– (red diagonal bar) larvae were 85% less responsive to light than were E+ (blue solid bar) larvae (significance was determined using a Kolmogorov–Smirnov test with significance set at $p< 0.01$).

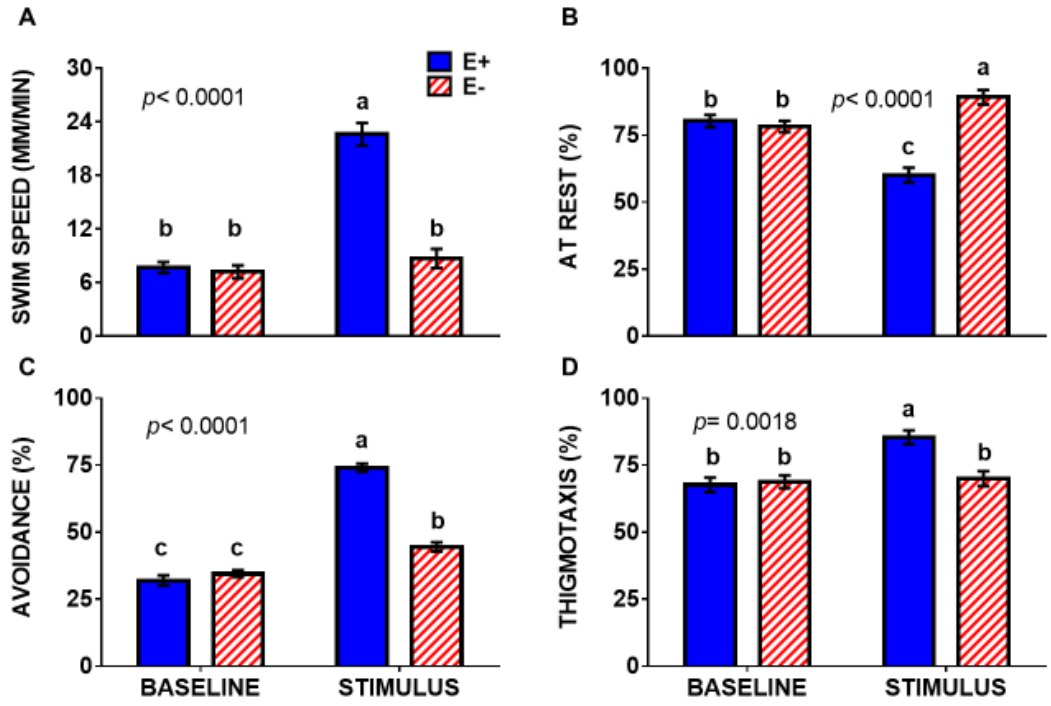

Figure 27. Perturbed responses of E– larvae to an avoidance assay are indicative of compromised neurodevelopment. Behaviors were quantified using a high-throughput response assay in which larvae (*n*= 40 per group; 3 replicate trials) were exposed to a blank background (baseline) and to a red moving bar (stimulus) in a PowerPoint presentation. A Average swim speed (mm/min). B Resting state (% of time that larvae move less than 1 mm/6 sec interval). C Avoidance of visual stimuli (larvae are in the bottom of the lane, away from the moving bar stimulus). D Edge preference or thigmotaxis (larvae are less than 3 mm from the lane perimeter). Shown are means ± SEM; 2-way ANOVA with Tukey's post-test for multiple comparisons; unique letters indicate significant differences ($p< 0.05$).

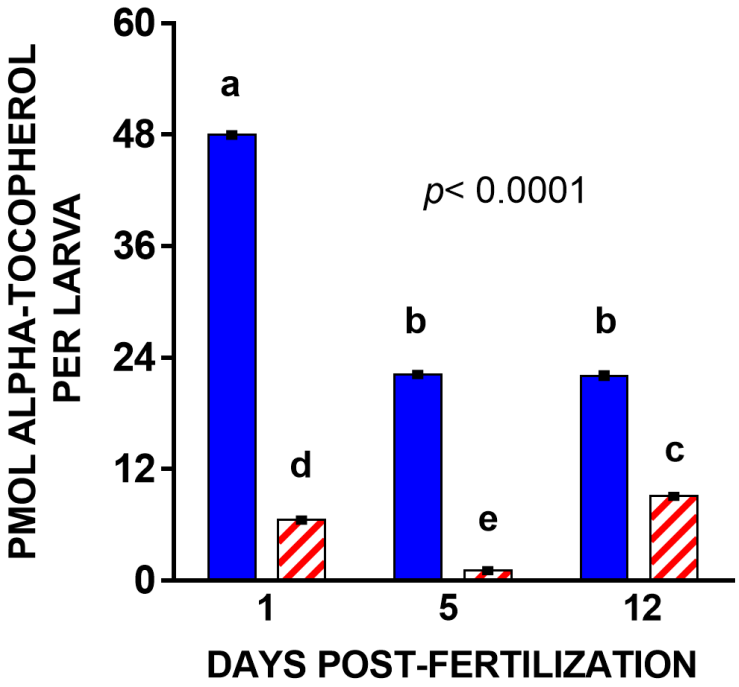

Figure 28. E– larvae remain VitE–deficient despite consuming an adequate diet for 7-days. Quantification of α-tocopherol in E– compared to E+ larvae (*n*=5 larvae per sample; 5 replicates per group) from a representative clutch. Shown are means ± SEM; *p*-values are for 2-way ANOVA of VitE x Age interactions, with Tukey's post-test for multiple comparisons, $p<0.05$ for bars bearing unique letters).

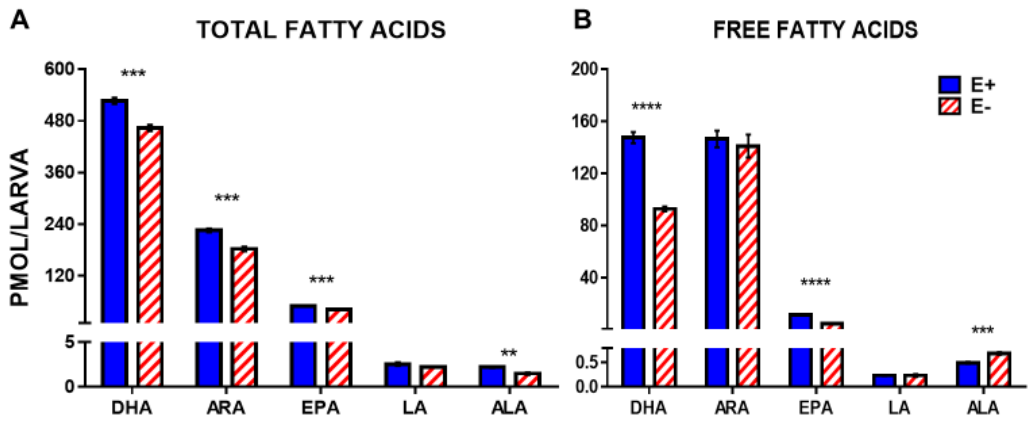

Figure 29. E– larvae have decreased DHA and other differences in ω-3 and ω-6 fatty acids during maintenance on a high-DHA diet. Quantified levels of total and free (unesterified) fatty acids in E– vs. E+ larvae were quantified from LC/MS-TOF area counts normalized using internal standards (*n*= 5 samples/group, with *n*= 5 larvae/sample for total lipids; *n*= 4 samples/group with *n*= 10 larvae/sample for free fatty acids). Total and free fatty acid measurements both were obtained from the same cohort of larvae. Shown are saponified A and extracted only B samples, means ± SEM. Statistical significance ($p<0.05$) was calculated using the Holm-Sidak method for multiple comparisons of normalized intensity values. Paired comparisons *p*-values are indicated as **<0.01, ***<0.001, **** <0.0001. Abbreviations: ALA (α-linolenic acid); LA (linoleic acid); EPA (eicosapentaenoic acid); ARA (arachidonic acid); DHA (docosahexaenoic acid).

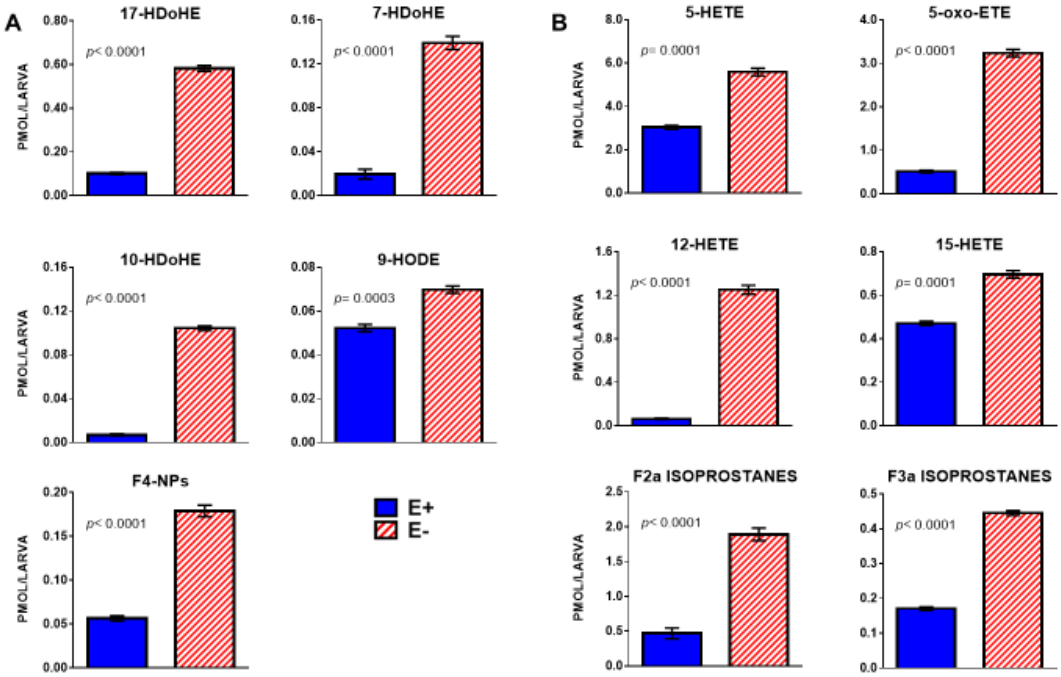

Figure 30. E– larvae have increased levels of oxidized ω-3 and ω-6 lipid derivatives. A oxidized ω-3 lipids, and B oxidized ω-6 lipids, analyzed as described for Figure 29B. Abbreviations: 5-, 12-, 15-HETE, 5-, 12-, 15-hydroxy-eicosatetraenoic acids; 5-oxo-ETE, 5-oxo-eicosatetraenoic acid; 17-, 7-, 10-HDoHE, 17-, 7-, 10-hydroxy-docosahexaenoic acids; 9-HODE, 9-hydroxy-docosahexaenoic acid; F4-NPs, shown as 7-series 9-3,5-Dihydroxy-2-2,5-octadien-1-yl cyclopentyl-7-hydroxy-4,8-nonadienoic acid.

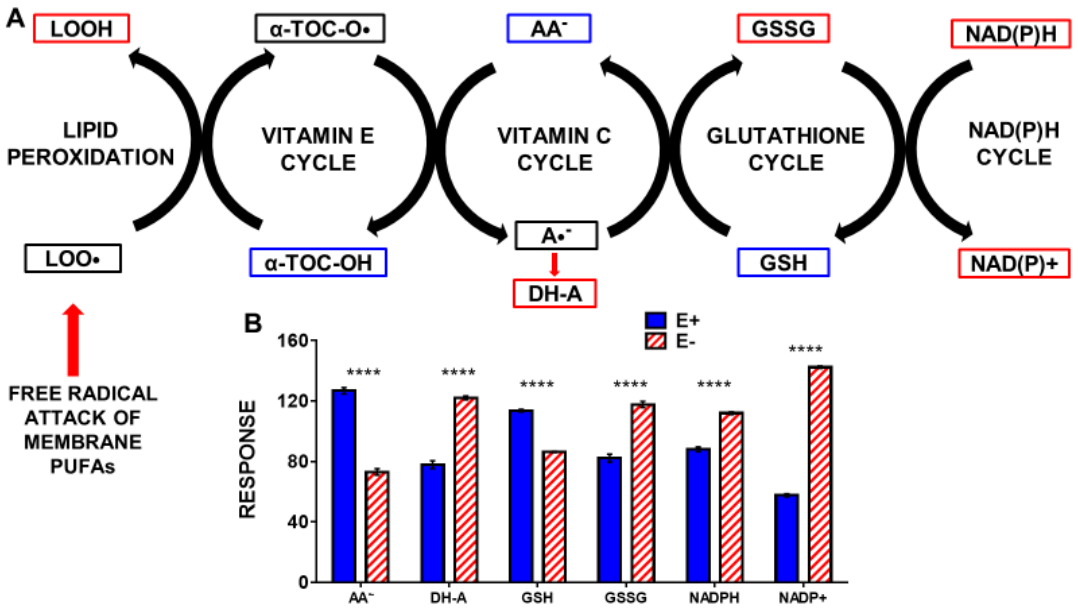

Figure 31. E– larvae have sustained metabolic perturbations to the cellular antioxidant network despite consuming adequate diets for 7-days. A Antioxidant network scheme showing interaction of antioxidants with lipid radicals and consumption or NAD(P)H. B. E– and E+ larvae (*n*= 10/sample; 4 samples/group) relative response data was normalized against QC sample intensities (*n*= 4) for each individual metabolite. Boxes shown in Red (increased in E– or Blue (increased in E+) represent metabolites that were higher in E– or E+ larvae, respectively. Black boxes indicate relative levels of a given metabolite are not shown in the figure. Statistical significance ($p<0.05$) was determined using the Holm-Sidak method for multiple comparisons of normalized intensity values. Shown are means ± SEM, *p*-values are indicated as **** <0.0001. Abbreviations: $LOO^{\cdot}$, lipid radical; LOOH, oxidized lipid; α-TOC-OH, α-tocopherol; α-TOC-O$^{\cdot}$α tocopheroxyl radical; AA^{-}, ascorbic acid; $A^{-\cdot}$ ascorbate radical; DH-A, dehydroascorbate; GSH, glutathione; GSSG, glutathione disulfide; PUFAs, polyunsaturated fatty acids.

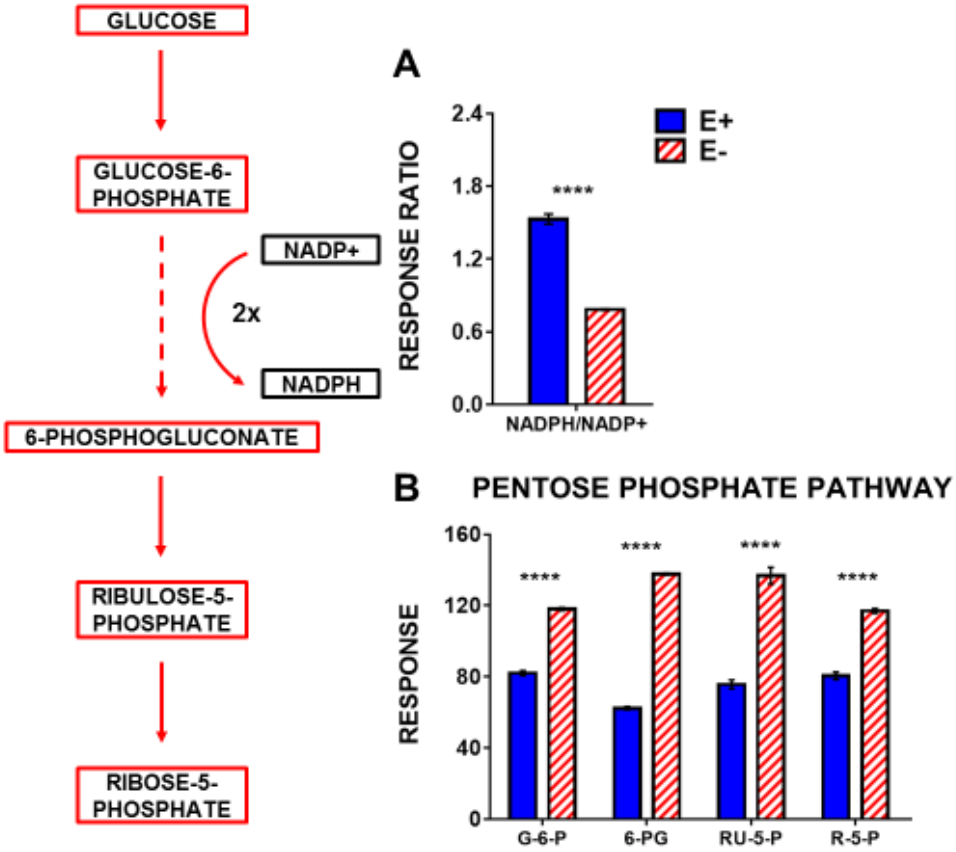

Figure 32. E– larvae contain higher levels of pentose-phosphate pathway intermediates and show increased NADPH utilization when compared to E+ larvae. The outlined metabolic pathway diagram (left) shows the relationship of the metabolites shown in the bar charts in A and B. Solid lines indicate direct reactions and dashed lines denote several reaction steps between metabolites. Boxes shown in Red (increased in E– or Blue (increased in E+) represent metabolites that were higher in E– or E+ larvae, respectively. Black boxes indicate relative levels of a given metabolite are not shown in the figure. Data were analyzed as in Figure 31. Abbreviations: 6-PG, 6-phosphogluconate; RU-5-P, ribulose-5-phosphate; R-5-P, ribose-5-phosphate

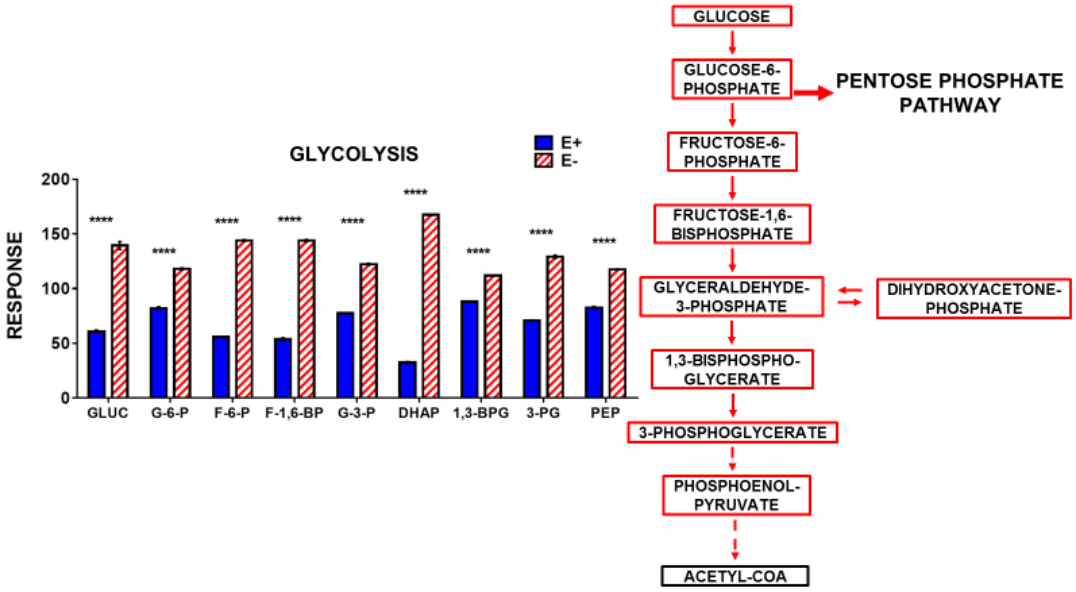

Figure 33. E– larvae contain increased levels of glucose, glycolytic intermediates, and ketogenic amino acid when compared to E+ larvae. The outlined metabolic pathway diagram (left) shows the relationship of the metabolites shown in the bar chart. Solid lines indicate direct reactions and dashed lines denote several reaction steps between metabolites. Boxes shown in Red (increased in E– or Blue (increased in E+) represent metabolites that were higher in E– or E+ larvae, respectively. Black boxes indicate relative levels of a given metabolite are not shown in the figure. Data were analyzed as in Figure 31. Abbreviations: GLUC, glucose; G-6-P glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-BP, fructose-1,6,-bisphosphate; G-3-P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; 1,3-BPG, 1,3-bisphosphoglycerate; 3-PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate

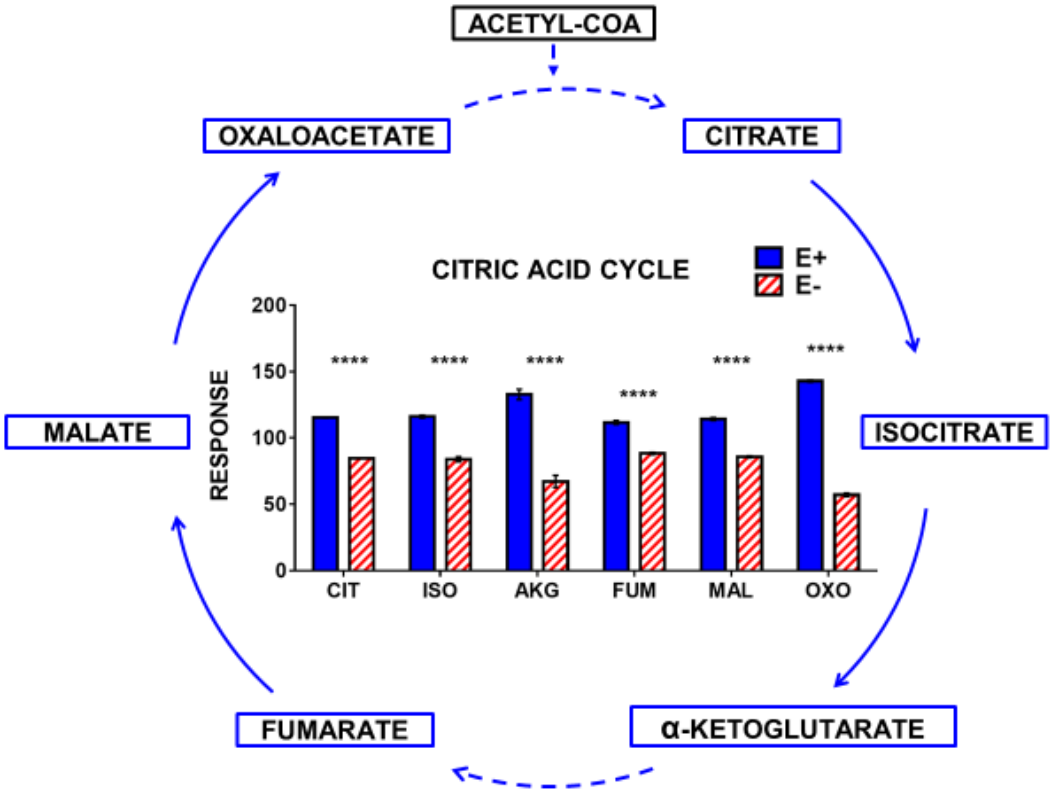

Figure 34. E– larvae have perturbed aerobic metabolism when compared to E+ larvae. The outlined metabolic pathway diagram (left) shows the relationship of the metabolites shown in the bar chart. Solid lines indicate direct reactions and dashed lines denote several reaction steps between metabolites. Boxes shown in Red (increased in E– or Blue (increased in E+) represent metabolites that were higher in E– or E+ larvae, respectively. Black boxes indicate relative levels of a given metabolite are not shown in the figure. Data were analyzed as in Figure 31. Abbreviations: CIT, citrate; ISO, isocitrate; AKG, α-ketoglutarate; FUM, fumarate; MAL, malate; OXO, oxaloacetate

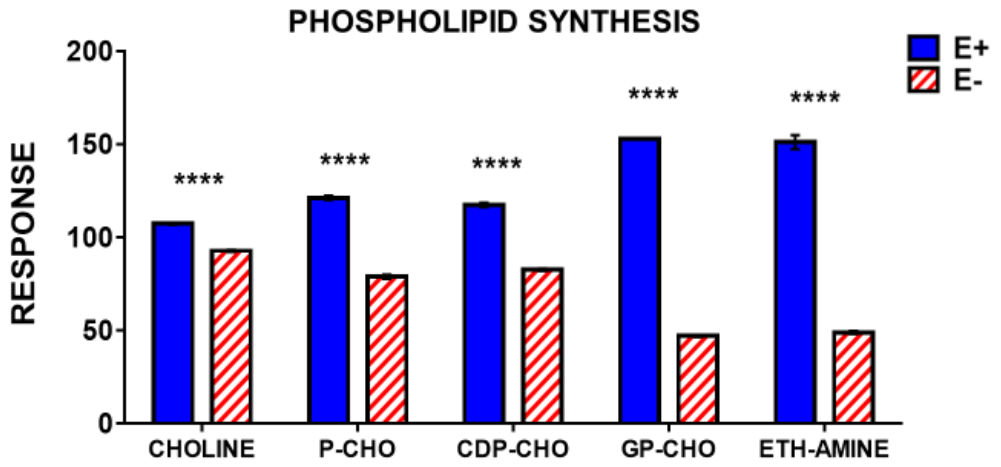

Figure 35. Phospholipid synthesis is lower in E– larvae despite maintenance on a choline-adequate diet. Relative response intensities of choline, CDP-choline pathway, and PEMT pathway metabolites. E– and E+ larvae (*n*=10/sample; 4 samples/group) data were normalized against QC sample intensities (*n*=4) for each individual metabolite. Statistical significance ($p<0.05$) was determined as described in Figure 31. Shown are means ± SEM, *p*-values are indicated as **** <0.0001.

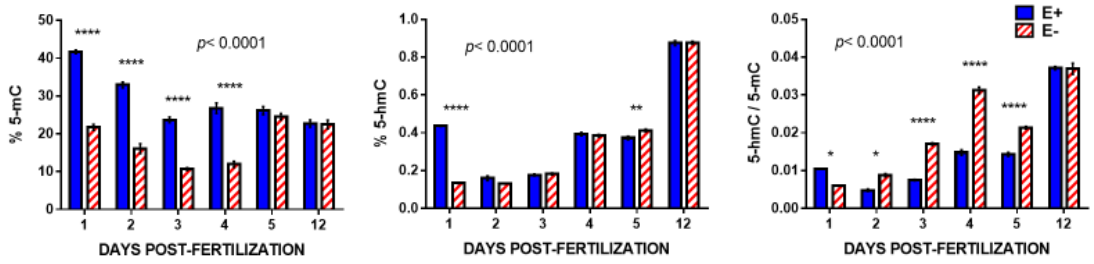

Figure 36. Minor decreases in choline levels at 12 days post-fertilization in E– compared to E+ larvae do not disturb global DNA methylation. Global (total) DNA methylation (5-methylcytosine content; 5-mC) and hydroxy-methylation (5-hydroxy-methylcytosine; 5-hmC) were quantified and expressed as percentages of total DNA cytosine content (19.3% for zebrafish [263]) in E– and E+ embryos/larvae from 1-5 and 12 dpf (*n*= 8-16 per sample; 4 sample replicates per group). Shown are means ± SEM; 2-way ANOVA with Tukey's post-test, statistical significance set at $p<0.05$. Paired comparison *p*-values are indicated as * <0.05, **<0.01, ***<0.001, **** <0.0001. **** <0.0001.

Supplementary Table 4

Click here to download **Supplementary Table 4**

CHAPTER SIX:

CONCLUSIONS AND SYNTHESIS

Vitamin E (α-tocopherol; VitE) first was discovered in 1922 as an essential nutrient for preventing fetal resorption in rats [78], and has since been linked with embryonic and neurological health in both numerous animal (including my own work with zebrafish) and human studies. However, nearly a century after its initial discovery, the underlying biological rationale explaining VitE's essentiality for (neuro)development and brain function remains unknown. The purpose of this project was to provide for an evidence-based assessment of metabolic interactions between VitE and specific membrane lipids to elucidate the biochemical basis underlying VitE's neurological function *in vivo* during neurodevelopment and into old age. To accomplish this overall aim, I exploited a zebrafish model and used both embryonic and adult conditions of VitE deficiency to publish compelling evidence that demonstrates the major role for VitE in the brain is to protect docosahexaenoic acid (DHA) and DHA-containing phospholipids (DHA-PLs) against oxidative stress, and without this antioxidant protection, ensuing secondary deficiencies in both DHA and choline coincide with increased morbidity, mortality, and/or cognitive impairments. Further, my work shows that VitE's antioxidant activity is vital for maintaining the cellular antioxidant network, and dysregulation of such aberrantly alters energy metabolism by severely compromising mitochondrial function. The studies included in this work, when considered together, provide insight as to how inadequate VitE perturbs DHA, phospholipid, and choline metabolism, resulting in dysregulation of other metabolic pathways and how disruption of these processes compromises neurological and cognitive outcomes both during neurodevelopment as well as in later life.

My primary research goal was to help elucidate the mechanism(s) through which VitE contributes to lifetime brain health. To achieve this, I evaluated the consequences of inadequate VitE from the earliest stages of brain development through mid-adulthood. My central hypothesis was that VitE protects DHA, a vital substrate for brain membrane phospholipid maintenance, and that dysregulation of DHA-PL status due to restricted dietary VitE severely perturbs critical events necessary for embryonic neurodevelopment that, ultimately, increase susceptibility for consequent, persistent cognitive impairments. I completed three Specific Aims to provide mechanistic support for this global hypothesis:

AIM 1: Elucidate the underlying mechanism(s) by which VitE deficiency impairs neurodevelopment in VitE-deficient (E–) vs. VitE-sufficient (E+) zebrafish embryos.

Hypothesis: Inadequate embryonic VitE perturbs DHA and DHA-PL status, which leads to dysregulation of membrane phospholipid remodeling and consequent metabolic changes that together severely disrupt neurodevelopment, thereby resulting in behavioral abnormalities as well as increased embryonic morbidity and mortality.

AIM 2: Determine the extent to which chronic dietary VitE deficiency compromises neurological outcomes in E- vs. E+ adult zebrafish.

Hypothesis: Inadequate dietary VitE during adulthood causes depletion of brain DHA and DHA-PLs, which leads to nervous system aberrations that result in significant functional and cognitive impairments.

AIM 3: Evaluate the lifetime neurological consequences of developmental VitE deficiency in a cohort of E- vs. E+ zebrafish embryos raised to the larval stage.

Hypothesis: The dysregulation of biochemical processes and resulting neurological perturbations due to inadequate VitE during critical periods of neurodevelopment are manifest beyond the embryonic period and are irreversible despite ensuing VitE and DHA repletion.

In *Chapter Two*, as partial fulfillment of **Aim 1**, I present studies in which I hypothesized that VitE is required by the developing embryonic brain to prevent depletion of highly polyunsaturated fatty acids, especially DHA, the loss of which I predicted would underlie abnormal morphological and behavioral outcomes. The E- compared with E+ embryos demonstrated impaired locomotor behavior, even in the absence of gross morphological defects. Evaluation of phospholipid (PL) and lysophospholipid (lyso-PL) composition using untargeted lipidomics in E- compared with E+ embryos over 120 hours post-fertilization (hpf) showed that four PLs and three lyso-PLs containing DHA, including lysophosphatidylcholine (LPC 22:6, required for transport of DHA into the brain), were at lower concentrations in E- throughout development. Additionally, $H_2{}^{18}O$ labeling experiments revealed enhanced turnover of LPC 22:6 and three other DHA-containing PLs in the E- compared with the E+

embryos, suggesting that increased membrane remodeling is a result of PL depletion. Together, these data indicate that VitE deficiency in the zebrafish embryo causes the specific depletion and increased turnover of DHA-PL and lyso-PLs, which may compromise DHA delivery to the brain and thereby contribute to the behavioral impairments observed in E- embryos.

The alterations in both PL and lyso-PL levels and label incorporation I observed in the E- relative to the E+ embryos in this study were hypothesized to be the result of a depletion of DHA-containing lipids and an increased requirement for DHA to remodel and repair affected PLs. Considering my lab group's recent finding of increased DHA peroxidation in adult E- zebrafish brains [72], insufficient VitE concentrations during development likely allows lipid peroxidation to deplete not only brain DHA-PLs, but DHA throughout the body, thereby limiting DHA delivery (*e.g.* via LPC 22:6) to the brain. Evidence reported in *Chapter Two* alone was insufficient to substantiate this claim, and therefore necessitated additional research to more fully elucidate the mechanism(s) underlying DHA depletion and perturbed PL remodeling, and how these factors contributed to the phenotypic aberrations observed in E- embryos. Thus, to fulfill my **Aim 1** goals, I performed additional embryo studies to measure not only DHA peroxidation, but other metabolic disturbances consequent to VitE deficiency as well.

In *Chapter Three*, I completed **Aim 1** by investigating the underlying mechanisms causing VitE deficiency-induced embryonic lethality using targeted lipidomic and metabolomic analyses of E- compared to E+ embryos over 120 hpf, which again coincided with increased morbidity and mortality in the E- group. Lipidomic analyses revealed that VitE deficiency resulted in significant peroxidation of DHA, which metabolomic analyses showed led to altered especially PC metabolism and choline depletion. Reduced choline levels consequently decreased available methyl-donor metabolites, including betaine and SAM, hypothetically impacting important methylation reactions like the epigenetic methylation of DNA and histones. Increased lipid peroxidation also depleted the E- embryo of glucose by shunting it to the pentose phosphate pathway rather than through glycolysis and the citric acid cycle. Further, VitE deficiency was associated with mitochondrial dysfunction with concomitant impairment of energy homeostasis, as observed in bioenergetic profiling assays – deranged energy metabolism showing a "metabolic switch" between 24 and 48 hpf, which suggested reliance on anaerobic glycolysis (the Warburg effect [418, 419]) in

the E- embryo, with an inability to convert to primarily aerobic metabolism as a consequence of mitochondrial damage. The observed morbidity and mortality outcomes could be attenuated, but not fully reversed, by glucose injection into VitE-deficient embryos at developmental day one, indicating that while perturbed energy homeostasis contributed to embryonic morbidity and lethality, other underlying mechanisms – choline and/or DHA depletion, for example – also played a vital role in VitE deficiency-induced consequences. Overall, this study provided novel evidence showing that embryonic VitE deficiency in vertebrates leads to a metabolic reprogramming that adversely affects antioxidant, DHA, and methyl-donor status, as well as cellular energy homeostasis, with ultimately lethal outcomes.

The studies presented in *Chapters Two* and *Three*, in execution of **Aim 1**, show that the mechanisms underlying VitE's essentiality during neurodevelopment include its antioxidant protection of DHA and DHA-PLs, which prevents lipid peroxidation and aberrant membrane PL turnover, additional antioxidant depletion, and metabolic dysregulation with secondary decreases in both choline (methyl-donors) and glucose. Such pleiotropic outcomes presumably cause death both by compromising the structural integrity of brain membrane PLs and by starving the embryonic brain not only of vital nutrients, namely DHA and choline, but also of the energy (glucose) needed for its growth and function. These findings acquire even greater significance upon consideration that – nearly a century after its discovery – they provide mechanistic insights into why the vertebrate fetus requires VitE, and, as a corollary, expand the role VitE deficiency plays in human neurodevelopment. However, questions remained following these investigations, especially concerning: (i) the impact secondary choline and methyl-donor deficiencies may have on DNA methylation, and how such may relate to VitE deficiency-induced neurological impairments; (ii) the specific nature of E- embryo behavioral abnormalities (*i.e.* functional locomotor, perception/vision, cognitive, or a combination); and (iii) whether the detrimental effects of embryonic VitE deficiency may be prevented or resolved by providing adequate levels of depleted nutrients, given that immediate VitE supplementation at 0 hpf, but not glucose supplementation at 24 hpf, rescued E- embryos. Such questions sparked significant motivation for the research outlined in *Chapter Five*, and provided the foundation necessary to link **Aim 1** endpoints with the rationale and design for **Aim 3** studies (discussed below).

Another extremely important remaining question concerned whether the lipid and metabolite differences found in E- compared to E+ embryos were truly manifest in their brains; each analysis used samples of whole embryos because isolation of specific embryo organs required micro-dissection techniques as well as prodigious samples numbers that were not feasible with available resources. Therefore, any speculation of perturbations in brain metabolism or neurodevelopment due to VitE deficiency, at least in the E- embryo, must take this caveat into consideration. To better determine brain-specific (and, hence, neuropathology-specific) consequences of VitE deficiency, as well as to evaluate how effects of too little VitE during neurodevelopment compared with those of chronic "lifetime" VitE inadequacy, I performed similar lipidomic and metabolomic assessments in adult zebrafish brain tissue. The studies conducted in *Chapter Four* investigated behavioral perturbations due to isolated, chronic VitE deficiency in adult zebrafish fed diets that were either VitE-deficient (E- group) or sufficient (E+ group) for up to 18-months of age, in fulfillment of **Aim 2**. I hypothesized that E- adult zebrafish would display significant cognitive impairments associated with elevated lipid peroxidation and additional metabolic disruptions in the brain; somewhat analogous to observations in E- embryos. Using assays of both associative (avoidance conditioning) and non-associative (habituation) learning, I found E- adults were learning impaired relative to E+ fish, and that these functional deficits occur concomitantly with the following observations in adult E- brains: decreases in and increased peroxidation of polyunsaturated fatty acids (especially of DHA); altered brain phospholipid and lysophospholipid composition with depletion of DHA-containing PLs and lyso-PLs; dysregulation of the cellular antioxidant network; and perturbed energy (glucose/ketone), PC, and choline/methyl-donor metabolism. These data collectively indicated chronic VitE deficiency leads to cognitive dysfunction through multiple potential mechanisms, including decreases in DHA, antioxidants, glucose, and choline, as well as corresponding dysfunction in related metabolic pathways (*e.g.* energy/NAD(P)H and methyl-donor metabolism) in the brain.

The similarities between "omics" results in E- embryos and E- adult brains suggest that the behavioral disruptions observed in both groups may be attributed to shared underlying mechanisms, as the same metabolic pathways (*i.e.* PC synthesis via the PEMT pathway, the cellular antioxidant network, glucose metabolism) were affected. Although a more exhaustive evaluation of untargeted metabolomics studies

in E- embryos and adults – perhaps using several different tissues from the latter (liver, brain, muscle, etc.) is necessary to conclude that the effects of VitE deficiency truly are “the same” for each, my findings in *Chapter Four* and completion of **Aim 2**, when considered with studies supporting **Aim 1**, demonstrate VitE’s critical role in maintaining neurological health, both during development and during adulthood, may be attributed to several functions that likely converge and interrelate to explain how VitE deficiency contributes to cognitive impairments. A noteworthy result of the adult investigation also was the revelation that isolated VitE deficiency (with adequate vitamin C) does not compromise motor/muscle function, indicating that the effects of chronic inadequacy may be strictly neurological. Histological evaluations of neuronal structure, both in the brain and the periphery, are warranted to determine if the “hallmark” retrograde dying-back and demyelination of large-caliber sensory neurons that presents with overt human VitE deficiency occurs also in the E- adult zebrafish. Further, the adult model provides sufficient tissue for separate evaluations of neuronal mitochondria and synapse architecture, respectively, which may yield additional insights as to how VitE deficiency-induced metabolic disruptions affect neuronal structures, and, ultimately, neuron function. Such would enhance **Aim 2** findings presented in *Chapter Four* in part by bolstering the hypothesis that DHA-PL depletion compromises synaptic membrane integrity and that oxidative stress damages mitochondria in the E- condition; regardless, these analyses provide an intriguing area of continued research.

Taken together, the outcomes of **Aims 1** and **2** demonstrate that both developmental and chronic adult VitE deficiency are deleterious to the brain. However, they did not answer the other questions mentioned above; most importantly, if and how the neurological consequences of inadequate VitE may be prevented or attenuated – in summary, does developmental VitE deficiency have *lasting*, possibly irreparable effects that contribute to cognitive decline? To address such unresolved queries, I performed studies described in *Chapter Five*, in which I completed **Aim 3** by investigating the long-term effects of embryonic VitE deficiency, and whether they are ameliorated by feeding VitE–adequate diets, using my zebrafish model of developmental VitE deficiency followed by dietary remediation. I evaluated E- and E+ embryos/larvae up to 12 days dpf. The E– group suffered significantly increased morbidity and mortality as well as altered DNA methylation status through 5 dpf when

compared to E+ larvae, but upon feeding with a VitE-adequate diet from 5-12 dpf both the E– and E+ groups survived and grew normally; the DNA methylation profile also was similar between groups by 12 dpf. However, 12 dpf E– larvae still had behavioral defects. These observations coincided with sustained VitE deficiency in the E– vs. E+ larvae despite adequate dietary supplementation. I also found in E– vs. E+ larvae continued DHA depletion and significantly increased lipid peroxidation of several polyunsaturated fatty acids. Further, targeted metabolomics analyses revealed persistent dysregulation of the cellular antioxidant network, the CDP-choline pathway, and glucose metabolism. While anaerobic processes were increased, aerobic metabolism was decreased in the E– vs. E+ larvae, indicating mitochondrial damage. These outcomes suggest embryonic VitE deficiency causes lasting behavioral impairments due to persistent lipid peroxidation and metabolic perturbations that are not resolved via later dietary VitE supplementation.

Additionally, although not different at 12 dpf, global DNA methylation was altered in E- compared to E+ embryos in 24-120 hpf assessments, indicating that epigenetic changes in gene expression established during earlier stages of neurodevelopment may contribute to later cognitive deficits. The logistics of growing E- and E+ embryos past the larval stage and all the way to adulthood, as initially intended, proved untenable herein; however, for the purposes of **Aim 3** and the questions it was designed to answer, the results presented in *Chapter Five* still yielded valuable knowledge regarding the possibility of "rescuing" the E- group: provision of a nutritionally adequate diet containing not only VitE but also sufficient DHA and choline to support normal E+ embryo/larval development, does not save E- larvae. Thus, completion of **Aim 3** demonstrates that embryonic VitE inadequacy has long-term – potentially irreparable – adverse effects on the brain, as observed with prenatal DHA and choline deficiencies, respectively, during critical periods of brain development.

Such findings substantiate the provocative contention that VitE also is a nutrient of similar status and significance, both for the fetal brain and for long-term cognitive function, and provide evidence to encourage future reevaluation of recommended VitE intake levels to promote optimal neurodevelopment and cognitive health in humans. Adjustments to the formulation and timing of nutrient remediation – for instance, micro-injection into the yolk of VitE and/or choline at earlier embryonic stages of development, reduced diet polyunsaturated fatty acid content to mitigate lipid

peroxidation, and so on – likely would significantly impact the behavioral effects I found in my **Aim 3** studies (*Chapter Five*), and constitute an important focus for continued research, as do similar dietary remediation studies in the E- adult model to compare embryo vs. adult outcomes to determine which period(s) of VitE deficiency are most lasting and damaging. However, the general implications that these larval outcomes convey remains unchanged: the brain requires VitE, it does so throughout life, and there are sensitive – I would argue *critical* – periods during which VitE is essential for healthy, normal brain development and function. Future studies are necessary to more specifically define these periods, but my *Chapter Five* data strongly suggest, if not confirm, their existence.

Collectively, the outcomes of these completed Aims provide mechanistic evidence to explain VitE's essentiality for human neurodevelopment and adult brain function, and yield new insights regarding the impact early-life VitE deficiency has on embryonic (neuro)development as well as on the inception of cognitive decline and ensuing neurological disorders.

Future Directions

It is perhaps naïve to use the term "completed" for my research Aims, as they include what may be characterized better as a collection of studies that exist within a continuum of research endeavored to elucidate details of the biological need for VitE, rather than a separate, self-contained project unto themselves. Thus, there are myriad possibilities for continued research; the following suggestions represent those that I believe represent the most immediately relevant and logical next steps to further defined when and why the vertebrate brain, and by extension the entire organism, requires VitE.

Genetic/Epigenetic Analysis and Secondary Nutrient Deficiencies

First, targeted gene analyses in the E- compared to E+ embryo conditions are necessary to determine how the global DNA hypomethylation I report impacts their (neuro)development and cognitive outcomes. Specifically, using methods such as bisulfite sequencing [420, 421] to evaluate the methylation status of individual genes (and their promoters/regulators) posited to play a role in mediating the downstream effects of VitE deficiency will provide knowledge concerning the impact of choline and methyl-donor depletion. By mining previous microarray data [285] from 36 hpf E- vs. E+ embryos – a timepoint between the "metabolic switch" that occurs over 24 to 48

hpf – I have found that gene expression of several enzymes involved in choline metabolism, including choline phosphotransferase (*CHPT1*), CTP-phosphocholine cytidyltransferase (*PYCT1A*), and phosphatidylethanolamine N-methyltransferase (*PEMT*), all are decreased significantly by approximately 1.5-fold in the E- condition; whether choline availability modulates its own metabolism via methylation/demethylation of any of these targets, particularly *PEMT* given its importance for generating DHA-PLs, remains an area of active investigation, and may be included in initial follow-up gene studies.

Additionally, this same data-set shows differential expression of many genes linked to neurogenesis, synaptogenesis, neuronal signaling, and glial function, which indicates that brain development is perturbed by 36 hpf in the E- embryo. Creating a targeted array to include the most outstanding of these, as well as other neurodevelopment and behavior-related genes known to be regulated via choline status and methylation (*e.g. BDNF* [40, 422]) is a feasible starting point to begin investigating the mechanistic details of how VitE deficiency-induced choline depletion results in neurological impairments; further, including samples from embryos throughout the developmental time-course (24-120 hpf) is essential to link potential differences between E- and E+ groups with the onset of morbidity/mortality and behavioral impairments. Investigation of the same targets in E- compared to E+ adult brains also would yield helpful insights concerning brain-specific gene effects of VitE deficiency, while analyses using 12 dpf larval samples would provide important evidence of lasting genetic changes due to developmental VitE (and choline) deficiency that persist even after the restoration of methyl-donor status.

However, choline and methyl-donor availability are not the only means through which DNA and/or histone methylation and gene expression may be disrupted in the E- condition. Numerous studies focused on the redox-control of energy metabolism [400] show that DNA/histone methylation (epigenetic) events mediate cellular bioenergetics [401], including glucose metabolism via “anaerobic glycolysis” (Warburg effect) vs. the citric acid cycle [423, 424], and even through relationships with the endogenous availability of sulfur groups to maintain both cellular antioxidant (glutathione) and methyl-donor (*e.g.* methionine and SAM) pools [425, 426]. This information represents an exciting potential intersection of redox-mediated pathways that connect oxidative stress, methylation cycle disruptions, and altered energy

metabolism – all factors demonstrated in my zebrafish work. My findings showing elevated levels of global DNA hydroxymethylation by 5 dpf are indicative of oxidative stress-induced chemical changes to DNA [401], which suggests that gene expression changes due to redox mechanisms, not necessarily choline deficiency, are responsible for the developmental perturbations I found, and may partially underlie the continued behavioral disruptions in E- larvae, as evidenced in autism models [402]. Both DNA methylation and hydroxymethylation are known to regulate neural crest formation [403] and other neurodevelopmental processes [404]; thus, future studies focused on brain and gene-specific methylation status pursuant to enhanced oxidative stress (again, rather than global DNA methylation alone) are warranted to investigate the possibility of altered methylation and hydroxymethylation profiles that specifically, and potentially permanently, affects cognitive development. Notably, embryonic rescue interventions (*i.e*. utilizing choline supplementation via micro-injection into the yolk prior to choline depletion) are needed to facilitate determination of which specific epigenetic/methylation outcomes are a result of choline deficiency, and which may be attributed to disturbed redox biology. Such experiments may be conducted once individual gene methylation targets are identified. Taken together, investigating the details of the global DNA methylation differences I found, both in terms of choline/methyl-donor availability as well as altered cellular redox/antioxidant homeostasis, represent intriguing avenues for continued research to better explain the mechanistic underpinnings of my present data, and to more generally advance knowledge of the biological rationale for VitE's essentiality.

Next, it is also necessary to consider how the depletion of DHA affects gene expression and cellular signaling pathways involved in brain development and function. For example, DHA-influenced changes in gene expression could contribute to perturbed brain function in the E- embryo and adult groups: DHA modulates gene transcription by activating peroxisome proliferator-activated receptor (PPAR) family members [133]; hence, DHA alters expression of genes involved in energy metabolism within the brain [383] and the mRNA stability of several enzymes associated with glucose and lipid metabolism [384]. Microarray data showing altered PPAR-gamma coactivator (*PGC1a*) (a "master-regulator" of cellular energy metabolism and mitochondrial biogenesis [427, 428]) expression in E- vs. E+ embryos suggests that VitE deficiency-induced alterations in DHA availability may influence some of the

metabolic outcomes (particularly those related to mitochondrial metabolism) found in E- individuals, as supported by various reports from the literature [429, 430]; such also may be important for brain function in the adult [33, 431-433].

Further, membrane DHA is suggested to facilitate N-methyl-D aspartate (NMDA) receptor responses [379], which result in long-term potentiation, a crucial process for synaptic modifications underlying long-term memory and learning in vertebrates [380], including zebrafish [381]. DHA supplementation also has been shown to increase the levels of hippocampal BDNF [382], as well as promote the activation of protein kinase B (PKB; or Akt), a crucial signaling protein for neuronal survival, via alterations to the membrane PL profile [300]. Oxidized DHA derivatives (and oxylipins in general) also are capable of modulating gene expression and cellular signaling (as reviewed [132]), thus, increased DHA and polyunsaturated fatty acid peroxidation in E- zebrafish, as well as indications of increased lipoxygenase (*ALOX5*) activity in E- embryos at 36 hpf, motivate continued studies to deduce how oxidized DHA and oxylipins influence the metabolic and neurobehavioral outcomes observed in E- embryos and adults, respectively. Analogous to the choline rescue model suggested above, rescues experiments including administration of DHA – perhaps utilizing synthetic deuterated-DHA compounds resistant to peroxidation [356, 434] – would provide evidence for a better understanding of how DHA depletion and its enhanced peroxidation alters metabolism and brain development/function following primary VitE deficiency. In summary, the numerous and varied functions of DHA, as an integral part of brain PLs, constitute several, probably cumulative, processes through which VitE deficiency-induced decreases in brain DHA and DHA-PLs adversely impacted E- individuals across the lifespan.

Another avenue for exploration is focused “omics” and gene expression (*e.g.* as related to DNA and/or histone methylation status) of E+ and E- embryos from time zero (fertilization) to 48 hpf (time-point by which the most E- embryos are malformed or dead). Given that classical epigenetics represents *heritable* changes in the epigenome [233], assessments aimed at defining the (epi)genetic origins (*i.e.* maternal vs. acquired during embryonic development) of the metabolic perturbations I report in the developing E- embryo are required to elucidate the exact sequence of events resulting in their neurodevelopmental impairments and, ultimately, generate a more comprehensive time-course of how their death is caused by VitE deficiency. Previous

work shows that E- embryos have higher levels of DHA at fertilization [286], which continues until 24 hpf [306, 307]. Other metabolites (*e.g.* glucose and glycolytic/citric acid cycle intermediates, vitamin C, NADPH) also are elevated in E- embryos at 24 hpf, yet choline is conspicuously decreased [307]. I did not conduct lipidomic, metabolomic, or methylation profile analyses using zero hpf eggs, which limits my ability to differentiate potentially inherited metabolic differences compared to those that occur by 24 hpf, as the individual E- embryo grows.

The case of choline depletion (as well as an evident trend for early depletion of glutathione [307]) at 24 hpf indicates that VitE deficiency-induced aberrations exist prior to 24 hpf; as 0-24 hpf is such a dynamic period of (neuro)development – recall that neurulation, which required adequate choline [349], occurs between 12-15 hpf in zebrafish [252] – alterations in gene methylation and expression during this time-frame likely have significant consequences, and may represent key initiators of VitE deficiency pathogenesis. Knowledge of whether such differences are evident at fertilization, and therefore probably inherited from maternal/paternal sources, will provide insight necessary to guide future intervention/rescue experiments: can defects in the E- embryo be ameliorated at some point during its life by providing adequate nutrition, or are there inherited factors that must be resolved instead, perhaps though maternal supplementation, to facilitate normal embryonic development? Research addressing this question is vital to ascertain the specific “point of no return” for developmental VitE deficiency, and will yield valuable information concerning when VitE is most critical for neurodevelopment, as well as provide mechanistic understanding of the sequence of events causing the E- outcomes I report herein.

A final note is that all gene changes, and, indeed, any/all metabolic changes previously found between E+ and E- embryonic conditions, must be analyzed in a tissue-specific manner to obtain definitive evidence that VitE deficiency outcomes are neurological. Assessing outcomes in adult E- brain tissue yielded helpful information (as indicated regarding the transition from **Aim 1** to **Aim 2** studies), but such is not necessarily applicable to the embryonic model; this is primary and highly significant limitation of my work. Thus, methods such as laser capture isolation of specific organs (brain) [435], use of transgenic zebrafish lines in which select neuronal populations fluoresce [436, 437], mass spectroscopy-based imaging techniques [438, 439], or, ideally, a combination of these protocols, are warranted to provide unequivocal

evidence that VitE deficiency is affecting metabolism, membrane dynamics/structure, and/or gene expression within the developing embryonic brain. Importantly, such methods also capitalize on the unique strengths of the zebrafish model, and therefore represent a means not only to advance the study of VitE biology, but also to promote the embryonic zebrafish as a translational system for use in nutritional science applications.

Centrality of Lipid Peroxidation in VitE Deficiency-Induced (Neuro)pathology

It is tempting to propose a unifying mechanism to link all the pathological developmental and brain-specific effects of VitE deficiency I report in this body of work – and, indeed, superficially "straight-forward" lipid oxidation may represent such a mechanism: inadequate VitE promotes lipid peroxidation, lipid peroxidation induces oxidative stress, which results in DHA depletion and alters PC/choline metabolism, as well as influences gene expression/methylation, mitochondrial function, and energy metabolism through redox-mediated mechanisms. Such an explanation is initially underwhelming, since VitE has been known to function as a lipophilic antioxidant for decades [440] and lipid peroxidation is an established causative event in many disease states, including neurological (*i.e*. neurodegenerative disorders; dementia) [201]. What *is* striking about my data and, therefore, is highly motivating for continued study, is the fact that lipid peroxidation due to VitE deficiency may have such pervasive and varied effects. Unraveling the pathways relating these seemingly disparate outcomes constitutes an exciting area for future research to elucidate the mechanism(s) via which VitE supports optimal human health.

To elaborate on one example of how lipid peroxidation may lead to neurological impairments, and even mortality, many of the metabolic perturbations reported herein (all of which may compromise brain function and impact survival), is *ferroptosis,* a process of non-apoptotic programmed cell death [405] that involves several key features of lipid peroxidation observed in my developmental E- model, most critically: 1) enhanced enzymatic lipid peroxidation [406], especially due to excessive arachidonic acid metabolism via 5- [407] and 15-lipooxygenase [408] activities; 2) glutathione depletion [410]; and 3) mitochondrial dysfunction [411]. I also found E- larvae had increased levels of glutamate relative to E+ larvae, which could signify glutamate toxicity, another metabolic perturbation associated with ferroptosis [407] via inhibition of the cellular cysteine/glutamate antiporter system X_c- [405]. Excessive

glutamate is neurotoxic [412] and may be a consequence of compromised mitochondria [413]; additional experiments regarding the regulation of neuronal intracellular glutamate levels are required to determine whether these mechanisms also apply to my VitE deficiency model. Importantly, VitE supplementation successfully prevents ferroptosis [339] and associated neurological impairments [414], while VitE deficiency exacerbates the latter [415], indicating that ferroptosis may constitute a biological rationale for the consequences of VitE deficiency I observed in E- conditions.

Follow-up experiments focused on VitE deficiency-induced lipid peroxidation, consequent defects in mitochondrial biogenesis and function (energy metabolism), and potential associations with ferroptosis, represent next steps that build from the data I report. Such studies to determine the point at which lipid peroxidation, should it indeed be the “unifying” initiator of all the downstream consequences of inadequate VitE (secondary nutrient deficiencies, disruption of the antioxidant network, altered cellular bioenergetics, etc.) may be conducted using my embryonic zebrafish model. Much of my findings from 24-48 hpf embryos may serve as preliminary evidence for appropriate paths of investigation. For example, my data shows that lipid peroxidation already occurs by 24 hpf [307] and that E- compared to E+ embryos are hypermetabolic [306]; as peroxidation ensures and antioxidants are depleted the E- embryos become hypometabolic by 48 hpf and demonstrate mitochondrial dysfunction that lasts even after dietary remediation (*Chapter Five*). This suggests energy metabolism is being disrupted by redox-mediated mechanisms, and that the aberrant “metabolic switch” between 24 and 48 hpf is driven by oxidative stress caused by pathological lipid peroxidation.

Interestingly, by probing previous microarray data [285], I found 36 hpf E- vs. E+ embryos have two-fold decreased expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), one of the most prominent cellular targets of oxidative modifications [441] when reactive oxygen species are formed during metabolism and under stress conditions [442]. GAPDH harbors a strictly conserved catalytic cysteine [443]; upon reversible oxidative thiol modification, glycolysis is inhibited leading to a diversion of metabolic flux through the pentose-phosphate cycle to increase NADPH production [442]. Furthermore, oxidized GAPDH may adopt new redox-dependent “moonlighting” functions in different cellular compartments, including the mitochondria and plasma membrane, and recently has been acknowledged as a sensor for redox

signals and a central “transducer” of these signals for subsequent metabolic responses [441]. Thus, increased VitE deficiency-induced lipid peroxidation and disruption of the cellular antioxidant network may create an environment that promotes protein oxidation as well; in the case of GAPDH, such would directly affect energy metabolism and, in so doing, may represent and important link between oxidative stress in the E- embryo and their resulting metabolic dysfunction.

Other relevant proteins known to upset mitochondrial function due to oxidation include the tublin-VDAC2 system, in which oxidized tubulin becomes unable to repress/modulate the opening of voltage-dependent anion channel 2 (VDAC2), located in the mitochondrial outer membrane [444]. VDAC2 is a metabolic link between glycolysis and oxidative phosphorylation in the Warburg/aerobic glycolysis phenotype [445] found in E- embryos; its unregulated function following tubulin oxidation causes increased mitochondrial metabolism [446], leading to decreased glycolysis and amplified oxidative stress that ultimately results in mitochondrial dysfunction, bioenergetic derangements, and cell death [445]. GAPDH and the tubulin-VDAC system indicate that increased lipid peroxidation and consequent antioxidant depletion pursuant to VitE deficiency may lead to protein oxidation of certain redox switches that link oxidative stress and the observed metabolic outcomes in E- compared to E+ embryos. Targeted proteomic analyses (*e.g.* including GAPDH, tubulin, and additional thiol/redox enzymes involved in antioxidant metabolism and lipid peroxidation biology such as glutathione peroxidase 4 [GPx4][447-449]), in addition to metabolomic and genetic/epigenetic analyses, are warranted to further define how VitE deficiency impacts enzymes and other proteins implicated in the redox control of metabolism. These experiments, when conducted concomitantly in zebrafish embryos – ideally, in a tissue-specific manner – as well as in adult zebrafish brains, will yield a truly comprehensive dataset to link VitE deficiency-induced lipid peroxidation and disrupted cellular redox homeostasis with redox-mediated metabolic dysregulation and epigenetic/genetic perturbations that together may explain how inadequate VitE impairs brain development and function, as well as why VitE is more generally required for normal vertebrate embryogenesis. Further, such studies may reveal how mitochondria serve as an initial site of and cellular “hub” for VitE-induced pathogenesis. Implications related to Warburg physiology and the disruption of normal cellular energy (mitochondrial) metabolism during development also create a

particularly attractive avenue for additional work utilizing combined zebrafish and cell/tissue-culture systems.

Continued investigations concerning a relationship between VitE deficiency and ferroptosis represent cutting-edge studies that truly would be on the forefront of redox biology research, since ferroptosis was only "discovered" in 2012 [450] and now has become an incredibly active scientific focus. Both VitE deficiency and ferroptosis share lipid peroxidation as a defining – perhaps initially inceptive/causative – mechanism; thus, experiments that compare outcomes of ferroptosis with those of VitE deficiency may provide information integral to elucidating the biological underpinnings of the vertebrate requirement for VitE. Incidentally, a hallmark of ferroptosis is iron-induced lipid peroxidation; the involvement of iron in VitE deficiency was not something I considered relevant until recent mining of the microarray data for 36 hpf zebrafish embryos, the results of which show that several genes important for intracellular iron trafficking are differentially expressed in E- compared to E+ embryos: frataxin [451], ferrochetalase [452], transferrin receptor [453], and GAPDH [441, 443]. Frataxin (approximately two-fold decreased expression in E- compared to E+ embryos), an essential mitochondrial protein that is highly conserved from bacteria to primates [454], is the most immediately provocative of these.

Frataxin is involved in synthesis of iron sulfur clusters and is necessary for mitochondrial biogenesis [455], though its primary function remains unknown. Intriguingly, the neurodegeneration observed in patients with heritable "Ataxia with vitamin E deficiency" (AVED, severe vitamin VitE deficiency in humans caused by a deficiency of the alpha-tocopherol transfer protein [α-TTP][67]) with that found in Friedreich's ataxia (FA), the most common genetic cause of ataxia in humans (current estimated prevalence is 2-4/100,000 individuals)[456], due to a deficiency in frataxin, likely arise from shared underlying pathophysiological mechanisms. The neurologic symptoms in AVED and FA are almost identical [457] and, in fact, genetic testing is recommended for a differential diagnosis [458]. Both disorders feature a progressive, degenerative ataxia usually diagnosed in childhood, that in FA likely is caused by inadequate protection against lipid peroxidation due to lack of mitochondrial iron homeostasis [459], while antioxidant protection against lipid peroxidation from VitE [44], is lacking in AVED. The primary cause for the retrograde "dying back" of nerves that initially occurs in peripheral sensory neurons and leads to progressive

spinocerebellar neurodegeneration, has yet to be demonstrated for either disorder, but lipid peroxidation represents a highly feasible explanation.

The quantity of new research surrounding FA and ferroptosis provides an impetus to investigate whether potentially ferroptosis-induced lipid peroxidation in FA, due to frataxin deficiency and dysregulated iron metabolism, and VitE deficiency-induced lipid peroxidation in my E- zebrafish model, constitute an underlying oxidative pathology with common molecular mechanisms. VitE and antioxidant supplementation has been used to mitigate symptoms of FA in humans [460], so such research has possible clinical applications. Hypothetically, performing experiments as described herein to compare outcomes in transgenic frataxin knock-out models (available commercially) with those of VitE deficiency, may be used to determine which pathological consequences of FA and VitE deficiency depend on lipid peroxidation, and how iron redox status (*e.g.* regulation of the cellular "labile iron pool" and reactive free iron [411, 461]) and metabolism may impact observed defects in E- zebrafish.

Iron is crucial for healthy fetal neurodevelopment [462, 463], alters redox-mediated epigenetic mechanisms [14, 37, 464] that may be heritable [465] and/or relevant to choline metabolism [17], and dysregulated iron homeostasis has been implicated in many neurodegenerative pathologies including dementia [15, 205, 227, 466]. Thus, studies involving not only FA in particular, but also iron physiology and metabolism in general, represent areas for interesting, innovative continued investigations to uncover the role VitE has in brain function throughout life. The potential for discovery of new relationships between VitE and iron nutrition highlights the need for future research to focus on connections and interactions between multiple nutrients, as do my studies demonstrating that VitE deficiency causes secondary deficiencies in other essential nutrients like choline and DHA, in embryos and adults. Collectively, both my findings in this work, as well as my suggestions for future study, indicate that VitE's function is a nexus of multiple, varied, and complex biological processes; and that VitE, as a "critical period" nutrient during vulnerable life-stages (*e.g.* neurodevelopment and older-age) represents a compelling example to motivate future research in the nutritional sciences that will shifts focus toward new horizons exploring nutrient-nutrient interactions and their implications for human health.

Final Remarks

Adequate nutrition is essential for neurodevelopment and brain function throughout life, and VitE is especially significant in this regard. My modest body of work, by exploiting the versatility and strengths of the embryonic and adult zebrafish model, provides a small contribution to the greater body of nutritional science aimed at elucidating the mechanisms to explain VitE's neurological essentiality. The numerous avenues for continued study touched upon in the previous paragraphs provide exciting opportunities to extend the experiments I conducted into diverse fields that range from clinical-translational research of VitE as a therapeutic agent for neurodegeneration in humans, to basic systems biology research of lipid peroxidation as a foundational, principal contributor to myriad disease states. Possible public health implications of my VitE research also may prove substantial: for instance, prenatal vitamins almost always include high amounts of supplemental iron, but inclusion of VitE, choline, and/or DHA is inconsistent – women of child-bearing age (as well as older adults) frequently do not consume adequate levels of these latter nutrients [24, 79, 467], and therefore may have some degree of deficiency during pregnancy. My findings suggest this may have relevant impacts on redox homeostasis and neurodevelopmental outcomes; thus, there is potential to apply additional zebrafish work towards establishing specific life-stage appropriate nutrient recommendations that support optimal brain development and function; such may begin with more nuanced VitE dose-response analyses to determine the VitE intake at which an individual organism, either during development or adulthood, becomes VitE "deficient" to the point that cognitive impairment ensues. Likewise, possible effects of VitE deficiency on gene expression and DNA methylation indicate that personalized VitE dietary recommendations based on an individual's genetic profile and diet-gene interactions could be necessary; relatedly, if such alterations are heritable and/or established in early life, they could represent targets for intervention and prevention of cognitive decline in mid-adulthood. It is my hope that my work ultimately will progress research in one, if not several, of these domains, and that my findings in some way help to improve human well-being by increasing the present understanding of how and why certain nutrients, most of all VitE, are essential for life-long neurological health.

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