Effects of dietary n-3 and n-6 fatty acids and sex on the heart and brain oxylipin profiles in healthy rats

by

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Abstract

Although the effects of dietary polyunsaturated fatty acids (PUFA) are well-known in the heart and brain, little is known about oxylipins, their bioactive lipid metabolites. To provide fundamental data on these oxylipin profiles, and the effects of dietary PUFA and sex on these profiles, weanling female and male Sprague-Dawley rats were given diets modified in oil composition to provide higher levels of α-linolenic acid, eicosapentaenoic acid, docosahexaenoic acid or linoleic acid, compared to control diets. Dietary PUFA mainly altered the levels of their own oxylipins, but also affected others. N-6 PUFA derived oxylipins were reduced by dietary n-3 PUFA in the heart, but not the brain, which generally was less resistant to diet-induced changes in oxylipins. Oxylipins were generally higher in females in heart but higher in males in brain. These data provide fundamental data that will inform studies on the roles of oxylipins in heart and brain.

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DEDICATION

I would like to dedicate this thesis to

My parents, **Md Ataur Rahman** and **Shirin Aktar**

and

my husband, **Md Monirujjaman**

THESIS ORGANIZATION

This thesis was organized following a manuscript format and is composed of two manuscripts. The thesis begins with a general introduction (chapter 1) followed by literature review, hypothesis and objectives (chapter 2). Manuscripts 1 and 2 appear in chapters 3 and 4, respectively. Manuscript 1 has been submitted to the British Journal of Nutrition and manuscript 2 has been submitted to Lipids. The thesis ends with a general discussion, conclusions and future directions (chapters 5).

CONTRIBUTIONS OF AUTHORS

For both manuscript 1 and 2: Afroza Ferdouse, Shan Leng, Harold M. Aukema designed the research. Afroza Ferdouse, Shan Leng and Tanja Winter conducted the research. Specifically, Shan Leng carried out the animal study and provided the tissues and Tanja Winter ran the HPLC/MS/MS; Afroza Ferdouse prepared the samples for analysis of oxylipins by HPLC/MS/MS and for analysis of fatty acids by GC (samples were run on the GC by the department technicians); Afroza Ferdouse and Harold M. Aukema analyzed the data, wrote the manuscripts and had primary responsibility for final content.

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LIST OF ABBREVIATIONS

AdA, adrenic acid (22:4n-6)

ALA, α-linolenic acid (18:3n3)

ARA, arachidonic acid

CNS, central nervous system

COX, cyclooxygenase

CVD, cardiovascular disease

CYP, cytochrome P450

DGLA, dihomo-gamma-linolenic acid

DHA, docosahexaenoic acid (22:6n3)

DiHETrE, dihydroxyeicosatrienoic acid

DiHODE, dihydroxy-octadecadienoic acid

DiHOME, dihydroxy-octadecenoic acid

DPAn-3, docosapentaenoic acid (22:5n-3)

EFA, essential fatty acids

EPA, eicosapentaenoic acid (20:5n3)

EpDPE, epoxy-docosapentaenoic acid

EpETrE, epoxy-eicosatrienoic acid

GC, gas chromatography

HDoHE, hydroxy-docosahexaenoic acid

HETE, hydroxy-eicosatetraenoic acid

HODE, hydroxy-octadecadienoic acid

HOTrE, hydroxy-octadecatrienoic acid

HpETE, hydroperoxy-eicosatetraenoic acid

HpOTrE, hydroperoxy-octadecatrienoic acid

HPLC/MS/MS, high performance liquid chromatography tandem mass spectrometry

IL, interleukin

k, keto

LA, linoleic acid

LOX, lipoxygenase

LPS, lipopolysaccharide

NL, neutral lipid

PD, protectin D

PG, prostaglandin

PGDH, hydroxyPG dehydrogenase

PL, phospholipid

PUFA, polyunsaturated fatty acid

Rv, resolvin

sEH, soluble epoxide hydrolase

Tx, thromboxane

Chapter 1

1.1 General Introduction

Oxylipins are oxygenated metabolites of polyunsaturated fatty acids (PUFAs) synthesized in the body upon physiological stimuli. The PUFA precursors contain 18-, 20- and 22- carbon fatty acids that produce oxylipins called octadecanoids, eicosanoids and docosanoids, respectively. Eicosanoids are the most well-known class of oxylipins produced from arachidonic acid (ARA, 20:4n6). Other precursor PUFAs mostly come from plant derived 18-carbon linoleic acid (LA, 18:2n6) and alpha-linolenic acid (ALA, 18:3n3), the 20-carbon ARA analogue eicosapentaenoic acid (EPA, 20:5n3) and the 22-carbon docosahexaenoic acid (DHA, 22:6n3). Oxylipins are synthesized from free fatty acids released from the sn-2 position of membrane phospholipids by the enzymatic action of cytosolic phospholipase A_2 [(1)]. The liberated free fatty acids undergo an oxygenation process via one of three main enzymatic pathways, namely the cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) pathways [(2, 3)].

Oxylipins are involved in many physiological processes and inflammatory responses in different tissues in human and other animals. Although their effects are pronounced in peripheral tissues [(4)], but much less is known in the heart and the brain. For example, ARA derived oxylipins have effects on regulating cardiac contractility, hypertrophy and cardiac dysfunction reported in several *in vivo* and *in vitro* studies [(5-11)]. However, effects of oxylipins derived from other PUFAs such as from EPA and DHA were studied less although their important role in heart functions were reported [(12, 13)]. On the other hand, ARA oxylipins have both neuroprotective [(14-16)] and detrimental effects [(17-20)] in regulating brain functions. In the brain, neuroprotective effects of DHA derived oxylipins [(21, 22)] and anti-inflammatory effects of EPA derived oxylipins have also been reported [(23)]. Little is known about the existence and functions of heart and brain

oxylipins derived directly from 18-carbon PUFAs (LA and ALA) although there is some evidence that LA affects heart function indirectly by altering ARA oxylipins [24] and increased oxidative stress in aging brain by increasing its own oxylipins [25], but any precise effects of LA oxylipins have not been reported yet in these tissues. Along with these already reported, whether or not other unreported oxylipins (derived from ARA, EPA, DHA, LA and ALA) have effects in these tissues remain to be elucidated. For this investigation, a comprehensive oxylipin profile in the heart and brain is required which is currently lacking with little evidence in the literature [26-33].

Previously, dietary PUFA effects on oxylipins were mainly predicted based on the tissue PUFA composition. However, recent studies in the kidney, liver and adipose tissues in rats demonstrated that oxylipin profiles are not always reflected by the precursor PUFA composition and their composition can be modulated not only by their precursor PUFA but also by other PUFAs [34-36]. For example, in the kidney and some adipose depots, DHA oxylipins were higher in rats given either ALA, EPA or DHA diets, and some ALA oxylipins were lower in rats given EPA and/or DHA diets. In other studies, ARA oxylipins were decreased by dietary n-3 PUFAs [37], or increased by dietary LA in the heart [24], and LA and ARA oxylipins were increased and EPA oxylipins were decreased by dietary LA in cerebral cortex of rat brain [38]. Such dietary effects on the entire oxylipin profile in heart and brain are lacking, however.

Higher levels of renal, hepatic and serum oxylipins in male rats have been reported in previous oxylipin profiling studies [34, 35]. In some adipose depots, sex differences varied depending the dietary treatment: for example, dietary DHA resulted in higher adipose oxylipins in males, while dietary ALA or EPA resulted in higher oxylipins in females [36]. Existing literature provided little evidence on oxylipin sex differences in heart and brain. For example,

ARA oxylipins were higher in post-traumatic human brain in males [39] and were higher in females in vascular tissue [40, 41]. So, examining sex differences in brain and heart tissues will also be relevant. Therefore, the data presented herein aims to provide comprehensive oxylipin profiles, and to evaluate the effect of different dietary oils in the heart and the brain of male and female rats.

1.2 References for Chapter 1

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Chapter 2

Literature Review

2.1 Fatty acids, polyunsaturated fatty acids (PUFA) and their importance in health

2.1.1 Overview

Fatty acids are the simplest component of heterogeneous lipid molecules having a hydrocarbon chain with a carboxylic group at one terminal and a methyl group at the opposite terminal. The common chain length of fatty acids varies from 4 to 28 carbon atoms. Fatty acids are classified in several ways. Based on the presence of double bonds, they are classified as saturated (having no double bond), monounsaturated (having one double bond) and polyunsaturated (having two or more double bonds) fatty acids [(1)]. Polyunsaturated fatty acids (PUFA) can be classified as n-3, n-6, n-7 or n-9 PUFA considering the position of the terminal double bond from the methyl end. Both saturated and unsaturated fatty acids have important functional roles in the body [(2-4)].

2.1.2 Essential fatty acids

Essential fatty acids (EFAs) are the PUFA that human and other mammals cannot synthesize *de novo* within the body and must be obtained from the diet. There are 2 classes of EFA: n-6 and n-3 PUFA. Linoleic acid (LA; C18:2n-6) and α –linolenic acid (ALA; C18:3n-3) are the predominant dietary sources of n-6 and n-3 PUFA, respectively. Human can convert LA to arachidonic acid (ARA; 20:4n-6) and ALA to eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), respectively. Sometimes, EPA and DHA (rich in fish oils) become conditional EFA when ALA is deficient in diets. Metabolic conversions of EFA to long chain PUFA are shown in Fig. 2.1 [(5)]. In particular, LA, ALA and their long chain

derivatives such as ARA, EPA and DHA are important constituents of cell membranes for both animal and plants.

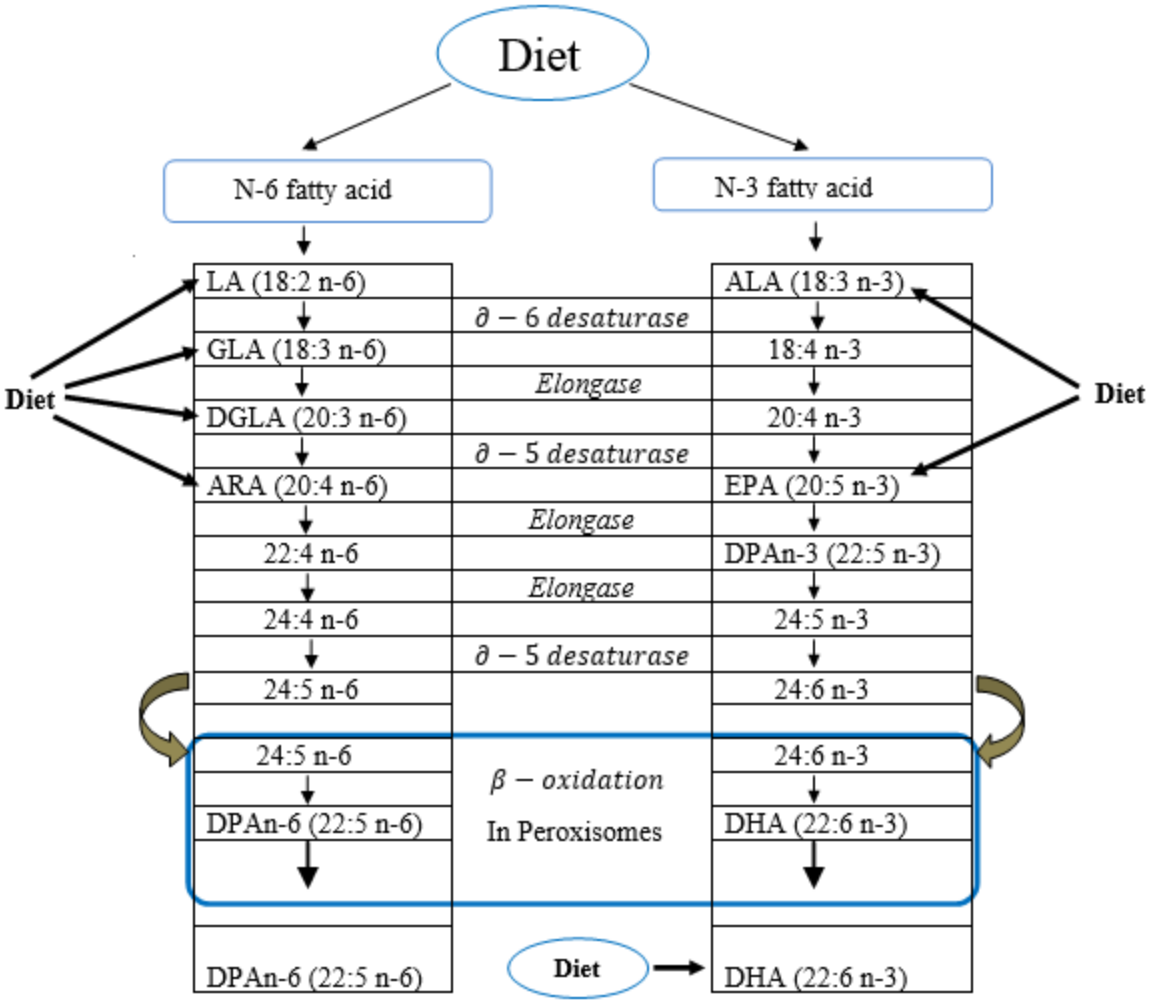

Fig. 2.1 Metabolic conversion of dietary PUFA to long chain PUFA in human. GLA, gamma-linoleic acid; DGLA, dihommo-gamma-linoleic acid; DPA, docosapentaenoic acid.

2.1.3 Importance of n-3 and n-6 PUFA in the body

Both n-6 and n-3 PUFA play important physiological roles in the body [6] such as in promotion of growth and development, prevention and management of coronary diseases, cancer, arthritis, diabetes, hypertension and autoimmune diseases [7-9]. A minimum dietary intake of different PUFA is required for optimal nutrition although it may vary between different age groups, sex and physiological conditions [10, 11]. Due to the structural similarities and competition between n-6 and n-3 PUFA for desaturase and elongase enzymes, higher dietary LA decreases or inhibits the biosynthesis of long chain n-3 PUFA from dietary ALA, and vice versa [12]. Evidence suggests that humans evolved on a diet with a ratio of n-6 to n-3 PUFA of 1:1; whereas the ratio is 15/1 to 16.7/1 in western diets since this diet has limited n-3 PUFA [13]. Diets rich in n-6 PUFA with a ratio of n-6/n-3 on an average closer to 10:1 eventually shifts the physiological condition into prothrombic and proaggregatory state via formation of more lipid mediators from ARA [14, 15]. Moreover, high n-6 PUFA intake also decreases synthesis of long chain PUFA and lipid mediators derived from n-3 PUFA [16-18]. A high ALA diet with a ratio of ~1.5 for n-6/n-3 PUFA exerted anti-inflammatory effects by reducing the production of inflammatory markers in cultured peripheral blood mononuclear cells of hypercholesterolemic patients compared to patients fed with high LA diets with a ratio of ~3.5 for n-6/n-3 PUFA [19]. An ALA enriched flax oil diet significantly lowered both systolic and diastolic pressure in cardiovascular disease (CVD) patients compared to those who fed with LA enrich safflower oil diets [20]. A ratio of 21:1 for n-6 to n-3 PUFA is linked to increased prevalence of type-II diabetes whereas, a lower ratio ~6:1 showed the opposite effect [21]. In asthmatic patients changing the ratio of n-6 to n-3 from ~10:1 to 5:1 improves respiratory distress in methacholine responders compared to non-responders [22]. However, an absolute amount of either n-3 or n-6 PUFA alone cannot ameliorate

chronic disease conditions [23]. Therefore, an optimal amount of n-6 and n-3 PUFA intake should be maintained for better physiological condition.

2.1.4 PUFA composition in the heart

Cardiac physiology is regulated by several cellular mediators both in the normal and diseased condition. Since PUFA are the source of the lipid mediators, the PUFA profile in heart is also important. The heart PUFA profile in rats has been reported in several studies [24,25]. ARA is the major PUFA in the heart followed by LA and DHA, but the level of ARA was drastically reduced more than 10 times after n-3 PUFA supplementation compared with the control group [24]. However, no EPA or ALA was detected in either supplement or control groups. Another study reported that feeding higher level of LA and ALA alters PUFA composition in the heart and increases LA and ALA along with other major PUFA [25]. So, dietary supplementation of PUFA can alter the PUFA profile in the heart of rats.

2.1.5 PUFA composition in the brain

DHA is the major PUFA in rat whole brain followed by ARA and LA, while ALA and EPA are present in trace amounts as reported [25]. The distribution of PUFA in brain is not even, but varies with diet, brain region and age. For example, dietary intervention for 17 weeks in aged (70 weeks) Wister rats with or without fish oils (e.g. EPA and DHA) resulted in variation of PUFA composition in cerebral cortex and hippocampus of brain [26]. The DHA and LA levels in the cerebral cortex were increased in the EPA and EPA+DHA group compared to the control group, whereas the ARA levels were decreased in the EPA and EPA+DHA group compared to the control group. A regional difference in PUFA distribution was also observed in cerebral cortex, hippocampus and cerebellum of brain in weanling rats aged 21 days with DHA followed by

ARA being the most highly concentrated in cerebral cortex among the 3 regions examined [27]. LA was below 1% in all 3 parts of brain, while ALA and EPA were not detected in any of the examined brain parts. PUFA composition in different parts of rat brain is altered after n-3 PUFA supplementation compared with the control rats [28]. This supplementation resulted in significantly higher levels of DHA in the cerebral cortex, hippocampus and cerebellum; and reduced ARA levels in these regions compared with control groups [28]. Trace amount of LA were detected but ALA and EPA were not detected in examined brain parts under this condition. Therefore, diets nutritionally adequate but rich in fish oil can increase the n-3 PUFA (mostly DHA) composition in different parts of adult rat brain such as in cerebral cortex, hippocampus and cerebellum. However, the effects of dietary LA, ALA and EPA on PUFA composition in different parts of rat brain remain to be elucidated.

2.2 Oxylipin formation and their functions

2.2.1 Generation of oxylipins from the precursor PUFAs

Oxylipins are the bioactive lipid mediators synthesized from PUFA in the body upon physiological stimuli [29]. Oxylipin synthesis requires free fatty acids to be liberated from membrane phospholipids (sn-2 position) by the enzymatic action of cytosolic phospholipase A_2 [30]. The liberated free fatty acids undergoes at least one step of mono or dioxygen dependent oxidation via one of three main enzymatic pathways, namely the cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) pathways [31, 32].

The COX pathway generates prostaglandins (PG) and thromboxanes (Tx), collectively called prostanoids [33]. The LOX pathway generates hydroxy-fatty acids such as 5-hydroxy-eicosatetraenoic acid (5-HETE). LOX activity combined with epoxygenase and hydroxylase activity also result in formation of di- and tri-hydroxy FAs such as leukotrienes (LT), lipoxins

(LX), resolvins (Rv), protectins (PD) and maresins [34, 35]. Many of the same oxylipins can also be formed non-enzymatically. Along with ARA, other precursor PUFAs such as, 18-carbon ALA and LA; 20-carbon (n-3 analogue of ARA) EPA and 22- carbon DHA also synthesize oxylipins using LOX pathway [29]. CYP epoxygenase enzymes generate epoxy-derivatives of ARA, EPA and DHA such as epoxy-eicosatrienoic acid (EpETrE), epoxy-eicosatetraenoic acid (EpETE) and epoxy-docosapentaenoic acid (EpDPE), respectively; and CYP ω-hydroxylase enzymes generate hydroxy-derivatives of ARA, EPA and DHA such as HETE, hydroxy-eicosapentaenoic acid (HEPE) and hydroxy-docosahexaenoic acid (HDoHE), respectively [29]. LA and ALA also produce CYP products via epoxygenase pathway. LA can be oxidized via the CYP epoxygenase enzymes and resulted in epoxygenated FAs such as 9, 10-epoxy-octadecenic acid (9, 10-EpOME), which is further metabolized via soluble epoxide hydrolase (sEH) to form dihydroxy FAs such as 9,10- dihydroxy-octadecenoic acid (9,10- DiHOME) [29]. Similar to LA, ALA also is oxidized via CYP epoxygenase enzymes and results in epoxygenated FAs, such as 12,13-epoxy-octadecadienoic acid (12,13-EpODE), which can be further metabolized to dihydroxy FAs such as 12,13-dihydroxy-octadecadienoic acid via sEH [36].

2.2.2 Oxylipins in the heart and their functions

Oxylipins play a significant role in maintaining normal physiology. Eicosanoids are the most well-known class of oxylipins produced from ARA and many of the PUFA effects of ARA are mediated through these oxylipins in the body in normal health and diseased conditions [37-40]. ARA produces PGs, LTs and other oxylipins that display primarily inflammatory and immune-stimulatory effects. Prostanoids as a result of cardiac ischemia, induce apoptosis on cardiac myocytes and contribute to the cardiac cell loss followed by myocardial infarction [41]. Selective COX-2 inhibition improved cardiac dysfunction and decreased infarct size in a chronic model of

myocardial infarction [42]. Although this inhibition worsens ischemia/reperfusion injury in guinea pig [43], long-term exposure of COX-2 pathway derived oxylipins in the ischemic myocardium may have harmful effects on cardiomyocyte by inducing inflammation and vasoconstriction [44, 45]. Recently discovered Rvs derived from EPA and DHA, and PDs derived from DHA are reported to exert anti-inflammatory and protective roles which oppose the inflammatory effects of ARA derived PGs and LTs [46, 47].

In cardiac tissues of CVD patients, 12/15-LOX has higher activity compared to healthy individuals with increased production of 12-HETE and 15-HETE [48-50]. Moreover, 12-HETE and 15-HETE enhance development of myocardial fibrosis, oxidative stress and atherogenesis [51]. On the other hand, EPA derived 18-HEPE inhibits cardiac fibrosis and inflammation in cultured cardiac fibroblasts and prevents cardiac fibrosis in mice [52].

In the heart, epoxy- and hydroxy-metabolites of ARA generated by the CYP pathway regulate cardiovascular function, while the analogous EPA and DHA metabolites generated by the same pathway often exert similar or opposing biological effects beneficial for cardiovascular functions [53, 54]. 20-HETE and EpETrE are CYP pathway derived ARA metabolites that partially oppose each other's effects in the regulation of cardiac functions, such as EpETrEs reduce infarction injury after ischemia and induce functional recovery, while 20-HETEs oppose the recovery under the same pathological conditions [54]. Among CYP isoforms, the predominant forms in the heart belong to the CYP4A, CYP2C and CYP2J families and are especially associated with CVD [55]. CYP4A converts AA to 20-HETE, a potent vasoconstrictor responsible for regulation of myogenic tone and inflammation, while the other two epoxygenases CYP2C and CYP2J, generate EpETrEs with opposing effects to 20-HETE. Once EpETrEs form they are rapidly converted to dihydroxyeicosatrienoic acid (DiHETrE) by sEH which is highly expressed

in heart [56]. 20-HETE also promotes endothelial dysfunction by enhancing activation of pro-inflammatory transcription factors and vasoconstriction [57, 58].

Thus, several oxylipins derived from ARA, followed by DHA and EPA, have been shown to have biological functions in the heart. These oxylipins can have similar or opposing effects and similar oxylipins can be formed by different pathways. There is also evidence of the existence of LA and ALA oxylipins in the murine heart [59] but a complete oxylipin profile of these oxylipins is needed. Before characterizing their functions, comprehensive knowledge of the oxylipin profile in the heart is needed in order to know which oxylipins are present. Table 2.1 contains a list of functions of oxylipins primarily in the heart. For a more exhaustive list see [29, 37].

Table 2.1 Examples of oxylipins and heart related functions

ARA oxylipins	
PGE_2	Vasodilates cerebral arterioles in cat [60]
$PGF_{2\alpha}$	Induces inflammatory tachycardia in mouse [61]
PGI_2	Vasodilates coronary arteries in dogs [62]
TxA_2	Induces inflammatory tachycardia in mouse [61], mitogenesis in coronary artery of guinea pig model [63], hypertension in rats [64] and vasoconstriction in rabbits aorta [65]
2,3-dinor TxB_2	Probable marker of acute MI in human urine [66]
11-dehydro TxB_2	Probable marker of acute MI in human urine [66]
5-HETE	Inhibits production of PGI_2 in endothelial cells of porcine coronary artery and causes cellular hypertrophy of ventricular cardiomyocytes in human [67, 68]
12-HETE	Causes cellular hypertrophy of ventricular cardiomyocytes in human [68], increases oxidative stress and apoptosis in rat heart [69], induces aortic fatty streak formation by monocyte adhesion in human endothelial cells [70, 71], and stimulates erythrocyte adhesion to endothelial cells in bovine aorta [72]

15-HETE	Induces cellular hypertrophy of human ventricular cardiomyocytes [68], vasodilates or vasoconstricts isolated arteries of human, guinea pig, rabbit and rat based on species differences and physiological conditions [73], and stimulates adhesion of erythrocyte to endothelial cells in bovine aorta [72]
15-HpETE	Induces either dilation or constriction of isolated arteries in human and other vertebrates depending on species and experimental conditions [73], and stimulates erythrocyte adhesion to endothelial cells in bovine aorta [72]
20-HETE	Induces cardiac hypertrophy [74]
LTE_4	Constricts pig coronary arteries [75]
LxA_4	Induces vasorelaxation of aortic rings similarly as LxB_4 in rat or guinea pig [76]
LxB_4	Induces vasorelaxation of aortic rings in rat or guinea pig (similar to LxA_4) [76]
5,6- EpETrE	Stimulates in vitro and in vivo angiogenesis of endothelial cell proliferation in murine model [77], less potent vasodilator than its DiHETrE isomers in canine coronary arterioles [78] and in mouse preconstricted pressurized arteries [79]
8,9-EpETrE	Dilates coronary microvessels similarly as other epoxy-fatty acids derived from ARA, EPA and DHA in dog and pig [80], and reduces severe oxidative stress induced cell death in myocytes of rat heart [81]
11,12-EpETrE	Dilates coronary microvessels similarly as other epoxy-fatty acids derived from ARA, EPA and DHA in dog and pig [80], reduces severe oxidative stress induced cell death in myocytes of rat heart [81], dilates pre-constricted arteries less efficiently than its dihydroxy derivative in mouse [79] and acts similarly in pig coronary arteries [82]
14,15-EpETrE	Dilates coronary microvessels similarly as other epoxy-fatty acids derived from ARA, EPA and DHA in dog and pig [80], reduces severe oxidative stress induced cell death in myocytes of rat heart [81], dilates pre-constricted coronary arterial rings more efficiently than its dihydroxy derivative in bovine [83], less potently in mouse arteries [79] and isolated coronary arterioles [82]
5,6-DiHETrE	Stimulates vasodilation of constricted arteries more efficiently than its epoxy isomer in

	mouse model [79] and induces hyperpolarization of vascular smooth muscle cells from coronary arteries of rats [84]
8,9-DiHETrE	Stimulates vasodilation of mouse arteries and canine coronary arterioles in isolation more efficiently than its EpETrE isomer [78, 79] and induces hyperpolarization of vascular smooth muscle cells in rats coronary arteries [84]
11,12-DiHETrE	Stimulates vasodilation of pre-constricted pressurized mouse arteries; dog and pig coronary arterioles more efficiently than its EpETrE isomer [78, 79, 82] and induces hyperpolarization of coronary arterial vascular smooth muscle in rats [84]
14,15-DiHETrE	Stimulates vasodilation of pre-constricted pressurized mouse arteries and isolated dog coronary arterioles more efficiently than its epoxy isomer [78, 79], and induces hyperpolarization of coronary arterial vascular smooth muscle in rats [84]
20-HETE	Induces vasoconstriction in porcine coronary arteries [85] and stimulates rapid increase of vascular smooth muscle cells in rats [86]
ALA oxylipins	
13-HpOTrE	Affects depression functionality of action potential parameters in rat cardiomyocytes [87]
9,10-DiHODE	Present at lower levels compared to dihydroxy derivatives of ARA in blood of hyperlipidemic vs. normolipidemic men [88]
LA oxylipins	
13-HODE	Prevents platelet adherence to vascular endothelium in human [89] and reduces in vitro platelet aggregation induced by thrombin [90]
9,10-EpOME	Induces heart failure in canine when injected intravenously [91]
9,10-DiHOME	Induces vascular resistance in the heart in mouse model and slows down recovery process after an onset of ischemia/reperfusion injury [92]
EPA oxylipins	
PGD_3	Reduces vascular resistance in the periphery and have beneficial effects in the physical properties of dog heart [93]
PGI_3	Induces vasodilation of bovine coronary arteries [94]

LTC_5	Prevents anaphylaxis in the isolated heart of guinea pig as efficiently as LtC4 [95]
8,9-EpETE	Dilates coronary microvessels in animals as efficiently as other EpETE, EpETrE and dihomo-EpETrE isomers [80]
11,12-EpETE	Dilates coronary microvessels in animals as efficiently as other EpETE, EpETrE and dihomo-EpETrE isomers [80]
14,15-EpETE	Dilates coronary microvessels in animals as efficiently as other EpETE, EpETrE and dihomo-EpETrE isomers [80]
17,18-EpETE	Dilates coronary microvessels in animals as efficiently as other EpETE, EpETrE and dihomo-EpETrE isomers [80] and vasodilates vascular smooth muscle cells in rats [96]
18-HEPE	Inhibits cardiac fibrosis and inflammation in vitro and prevents cardiac fibrosis and inflammation in vivo [52]
DHA oxylipins	
17S-HDoDE	Dilates coronary arterial smooth muscle cells in animal model [97]
7,8-EpDPE	Dilates porcine coronary arterioles [98]
10,11-EpDPE	Dilates porcine coronary arterioles [98]
13,14-EpDPE	Dilates porcine coronary arterioles [98]
13,14-DiHDoPE	Dilates porcine coronary arterioles less efficiently than 13,14-EpDPE [99]
16,17-EpDPE	Dilates porcine coronary arterioles [98]
19,20-EpDPE	Dilates porcine coronary arterioles [98]

2.2.3 Oxylipins in the brain and their functions

Recent findings on oxylipin function in brain revealed their importance in brain pathology. In rodent cerebral cortex and pyramidal neurons, PGE_2 regulate neurovascular coupling [100]. COX-2 is the inducible form of COX highly expressed in brain during neuroinflammation [101]. In cerebral ischemia injury, PGI_2 and TxA_2 are involved in pathological consequences mediated by the COX-2 pathway [102].

In the central nervous system (CNS), 5-LOX is highly expressed as an inflammatory enzyme and involved in tau phosphorylation, synaptic function, memory and plasticity in a mouse model which displays some common features of human prefrontotemporal dementia [103]. In ischemic stroke, 12/15 LOX acts as a central mediator of cultured neuronal cells *in vivo* [104]. In both human and mouse, 12/15 LOX is elevated in blood after stroke but what happens in brain is not clear [105].

Similar to COX, CYP pathway derived ARA oxylipins have a role in stroke [106]. For example, 20-HETE is a potent vasoconstrictor with detrimental effects on cerebral arteries during stroke [107-109]. On the other hand, EpETrEs such as 5, 6-EpETrE, 8, 9- EpETrE and 14, 15- EpETrE from the CYP pathway cause vasodilatation in neurons, astrocytes and cerebral blood vessels [110-112]. DHA derived Rv generated in mouse brain and human glial cells displays potential actions on leukocyte trafficking as well as down regulates cytokine expression on the respective cells [113]. This study has also illustrated that glial cells can release and transform DHA into novel docosatrienes. DHA derived PD_1 and RvD_1 provide neuroprotection [26, 114-116]. Other minor PUFAs such as EPA and LA also synthesize oxylipins and few of them are reported to have functional effects in the brain and CNS. For example, EPA derived 18-HEPE promotes remyelination in CNS neurons after an injury [117] and LA derived HODEs possibly indicates increased oxidative stress if present at higher level in aging brain [118].

The role of many ARA and DHA oxylipins followed by few EPA and LA oxylipins in regulating brain and CNS functions have been reported, to our knowledge. However, functions of ALA oxylipin in the brain remain to be elucidated. Before this is done a comprehensive characterization of the oxylipin profile in the brain is needed to know which oxylipins are

present. Table 2.2 contains a list of functions of oxylipins primarily in the brain. For a more exhaustive list see [29].

Table 2.2 Examples of oxylipins and brain related functions

ARA oxylipins	
PGD_2	Induces and increases sleeping time in a dose-dependent manner in monkeys [40], stimulates both nerve growth and neurotrophic factor production in cultured mouse astrocytes [119], increases in brain microvessel after an onset of ischemic brain edema [120]
PGE_2	Vasodilates cerebral arterioles in cat [60], stimulates both nerve growth and neurotrophic factor production in cultured mouse astrocytes [119], restores microglial chemotaxis, suppresses toxic inflammation and prevents synaptic injury and memory deficits in murine models of Alzheimer's disease [39] and increases in brain microvessel after an onset of ischemic brain edema [120]
$PGF_{2\alpha}$	Vasoconstricts brain arterioles in rats [121]
15-d-PGJ_2	Induces antipyretic (fever reducer) effects associated with reduction in LPS-induced COX-2 expression in the hypothalamus [122]
Tx	Involves in the production of Aβ and amyloid plaques in the pathogenesis of AD [123]
12-HETE	A marker of brain injury [124]
16-HETE	Decreases stroke associated intracranial pressure in rabbit model [125]
20-HETE	Promotes detrimental effects in the brain after an onset of ischemic stroke [126]
LTB_4	Involves in the production of Aβ and amyloid plaques in the pathogenesis of AD [123]
LTD_4	Increases during cerebral ischemic injury in humans [127] and induces brain edema [128]
LxA_4	Mediates neuroprotective effects in brain ischemia in rats possibly by anti-inflammatory mechanism [129], confers neuroprotective effects and decreases inflammation in rat after cerebral ischemia reperfusion injury [130]
11,12-EpETrE	Vasodilates cerebral microcirculation [131]
14,15-EpETrE	Vasodilates cerebral microcirculation [131], enhances cell viability against oxidation

	induced ischemic injury in astrocytes [132, 133]
LA oxylipins	
9-HODE	Possible indication of increased oxidative stress if present at higher level in aging brain [118]
13-HODE	Possible indication of increased oxidative stress if present at higher level in aging brain [118]
9,10-DiHOME	Possible indication of increased oxidative stress if present at higher level in aging brain [118]
EPA oxylipins	
TxA_3	Increases catecholamines upon intracerebroventricular administration as potently as TxA_2 in rats [134]
18-HEPE	Promotes remyelination after toxic injury to CNS oligodendrocytes [117]
RvE_1	Promotes resolution of inflammation in microglial cells in vitro by decreasing inflammatory cytokine expression induced by lipopolysaccharide (LPS) [135]
DHA oxylipins	
7-HDoHE	Associates negatively with age-related memory decline [26]
10-HDoHE	Associates negatively with age-related memory decline [26]
17-HDoHE	Associates negatively with age-related memory decline [26]
PD_1	Associates negatively with age-related memory decline [26], inhibits neuronal cell death through reduction in caspase activation [136], induces survival of brain cell from neurotoxicity and promotes neuroprotection [137]
PDX	Inhibits leukocyte infiltration, COX-2 induction and elicits neuroprotection in mouse model of ischemic stroke [138]
RvD_1	Associates negatively with age-related memory decline [26], promotes resolution of inflammation in microglial cells in vitro by decreasing inflammatory cytokine expression induced by LPS [135]
RvD_2	Associates negatively with age-related memory decline [26]

19,20-EpDPE	Modulates beneficial effects in the nociceptive signaling [139]

2.3 Dietary PUFA modulates endogenous oxylipin formation

2.3.1 General effects of dietary PUFA on oxylipin synthesis

The main sources of dietary ALA are green leaves, flax oil, canola oil and walnut oil, while soybean oil and corn oil are the main sources of LA [140]. DHA and EPA are most abundant in marine fish and algae. However, long chain fatty acids such as EPA and DHA can be formed to some extent from ALA via further elongation and desaturation processes. ARA can also be formed to some extent from LA via the same elongation and desaturation process. As humans cannot synthesize LA *de novo*, dietary LA is the only source of blood and tissue LA; lowering dietary LA is highly associated with lower level of circulatory LA and LA derived oxylipins [141]. Although very little is known about LA and ALA oxylipins, a recent dietary intervention study with ALA and LA in obese rats demonstrated that ~ 60% of total renal oxylipins detected are derived from ALA and LA [142]. Dietary ALA in rats is associated with decreased levels of COX derived ARA oxylipins, increased levels of LOX derived n-3 oxylipins and increased levels of CYP derived EPA and DHA oxylipins [143]. An ALA enriched diet resulted in increased ALA oxylipins in blood [144]. Fish oil supplementation displayed upregulation of n-3 PUFA oxylipins which is an indication that long chain PUFAs in the diet also are a source of these oxylipins [145]. Recently published oxylipin profiling studies in kidney, liver and adipose tissues demonstrated that oxylipins are not only modulated by their precursor PUFA, but also by the other PUFAs present in the diet [146-148]. For example, in kidney and some adipose depots, DHA oxylipins were increased when provided diets enriched in either ALA, EPA or DHA; ALA oxylipins were decreased when provided diets enriched in either EPA and/or DHA [147, 148]. In the kidney and

liver, diet enriched in LA increased both renal and hepatic ARA and other n-6 oxylipins which are not evident from their respective tissue fatty acid composition alone [142, 146]. Therefore, dietary PUFAs can be metabolized to their respective oxylipins, can modulate the formation of oxylipins derived from other precursor PUFAs and PUFA effects in oxylipins are not always reflected by the PUFA effects in their precursor PUFA composition in that tissue.

2.3.2 Effects of dietary PUFA on heart oxylipins

There is substantial literature on the fatty acid composition in mammalian heart, but little is known about their bioactive lipid mediators called oxylipins. ARA derived eicosanoids are the most well studied class of oxylipins having numerous biological functions including effects on the heart in health and diseased conditions [37]. Several studies have reported functional effects of n-3 PUFA, including some beneficial effects on heart diseases, and particular focus has been given to ALA, EPA and DHA in terms of their effects via oxylipin formation [149]. Recently, ALA derived 9-hydroxy-octadecatrienoic acid (9-HOTrE), 13-HOTrE and 9, 16-DiHOTrE also received attention due to their potential anti-inflammatory and anti-thrombotic effects [150].

Effects of dietary PUFA in modulating endogenous oxylipin levels have already been reported in rat kidney, liver, serum and several adipose sites [146-148]. In these tissues, oxylipin levels were modulated not only by their direct dietary precursor PUFAs but also by other PUFAs that are not their direct precursors. For example, dietary ALA reduced ARA oxylipins to a varying extent in these tissues; it also increased some DHA oxylipins in kidney, serum and in some adipose depots without altering the DHA level. Dietary LA in these rats increased LA oxylipins in all sites examined and also ARA oxylipins in the kidney and liver. In rat heart, ARA oxylipins were reduced by dietary n-3 fatty acids such as EPA and DHA [151], or increased by dietary LA [152]. In these studies, changes in the oxylipin levels in different tissues were not

always reflected by the dietary effects on tissue fatty acids. Since dietary PUFA can modulate endogenous oxylipin levels differently than their PUFA levels in tissues including the heart, and dietary PUFA effects on the comprehensive heart oxylipin profile is still lacking in the existing literature, it is important to know how diet affects heart oxylipins. This will guide future research on their potential roles in heart health and physiology.

2.3.3 Effects of dietary PUFA on brain oxylipins

Brain being the most lipid rich organ in mammals with significantly higher level of PUFAs such as DHA and ARA [25], it will be interesting to know whether their respective oxylipins are also abundant in this tissue and how they are modulated by dietary PUFA. Although ARA derived eicosanoids are well evident for maintaining neural functions both in normal health and diseased conditions [153], DHA derived oxylipins have also been shown to provide functional effects in brain physiology [139, 154]. However, presence and functions of oxylipins from less abundant brain PUFA such as EPA, ALA and LA remain to be elucidated. An *in vitro* study in cultured human brain endothelium cells reported formation of plenty of oxylipins in the presence of long chain PUFA such as ARA, EPA and DHA [155]. Dietary EPA and EPA+DHA elevated the level of EPA-derived 5-HEPE and DHA-derived 7-, 10-, and 17-HDoHE, PD_1, RvD_1, and RvD_2; and decreased ARA-derived PGE_2, PGD_2, and $PGF_{2\alpha}$ in rat cerebral cortex [26]. Also, in the EPA rats, the level of ARA-derived 5-, 12-, and 15-HETE were increased and DHA-derived PD_1, RvD_1, and RvD_2 were decreased compared with the rats provided with mixture of EPA+ DHA [26]. So, different levels of dietary PUFA resulted in different levels of oxylipin formation in the brain. Although ALA deficiency in the diet caused depletion in DHA level in the brain of 5 week old female rats [27], no data on the effects of dietary ALA on the rat brain oxylipin profile is available, to our knowledge. Increased dietary LA compared to low level of dietary LA resulted

in an increased level of LA and ARA derived oxylipins but decreased level of EPA derived oxylipins in rat cerebral cortex [156]. Since, dietary PUFA effects on oxylipin levels is not always predictable by their PUFA levels, examining dietary PUFA effects on this profile will provide fundamental data to screen which oxylipins to target for dietary modulation in response to various physiological conditions. Along with DHA, effects of dietary EPA, LA and ALA on rat brain oxylipin profile needs further attention.

2.4 Effects of sex on oxylipin synthesis

Sex effects on PUFA have been reported in several studies but little is known about the sex effects on their oxylipins. Since oxylipin data cannot always be extrapolated from their precursor PUFA data, it will be interesting to investigate oxylipin sex differences in different tissues.

Few discrete studies reported oxylipin sex difference in different tissues and animals. For example, renal CYP derived oxylipins such as HETEs, EpETrEs and DiHETrEs associated with renal function and blood pressure are decreased in male rats after a high fat diet, while no such changes are observed in female rats [157]. Low dose of aspirin causes inhibition of platelet aggregatory TxA_2 and formation of anti-inflammatory 15-epi-LXA_4 which is more likely to occur in female compared to male human plasma [158]. In atherosclerosis, female $ApoE^{-/-}$ mice had elevated levels of TxA_2 and diminished levels of PGI_2 in serum, while males did not exhibit these effects [159]. The increased atherogenic activity in females was found to be associated with markedly increased production of TxA_2 and decreased production of PGI_2. Similarly, the level of PGE_2, TxA_2 and PGI_2 are higher in macrophages of arthritis–susceptible female compared to male rats [160].

Also, recent oxylipin profiling studies in healthy rats reported unique oxylipin sex differences depending on tissue type, where oxylipins were usually higher in males in serum,

kidney and liver [146, 147]. In several adipose depots oxylipins were higher in either males or females depending on which PUFA provided in the diet [148]. For example, rats provided a DHA enriched diet had higher adipose oxylipins in males, but those provided with either ALA or EPA diets had higher oxylipins in females [148].

However, effects of sex on oxylipin metabolism are not well understood. There is little evidence of sex effects on heart or brain oxylipins. Since oxylipin sex difference are obvious in most tissue types examined and diet may influence such differences sometimes, it will be interesting to explore oxylipin sex differences in the heart and brain and also to determine whether the diet has any influence in such differences in these tissues.

2.5 Study rationale and research gap

To date, a number of studies have characterized oxylipins in human serum [145, 161, 162] and plasma [165-167] . Recently, oxylipin profiling has been done in kidneys of diseased rats [142, 166, 167] and serum, kidney, liver and several adipose sites of healthy rats [146-148]. Oxylipin profiling in human plasma reveals that the highest level of oxylipins are those derived from ARA and LA followed by oxylipins derived from EPA, DHA and ALA [168]. The levels of LA and ALA oxylipins are also higher in liver and kidney of rats compared to oxylipins derived from other PUFAs [142, 166, 167], showing that the relative abundance of oxylipins is different in different tissues. However, information on heart [59] and brain [139, 169-174] oxylipin profiles are limited. Previously, oxylipins were traditionally analyzed by immunoassay technique which is not suitable to screen many oxylipins at a time. Thus, researchers focus was mostly on the oxylipins that had profound effects on physiology and those that were known to be present. With the advancement of technology such as HPLC combined with tandem mass spectrometry (MS/MS), biologists are now able to screen more than 160 oxylipins at a time. HPLC separates compounds

based on their retention time and mass spectrometry ionizes molecules for separation according to their mass to charge ratio by a mass analyzer. Since important functions of some oxylipins are evident in heart and brain, by using this advanced technology (HPLC/MS/MS), comprehensive oxylipin profile in these tissues can now be done to add more fundamental data to better understand which oxylipins to target for their functional effects in these tissues.

Dietary intervention of n-3 PUFA in humans results in elevated levels of plasma n-3 PUFA oxylipins and reduced levels of n-6 PUFA oxylipins [164, 165]. Dietary LA does not alter the level of ARA in blood [175] but can increase select kidney ARA oxylipins [167]. Dietary PUFA such as ALA, EPA, DHA and LA result in tissue specific fatty acid changes, so it is likely that oxylipins also are affected differently in different tissues [146-148]. Heart and brain oxylipins also may be amenable to dietary changes [152, 154, 176, 177] and fatty acid composition does not always reflect oxylipin composition. Thus, examining effects of individual dietary PUFA such as ALA, EPA, DHA and LA on oxylipin levels in the rat heart and brain will provide fundamental data that will inform which PUFA to target to modulate their endogenous oxylipins in different physiological conditions.

Examining sex differences may also be relevant along with oxylipin profiling in the heart and brain tissues. Previous oxylipin profiling studies have reported higher levels of oxylipins in kidney, liver and serum in male rats, but higher levels in females in several adipose depots [146, 147]. Although an explanation for such differences is not known, it is important to explore oxylipin sex differences in other tissues as well. Since oxylipin sex differences in the heart and brain is largely unexplored, examining such differences in these tissues will provide fundamental data on any differences in oxylipins and possibly point to their biological roles in males and females.

Thus, determining the oxylipin profile and how their levels are affected by diet and sex is of interest. For these fundamental studies animal models such as the rat will provide initial information on different tissues. A growing rat model also is expected to exhibit a greater change more rapidly, providing an advantage of using a young, yet sexually mature model.

2.6 Hypotheses

1. Rat heart contains quantifiable levels of ARA, DHA, EPA, LA and ALA derived oxylipins.
2. Rat brain contains quantifiable levels of ARA, DHA, EPA and LA derived oxylipins and trace amounts of ALA derived oxylipins.
3. Dietary supplementation of oils containing higher levels of ALA, LA, EPA and DHA will alter the oxylipin profile in the rat heart.
4. Dietary supplementation of oils having higher levels of DHA, EPA, LA and ALA will alter the oxylipin profile in the rat brain.
5. Oxylipin levels in brain and heart are different in male and female rats and diet may influence some of the differences.

2.7 Objectives

Objectives for hypothesis 1 and 2: To characterize the oxylipin profile in the 10 week old rat heart and brain by HPLC/MS/MS.

Objective for hypothesis 3 and 4: To determine how dietary oils containing higher levels of ALA, EPA, DHA and LA provided to weanling rats for 6 weeks affects oxylipin composition in the 10 week old rat heart and brain.

Objective for hypothesis 5: To compare sex differences in oxylipin profile and dietary effects on these profiles in 10 weeks old rat heart and brain.

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Transition to next chapter

As discussed in this chapter, oxylipins derived from different n-3 and n-6 PUFA may play functional roles in different tissues. They can be modulated by changes in dietary PUFA, but this is not always predictable based on their precursor PUFA levels. Further, their levels can be different between males and females. Although some oxylipins have been reported to have functional effects in cardiac physiology, information on the heart oxylipin profile is limited. Heart oxylipins may also be amenable to dietary changes and sex differences. Therefore, the next chapter will examine the effects of dietary PUFA and sex differences on the comprehensive oxylipin profile in rat heart, and will test hypotheses 1, 3 and part of 5 in section 2.6.

Chapter 3

3. Dietary n-6 and n-3 PUFA alter the heart oxylipin profile differently in male and female rats

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Running Head PUFA and sex effects on heart oxylipins

Keywords oxylipins, eicosanoids, heart, sex, dietary fat, ALA, EPA, DHA, LA, ARA

3.1 Abstract

Oxylipins are bioactive lipid mediators synthesized from polyunsaturated fatty acids (PUFA). Eicosanoids are the most well studied class of oxylipins derived from arachidonic acid (ARA), and many of them influence cardiac physiology in health and disease. Oxylipins are also formed from other n-3 and n-6 PUFA such as α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and linoleic acid (LA), but fundamental data on the heart oxylipin profile, and the effect of diet and sex on this profile, are lacking. Therefore, weanling female and male Sprague-Dawley rats were given AIN-93G based diets modified in oil composition to provide higher levels of ALA, EPA, DHA, LA and LA+ALA, compared to control diets. After 6 weeks, heart oxylipins were increased primarily by their precursor PUFA, except for EPA oxylipins, which were increased not only by dietary EPA, but also by dietary ALA or DHA. Dietary DHA had a greater effect than ALA or EPA on reducing ARA oxylipins. An exception to the dietary n-3 PUFA lowering effects on ARA oxylipins was observed for several ARA derived prostaglandin metabolites that were higher in rats given EPA diets. Higher dietary LA increased LA oxylipins, but it had no effect on ARA oxylipins. Overall, heart oxylipins were higher in female rats, but this depended on dietary treatment: the female/male oxylipin ratio was higher in rats provided the ALA compared to the DHA diet, with other diet groups having ratios in between. In conclusion, individual PUFA and sex have unique and interactive effects on the rat heart oxylipin profile.

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3.2 Introduction

There is substantial literature on fatty acid compositions in mammalian heart, but little is known about their oxygenated bioactive metabolites called oxylipins. The most well studied class of oxylipins are the eicosanoids generated by cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) oxygenase activities from arachidonic acid (ARA, 20:4n-6); these bioactive lipids are the major mediators of ARA effects in the body [1, 2]. Oxylipins also are synthesized from other PUFAs such as EPA, DHA, α-linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6). Oxylipins have many biological functions, and several effects on the heart in health and disease have been shown [3, 4]. For example, ARA derived PGE_2 modulates cardiac contractility depending on which receptor it activates [5], 19-hydroxy-eicosatetraenoic acid (19-HETE) reduces cardiomyocyte hypertrophy [6], 20-HETE and epoxy-eicosatrienoic acids (EpETrEs) induce cardiac hypertrophy [7], EpETrEs prevent lipopolysaccharides (LPS)-induced cardiac dysfunction [8] and 5-, 12-, and 15-HETEs induce cellular hypertrophy in human ventricular cardiomyocytes [9]. Oxylipins derived from other PUFAs also have effects on the heart, such as EPA derived 18-HEPE, which is anti-inflammatory in cardiac fibroblasts [10], and DHA derived 13,14-EpDPE, which potently dilates porcine coronary microvessels [11]. Little is known about the existence and functions of heart oxylipins derived directly from 18-carbon PUFAs (LA and ALA), although there is some evidence that LA affects heart function indirectly by altering ARA derived oxylipins [12]. LA derived oxylipins such as 13-hydroxy-octadecadienoic acid have been shown to attenuate platelet adhesion to endothelial cells [13], and dihydroxy-octadecenoic acids may induce oxidative stress and proinflammatory events in vascular endothelial cells [14], but direct effects of LA oxylipins on the heart have not been reported. Furthermore, there is little information on the heart oxylipin profile in the literature [15].

Thus, the first objective of the current study was to provide a comprehensive oxylipin profile of the rat heart.

Dietary PUFA effects on oxylipins in different tissues have historically been predicted based on their effects on tissue PUFA composition. However, comparisons of PUFA and oxylipin profiles in rat kidney, liver and adipose reveal that oxylipins do not always reflect their parent PUFA levels. Further, tissue oxylipin levels are not only modulated by dietary alterations in their direct fatty acid precursors [16-21], but they also can be altered by dietary fatty acids that are not their direct precursors, such as ARA oxylipins being reduced in the heart by dietary n-3 fatty acids [22], or being increased by dietary LA [12]. Thus, the second objective of the current study was to examine the effect of differing dietary oils on the heart oxylipin profile.

Oxylipin profiling in the heart also may be relevant to further understanding of sex differences in the normal and diseased heart [4]. Previous oxylipin profiling studies demonstrated higher levels of renal, hepatic and serum oxylipins in male rats [23, 24]. In several adipose depots, sex differences depended on the diet: for example, rats provided a diet rich in DHA had higher adipose oxylipins in males, while those provided diets enriched in either ALA or EPA had higher oxylipins in females [21]. There is little data on sex differences in heart oxylipins, but a few studies indicate that some individual ARA derived oxylipins may be higher in females in vascular tissue [25, 26]. Therefore, the third objective of the current study was to examine sex differences in the heart oxylipin profile.

To achieve these three objectives, female and male rats were provided diets that differed only in oil composition so that each test diet compared to control had higher levels of specific PUFA. Rats were used since collection of hearts from humans consuming specific diets is not

possible, and rats are a good model of human lipid metabolism for a whole body approach although lipoprotein metabolism differs between humans and rats [27].

3.3 Materials and methods

3.3.1 *Animals and Diets*

A total number of 72 female (36) and male (36) 3 week old Sprague-Dawley weanling rats were randomly provided six different diets as described in detail [21, 23, 24] and in appendix A, which describe diet and sex effects on kidney, liver, serum and adipose oxylipins in these rats. Briefly, the diets were based on the standard AIN93G diet but had 10g oil instead of 7g oil per 100g diet. Oil blends were designed for each diet so that the control diet had adequate levels of the essential fatty acids, ALA and LA, and high levels of MUFA. In the test diets, MUFA were replaced with oils high in ALA, EPA, DHA and LA so that these diets contained 3g (per 100g diet) more of each of these PUFAs, respectively, compared to the control diet. The LA+ALA diet also contained 3g more LA per 100g diet, plus had additional ALA so that the LA/ALA ratio was similar to the control diet. The percentage of energy from LA (4.6% to 11.8% of total energy) and ALA (0.6% to 7.6% of total energy) in all diets except the ALA diet is within the range of human consumption patterns. The higher amounts of ALA, EPA and DHA in the ALA, EPA and DHA diets, respectively, compared to control, were matched with the increased amount of LA in the LA compared to control diet so that direct comparisons could be made between PUFA. However, the levels of these n-3 PUFA in the ALA, EPA and DHA diets are not commonly achieved in the human population. The levels of unsaturated and saturated fatty acids were similar across all diets. All the components of diet were purchased from Dyets Inc (Bethlehem, PA) except the antioxidant (tert-butylhydroquinone), which was from Sigma-Aldrich (Oakville, ON), and purified EPA and DHA, which were from Larodan (Solna, Sweden).

The rats were housed individually with a 12 hour light/dark cycle, had free access to diet, were weighed weekly and the dietary intervention was carried out for all rats during the same six

week period. Rats were anesthetized using isofluorane before termination and harvested heart tissues were weighed, immediately frozen in liquid nitrogen and stored at -80^{0}C until analysis. Animal sacrifice took place between 9:00 am to 3:00 pm in a random order. The stage of the estrous cycle was not examined. All animal procedures were performed according to the Canadian Council for Animal Care guidelines and approved by the University of Manitoba Animal Care Committee, see appendix I.

3.3.2 *Oxylipin analysis*

Hearts were rinsed with ice-cold Tyrode's salt (pH 7.6) solution to remove blood before homogenization (appendices B and H) of the whole heart in fresh Tyrode's solution. An optimum amount of heart homogenate extract (400 µL containing 70 mg tissue) for oxylipin quantification was determined from a dose response curve (appendix D), and analysis of oxylipins was performed by HPLC/MS/MS as described [1, 23]. Briefly, deuterated internal standards (Cayman Chemicals, MI) were added to sample homogenate extracts containing antioxidants and adjusted to pH <3.0. Strata-X-SPE (Phenomenex, CA) columns preconditioned with methanol followed by pH 3.0 water were used for solid phase extraction. After column loading and washing, oxylipins were eluted with 100% methanol. Samples obtained were dried and re-suspended in the mobile phase (water/acetonitrile/formic acid, 70/30/0.02 v/v/v) for oxylipin analysis by HPLC/MS/MS (QTRAP 6500; Sciex, ON). For details oxylipin extraction protocol see, appendix C. Quantification of oxylipins was performed using the stable isotope dilution method [28]. Oxylipins screened but below the level of detection (<3 times above baseline) and those detected but below the level of quantification (>3 to <5 times above baseline) are provided in online Supplementary Table S3.1a and b, respectively. However, if an oxylipin

was quantifiable in samples in at least one group, but not in all other groups, these were quantified; those that were not detected in the other groups were then ascribed a value of zero. Further details of all oxylipins scanned, mass transitions, internal standards, standard curve slopes and retention times are provided in [1, 23] (appendix F).

3.3.3 *Fatty acid analysis*

Total lipids were extracted from 250 µL of the heart homogenate and fatty acids were analyzed as described [19, 29] (appendix G). Briefly, after solvent extraction of total lipids, total phospholipid (PL) and neutral lipid (NL) were separated by TLC (heptane/isopropyl ether/acetic acid, 60/40/3, v/v/v) [30]. Fatty acids were methylated using methanolic H_2S0_4, extracted in hexane and analyzed by GC as described [31, 32].

3.3.4 *Statistical analysis*

With an *n*=6 rats in each diet/sex group (total of 72 rats), this study had a power of 0.8 to detect an effect size of 0.45 at a significance level of $P<0.05$ (G*Power Software version 3.1.9.2). Statistical analysis was performed using SAS Software Version 9.3 (SAS Institute Inc, NC). The Shapiro-Wilk Test was performed to test for normality, followed by 1- or 2-way ANOVA if data were distributed normally. The Kruskal-Wallis test was performed when data were not normal even after transformation. Tukey's test was used for post hoc analyses. Observations greater than the mean ± 3SD of a group were removed as outliers. All data are reported as mean ± SEM.

3.4 Results

3.4.1 *General findings*

At the end of the feeding period there were no differences in body or heart weights between the dietary treatments. Heart and body weights were higher in males, while heart weights relative to body weight were higher in females (Supplementary Table S3.2). Out of 164 oxylipins scanned, 75 were detected and quantified in the rat hearts. Approximately two-thirds of the oxylipins were derived from n-6 PUFAs; two-thirds of these were formed from ARA. Approximately half of the remaining n-3 PUFA derived oxylipins were formed from DHA, one-third were from EPA and one-fifth were from ALA (Supplementary Tables S3.3 and S4).

The proportions of oxylipin mass did not necessarily reflect PUFA mass proportions in either the PL or NL fractions. For example, in the control group, the proportion of LA and ARA oxylipins was ~ two-thirds and one-third, respectively, while the proportion of LA and ARA was almost equally split between these two PUFA in the PL fraction, and skewed more towards LA in the NL fraction (Fig. 3.1(a)). Further, the distributions of oxylipin and PUFA mass also were different with the different dietary oil treatments; examples of the DHA and LA groups are shown in Fig. 3.1(b) and (c), respectively; values for all diets can be found in Supplementary Table S3.5.

3.4.2 *Effects of dietary PUFA on n-3 derived oxylipins*

Compared to the control group, ALA and DHA oxylipins were higher only in hearts from rats given diets that were higher in their specific precursor PUFA (Fig. 3.2(a); Supplementary Table S3.4). On the other hand, EPA oxylipins were higher not only in EPA rats, but also in rats given

ALA and DHA diets. ALA and DHA diets did not increase EPA oxylipins as much as with the EPA diet, however. The LA+ALA diet with higher levels of ALA than the control diet, but lower levels than in the ALA diet, also increased ALA and EPA oxylipins when compared to the control diet, but fewer oxylipins were affected and the effect was smaller than in hearts from rats given the ALA diet. The LA diet did not affect the n-3 oxylipin levels in the rat heart, except for 13-hydroxy-octadecatrienoic acid.

To compare the levels of oxylipins to their PUFA precursor, the fatty acid compositions of the PL and NL fractions were analyzed (Fig. 3.2(c); Supplementary Tables S3.6 and S7). Similar to oxylipin effects, n-3 PUFA in heart PL were increased in rats provided their specific precursor PUFA (Fig. 3.2(c); Supplementary Table S3.6). However, effects on other n-3 PUFA did not necessarily reflect oxylipin changes. For example, dietary EPA significantly reduced DHA in the PL fraction, even though all DHA oxylipins (except 13-hydroxy-docosahexaenoic acid) were not different in rats given this diet. The results of the NL PUFA analyses were similar to the PL results – i.e. n-3 PUFA in heart NL also were generally higher in rats provided their specific precursor PUFA, but other n-3 PUFA were not significantly altered, even if their oxylipins were higher (Fig. 3.2(c); Supplementary Table S3.7).

3.4.3 Effects of dietary PUFA on n-6 derived oxylipins

Compared to the control group, the LA diet increased 6 out of 10 LA derived oxylipins, with the remaining LA oxylipins following the same trend (Fig. 3.2(b); Supplementary Table S3.4**)**. The LA diet also increased the one oxylipin derived from gamma-linolenic acid, but very few other n-6 oxylipins were altered by dietary LA; only 1 of 3 dihomo-gamma-linolenic acid (DGLA) and 1 of 30 ARA oxylipins were higher, and only in female hearts. The LA+ALA diet had a similar

level of LA as the LA diet, but also had a higher level of ALA compared to the control diet to achieve the same LA/ALA ratio as the control diet. N-6 oxylipin levels in rats given this diet were similar to those given the LA diet, although there were fewer differences between the LA+ALA and the control diet than was the case for the LA compared to control diet.

Higher levels of dietary n-3 PUFA, however, resulted in lower levels of ARA derived oxylipins, with little effect on oxylipins derived from other n-6 PUFA (LA, DGLA) (Fig. 3.2(b); Supplementary Table S3.4). The effect of individual dietary n-3 PUFA on ARA oxylipins differed, with DHA having the greatest effect: 25 out of 30 ARA oxylipins quantified were lower in hearts from DHA compared to control rats, while only 9 and 10 were lower in hearts from rats given the ALA and EPA diets, respectively.

As was the case with the n-3 PUFA, the changes in n-6 PUFA in the PL and NL sometimes reflected n-6 oxylipin changes (e.g. ARA in the PL fraction in hearts from rats given EPA and DHA diets), but other times did not (e.g. ARA in the PL and NL fractions in hearts from rats given ALA diets followed the same lowering trend, but were not significantly different) (Fig. 3.2(a) and (c)). Another example of how oxylipin levels can differ from their precursor PUFA levels is illustrated by the higher levels of some ARA derived PG metabolites (i.e. 15-deoxy-PGD_2 and 15-keto-PGE_2) in hearts from rats given the EPA diet, while 12 other ARA oxylipins were lower (Fig. 3.2(b)).

3.4.4 *Sex effects in oxylipin and PUFA levels*

Previous studies in these rats showed that males generally had higher levels of oxylipins in kidney and liver, except for the DHA derived oxylipins in kidney, which were generally higher in females [23]. Hence sex differences in oxylipins were examined in the heart. These analyses

revealed that all 27 oxylipins with a main sex effect were higher in females, and that 21 of the 22 with a sex by diet interaction had higher oxylipins in females in at least one group. Only TxB_2 was higher in males given the LA+ALA diet (Supplementary Table S3.4). However, sex differences were often not consistent across all groups, even within significant main effects. To further examine these interaction effects, the ratio of female to male oxylipin mass was calculated for each oxylipin, and the effect of diet on this ratio was tested. This analysis showed an interaction between diet and oxylipins derived from different PUFA, so the effect of diet on the female/male ratio was examined separately for oxylipins grouped by their PUFA precursor. These analyses confirmed the generally higher levels of oxylipins in females (i.e. female/male ratios were predominantly greater than 1) and further demonstrated that the female/male ratio differed with different diets. Dietary ALA resulted in the highest female/male ratio (>2) for all oxylipin groups, and dietary DHA was always among the lowest (0.5-1.5), with the remaining groups being in between (Fig. 3.3).

Sex effects on PUFA, however, were not consistent with sex effects on oxylipins. Main effects of sex were observed in three PUFA that were oxylipin precursors in the PL fraction: LA and ALA were higher in males, DHA was higher in females. DGLA in the PL fraction also was higher in males, but only in rats given the control and ALA diets. In the NL fraction, the only oxylipin precursor with a sex effect was EPA, which was higher in females, but only in rats given the EPA diet. Details are provided in online Supplementary Tables S3.6 and S3.7.

3.4.5 *Oxylipin to PUFA ratios*

Oxylipin to PUFA ratios were calculated to compare the relative levels of oxylipins with their precursor PUFA in the PL fraction. In almost all cases, these ratios were higher in females

compared to males, reflecting the higher oxylipin levels in females. With few exceptions, oxylipin to PUFA ratios were higher for n-3 compared to n-6 PUFA, and the order of oxylipin to PUFA ratios by chain length was 18-carbon ≥20-carbon ≥22-carbon PUFA. A representative figure of these data is shown in Fig. 3.4 and data for all ratios can be found in Supplementary Table S3.8.

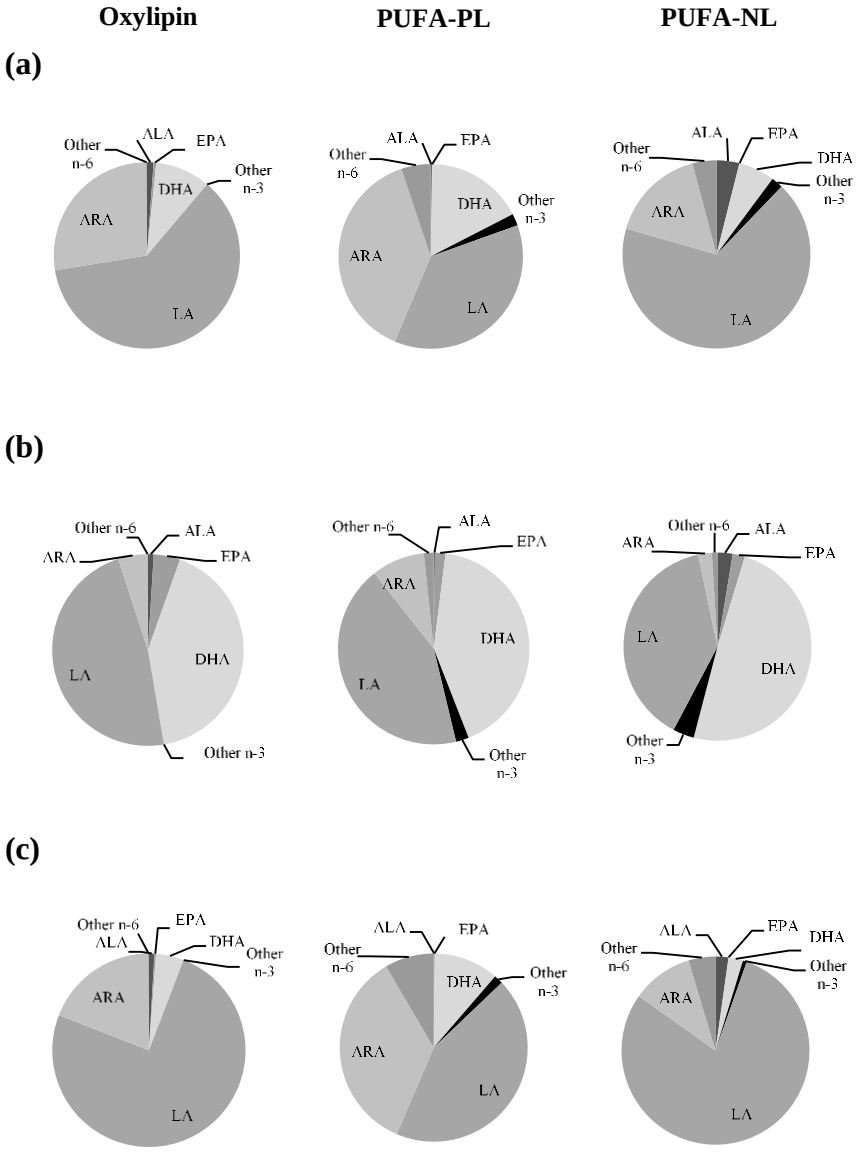

Fig. 3.1 Distribution of heart oxylipin and PUFA mass in PL + NL fractions in rats provided the control (a), DHA (b) and LA (c) diets. Data shown are for combined data from female and male rats. Separate female and male data for all diet groups are provided in online Supplementary Table S3.5. ARA, arachidonic acid; ALA, α-linolenic acid; LA, linoleic acid; NL, neutral lipid; PL, phospholipid.

(a) N-3 Oxylipins

Diet	ALA		EPA		DHA		LA		LA+ALA	
Sex	F	M	F	M	F	M	F	M	F	M
ALA Oxylipins										
9-HOTrE										
9-OxoOTrE										
13-HOTrE										
9,10-DiHODE										
12,13-EpODE										
12,13-DiHODE										
EPA Oxylipins										
Δ^{17}-6k-PGF$_{1\alpha}$										
5-HEPE										
8-HEPE										
9-HEPE										
12-HEPE										
15-HEPE										
14,15-EpETE										
17,18-EpETE										
18-HEPE										
DHA Oxylipins										
4-HDoHE										
7-HDoHE										
8-HDoHE										
10-HDoHE										
11-HDoHE										
13-HDoHE										
14-HDoHE										
16-HDoHE										
17-HDoHE										
PDX										
15*t*-PD1										
RvD5										
16,17-EpDPE										
19,20-EpDPE										
19,20 DiHDoPE										
20-HDoHE										

(b) N-6 Oxylipins

Diet	ALA		EPA		DHA		LA		LA+ALA	
Sex	F	M	F	M	F	M	F	M	F	M
LA Oxylipins										
9-HODE										
9-OxoODE										
13-HODE										
13-OxoODE										
9,10,13-TriHOME										
9,12,13-TriHOME										
9,10-EpOME										
9,10-DiHOME										
12,13- EpOME										
12,13-DiHOME										
GLA Oxylipins										
13-HOTrEγ										
DGLA Oxylipins										
PGF$_{1\alpha}$										
8-HETrE										
15-HETrE										
ARA Oxylipins										
PGD$_2$										
15d-PGD$_2$										
PGJ$_2$										
6k-PGF$_{1\alpha}$										
PGE$_2$										
15k-PGE$_2$										
TxB$_2$										
12-HHTrE										
5-HETE										
5-OxoETE										
5,15-DiHETE										
8-HETE										
9-HETE										
11-HETE										
12-HETE										
12-OxoETE										
15-HETE										
15-OxoETE										
6*t*,12epi-LTB$_4$										
LTB$_4$										
5,6-EpETrE										
5,6-DiHETrE										
8,9-EpETrE										
8,9-DiHETrE										
11,12-EpETrE										
11,12-DiHETrE										
14,15-EpETrE										
14,15-DiHETrE										
16-HETE										
18-HETE										

(c) Precrursor PUFA

Diet	ALA		EPA		DHA		LA		LA+ALA	
Sex	F	M	F	M	F	M	F	M	F	M
N-3 PUFA										
ALA - PL										
- NL										
EPA - PL										
- NL										
DHA - PL										
- NL										
N-6 PUFA										
LA - PL										
- NL										
GLA - PL										
- NL										
DGLA - PL										
- NL										
ARA - PL										
- NL										

Legend

Shading of relative differences in PUFA and oxylipins is as follows:

- higher than control + 1 other group
- higher than control + 2 or 3 other groups
- higher than all other groups
- lower than control + 1 other group
- lower than control + 2 or 3 other groups
- lower than all other groups

Fig. 3.2 Relative differences in heart n-3 oxylipins (a) and n-6 oxylipins (b), and their precursor PUFA (c) in rats provided ALA, EPA, DHA, LA and LA+ALA diets compared to control diets. N= (5-6) for each diet group; statistical analysis was carried out by 2-way ANOVA followed by Tukey's post hoc test. Cells are only colored when there is a significant statistical difference at ($P< 0.05$). Means, SEMs, p values and complete statistical analysis of diet, sex and interaction effects are provided in Supplementary Tables S3.3, S3.4, S3.6 and S3.7. ARA, arachidonic acid; ALA, α-linolenic acid; d, deoxy; DGLA, dihomo-gamma-linolenic acid; DiHDoPE, dihydroxy-docosapentaenoic acid; DiHETE, dihydroxy-eicosatetraenoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; DiHODE, dihydroxy-octadecadienoic acid; DiHOME, dihydroxy-octadecenoic acid; EpDPE, epoxy-docosapentaenoic acid; EpETE, epoxy-eicosatetraenoic acid; EpETrE, epoxy-eicosatrienoic acid; EpODE, epoxy- octadecadienoic acid; EpOME, epoxy-octadecenoic acid; GLA, gamma-linoleic acid; HDoHE, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HETrE, hydroxy-eicosatrienoic acid; HHTrE, hydroxy-heptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; k, keto; LA, linoleic acid; LT, leukotriene; NL, neutral lipid; oxoETE, oxo-eicosatetraenoic acid; oxoODE, oxo-octadecadienoic acid; oxoOTrE, oxo- octadecatrienoic acid; PD, protectin; PL, phospholipid; Rv, resolvin; *t*, trans; TriHOME, trihydroxy-octadecenoic acid; T_X, thromboxane; γ, gamma.

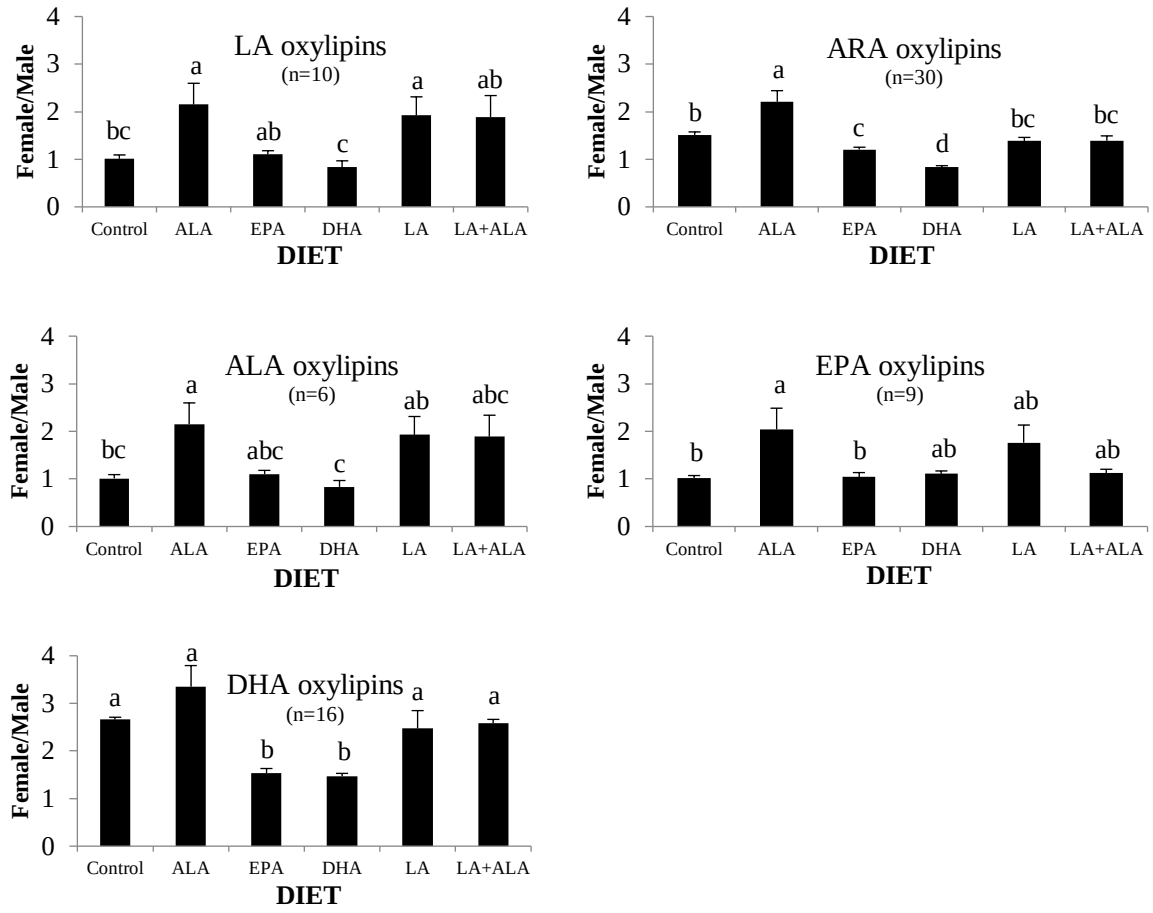

Fig. 3.3 Effect of diet on sex differences in oxylipin levels. The data obtained as the ratio of female/male from the mean individual oxylipin levels for females and males in each diet group using 1-way-ANOVA followed by Tukey's post hoc test, and the effect of diet was tested on ratios from oxylipins grouped by their PUFA precursor. Ratios with differing letters are significantly different from each other at ($P < 0.05$). ALA, α-linolenic acid; LA, linoleic acid.

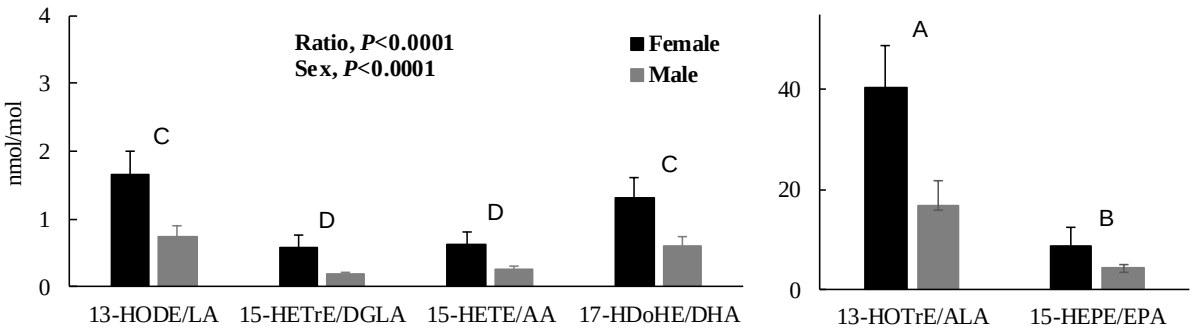

Fig. 3.4 Heart oxylipin to PUFA ratios for the 15-lipoxygenase enzyme in rats provided the LA diet. Statistical analysis was carried out using 2-way ANOVA followed by Tukey's post hoc test. Ratios with differing letters are significantly different from each other at ($P < 0.05$). All ratios for the 5-, 12-, 15-lipoxygenase, cytochrome P450 hydroxylase and epoxygenase enzymes for all diets are provided in Supplementary Table S3.8.

Supplementary Table S3.1a Oxylipins scanned but below the level of detection (0 to <3 times baseline)

ARA Oxylipins

2,3-dinor-11β-$PGF_{2\alpha}$; 2,3-dinor-TxB2; 5,6-dihydroxy eicosatetraenoic acid; 5-hydroxy eicosatrienoic acid; 5-iso-$PGF_{2\alpha VI}$; 6,15-diketo-13,14-dihydro $PGF_{1\alpha}$; 6k-PGE_1; 6-LXA_4; 6-trans-LTB_4; 11β-dihydroketo-$PGF_{2\alpha}$; 11β-PGE_2; 11β-$PGF_{2\alpha}$; 11-dehydro-TxB2; 12-epi-LTB_4; 12-oxo-LTB_4; 14,15-LTC_4 (EXC_4); 14,15-LTD_4 (EXD_4); 14,15-LTE_4 (EXE_4); 15-deoxy-PGA_2; 15-deoxy-PGJ_2; 15k-$PGF_{2\alpha}$; 19-HETE; 19oh -PGE_2; 19oh -$PGF_{2\alpha}$; 20-carboxy-LTB_4; 20-HETE; 20oh-LTB_4; 20oh-PGE_2; 20oh-$PGF_{2\alpha}$; bicyclo-PGE_2; dihydro-$PGF_{2\alpha}$; dihydroketo-PGD_2; dihydroketo-PGE_2; Hepoxilin A_3; Hepoxilin B_3; LTC_4; LTD_4; LTE_4; LXA_5; LXB_4; PGA_2; PGB_2; PGK_1; PGK_2; tetranor-12-hydroxy-eicosatetraenoic acid; tetranor-PGD metabolite; tetranor-PGE metabolite; tetranor-PGF metabolite.

Other Oxylipins

2,3-dinor 8-iso-$PGF_{2\alpha}$; 8,15-dihydroxy-eicosatetraenoic acid; 8-iso-15k-$PGF_{2\beta}$; 8-iso-$PGF_{2\alpha III}$; 8-iso-$PGF_{3\alpha}$; 9,10-EpODE; 9-Nitrooleate; 10-Nitrooleate; 11-hydroxy-eicosapentaenoic acid; 13-oxo-octadecatrienoic acid; 15,16-dihydroxy-octadecadienoic acid; 15,16-epoxy-octadecadienoic acid; 15-oxo-eicosadienoic acid; 17-hydroxy-eicosatetraenoic acid; 17k-DHA; 17k-DPA; dihomo-15-deoxy-PGD_2; dihomo-PGD_2; dihomo-PGE_2; dihomo-$PGF_{2\alpha}$; dihomo-PGJ_2; Protectin D_1; PGE_1; $PGF_{3\alpha}$; RvD_1; RvD_2; RvE_1; TxB_1; TxB_3.

Supplementary Table S3.1b Oxylipins detected but below the level of quantification (>3 to <5 times baseline)

2,3-dinor-6k-$PGF_{1\alpha}$; 7-Maresin-1; 15k-PGD_2; 15k-$PGF_{1\alpha}$; 15-LXA_4; 20-carboxy-ARA; Dhk-$PGF_{2\alpha}$; PGD_1; PGD_3; $PGE_{3;}$ $PGF_{2\alpha}$.

EX, eoxin; k, keto; LT, leukotriene; LX, lipoxilin; oh, hydroxy; PG, prostaglandin; Rv, resolvin; Tx, thromboxane.

Supplementary Table S3.2 Heart weights at termination in rats given control, ALA, EPA, DHA, LA and LA+ALA diets for 6 weeks

Diet	Control		ALA		EPA		DHA		LA		ALA+LA		P Value		
Sex	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Diet	Sex	Int.[#]
Heart, g	1.09±0.04[b]	1.47±0.02[a]	1.03±0.04[b]	1.52±0.05[a]	0.99±0.05[b]	1.56±0.02[a]	1.12±0.04[b]	1.45±0.02[a]	0.99±0.03[b]	1.53±0.05[a]	1.03±0.04[b]	1.49±0.06[a]			0.0411
Heart/Body, mg/g	3.44±0.12	2.89±0.06	3.41±0.10	3.07±0.08	3.23±0.05	3.04±0.06	3.29±0.06	3.15±0.05	3.41±0.05	3.11±0.11	3.19±0.12	3.02±0.09	0.37	<0.0001	

Data were analyzed by 2-way ANOVA when normally distributed and by Kruskal-Wallis when not, followed by Tukey's post hoc test. Mean values within a row with differing superscript letters are significantly different ($P<0.05$). Only significant P values were reported. P value for sex effect is shaded in pink when oxylipins are higher in female hearts. [#]Int. represents interaction between diet and sex ($P<0.05$). Body weight data have been published in references 23 and 24. ALA, α-linolenic acid; LA, linoleic acid.

Supplementary Table S3.3 Diet and sex effects on heart n-3 oxylipins in rats given control, ALA, EPA, DHA, LA and LA+ALA diets for 6 weeks

Diet	Control		ALA		EPA		DHA		LA		LA+ALA		P value		
Sex	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Diet	Sex	Int.[#]
							ng/g								
ALA Oxylipins															
9-HOTrE	60.6±9.62^{C}	62.8±11.6	1015±118^{A}	650±95.8	85.9±14.6^{C}	63.9±11.0	99.8±11.6^{C}	87.2±20.4	116±5.25^{C}	57.5±8.38	200±40.6^{B}	156±37.9	<0.0001	0.0018	
9-oxoOTrE	106±16.6^{cd}	125±24.8b^{cd}	1428±155^{a}	887±130^{a}	127±24.4^{bcd}	139±22.2^{bcd}	84.2±14.1^{d}	119±14.4^{bcd}	174±25.1^{bcd}	83.0±12.6^{d}	289±67.4^{b}	259±52.3^{bc}			0.0251
13-HOTrE	54.9±7.77^{D}	55.8±11.2	1056±117^{A}	765±149	84.9±8.01^{D}	51.8±8.71	70.7±6.80^{CD}	79.9±13.8	151±14.4^{BC}	81.8±13.5	188±22.6^{B}	157±40.9	<0.0001	0.0022	
9,10-DiHODE[†]	0.32±0.06	0.23±0.03	0.36±0.06	0.21±0.04	0.50±0.16	0.33±0.06	0.21±0.04	0.43±0.10	0.46±0.13	0.38±0.06	0.72±0.23	0.38±0.08	0.20	0.33	
12,13-EpODE	2.84±0.83^{c}	2.55±0.59^{c}	65.9±5.84^{a}	15.5±1.84^{b}	6.74±2.02^{bc}	3.26±0.48^{c}	3.68±0.69^{c}	3.82±1.01^{c}	3.57±1.13^{c}	2.10±0.51^{c}	9.48±2.79^{bc}	6.10±1.68^{c}			<0.0001
12,13-DiHODE	0.29±0.03^{C}	0.29±0.04	2.14±0.23^{A}	1.29±0.13	0.30±0.04^{C}	0.31±0.05	0.47±0.11^{BC}	0.32±0.06	0.45±0.05^{BC}	0.41±0.03	0.65±0.09^{B}	0.45±0.05	<0.0001	0.0188	
EPA Oxylipins															
Δ^{17}-6k-PGF$_{1\alpha}$	0.51±0.16^{C}	0.53±0.12	2.86±0.49^{B}	2.64±0.69	14.2±1.64^{A}	18.1±2.45	2.54±0.49^{B}	1.91±0.39	0.53±0.14^{C}	0.30±0.06	0.90±0.39^{C}	0.79±0.21	<0.0001	0.52	
5-HEPE	27.8±3.92^{C}	29.7±5.71	515±110^{B}	323±33.9	3840±282^{A}	3121±565	364±17.0^{B}	353±57.0	30.4±6.02^{C}	22.5±1.83	40.5±4.72^{C}	39.3±3.56	<0.0001	0.06	
8-HEPE	14.0±2.14^{CD}	13.6±3.42	170±23.1^{B}	127±17.4	931±18.3^{A}	915±169	153±9.69^{B}	133±20.0	10.57±1.82^{D}	14.8±1.78	19.8±1.52^{C}	18.8±2.66	<0.0001	0.46	
9-HEPE	34.8±6.12^{D}	31.9±4.17	656±141^{B}	503±93.4	3340±129^{A}	4017±386	459±48.7^{B}	474±81.7	57.1±11.3^{CD}	27.3±5.38	61.7±11.5^{C}	53.6±6.93	<0.0001	0.12	
12-HEPE	8.22±1.13^{D}	7.04±1.60	129±15.5^{B}	99.0±13.3	749±43.4^{A}	572±134	114±9.40^{B}	101±14.6	8.35±1.48^{D}	5.22±0.77	13.0±1.57^{C}	12.4±2.12	<0.0001	0.0041	
15-HEPE	4.49±0.49^{D}	4.69±0.93	103±10.9^{B}	94.9±16.8	745±41.4^{A}	782±103	94.9±11.5^{B}	96.8±14.8	6.16±0.87^{D}	5.26±0.71	11.4±1.29^{C}	9.38±1.82	<0.0001	0.33	
14,15-EpETE	_¥^{b}	_^{b}	2.06±0.48^{b}	0.52±0.11^{b}	24.8±8.13^{a}	15.8±4.07^{a}	1.40±0.32^{b}	1.51±0.40^{b}	_^{b}	_^{b}	_^{b}	_^{b}			<0.0001[$]
17,18-EpETE	0.28±0.06^{d}	0.39±0.06^{d}	5.18±0.93^{b}	1.53±0.42^{c}	38.5±8.43^{a}	40.9±7.90^{a}	3.04±0.69^{bc}	3.06±0.42^{bc}	0.20±0.05^{d}	0.16±0.04^{d}	0.39±0.06^{d}	0.33±0.07^{d}			0.0093
18-HEPE	4.42±0.54^{d}	3.79±0.25^{d}	102±10.8^{b}	44.7±4.89^{c}	871±57.9^{a}	1230±218^{a}	86.8±12.3^{bc}	59.7±12.5^{bc}	3.86±0.67^{d}	0.93±0.26^{e}	5.36±0.92^{d}	3.34±0.33^{d}			0.0002
DHA Oxylipins															
4-HDoHE	585±86.9^{B}	236±40.3	694±99.1^{B}	194±22.7	416±45.1^{B}	253±50.9	2417±246^{A}	2008±268	277±45.9^{B}	182±37.0	503±110^{B}	198±47.3	<0.0001	<0.0001	
7-HDoHE	318±34.7^{bcd}	119±9.91^{e}	443±42.6^{b}	163±28.6^{de}	266±19.8^{bcd}	190±43.8^{cde}	1558±174^{a}	998±121^{a}	390±67.3^{bc}	97.4±18.4^{e}	369±62.8^{bc}	163±35.7^{de}			0.0245
8-HDoHE	180±35.8^{B}	96.8±31.0	307±30.0^{B}	93.8±19.6	149±13.9^{B}	124±30.7	960±114^{A}	534±62.9	202±48.2^{B}	81.5±24.0	227±42.3^{B}	92.6±26.5	<0.0001	<0.0001	
10-HDoHE	165±24.4^{c}	86.4±34.8^{c}	234±29.2^{c}	97.1±19.9^{c}	139±13.7^{c}	91.6±25.5^{c}	838±81.6^{a}	440±33.4^{b}	229±46.4^{c}	83.4±22.4^{c}	189±35.7^{c}	85.8±28.9^{c}			0.0005
11-HDoHE	170±23.2^{cd}	86.4±27.0^{d}	288±40.7^{c}	89.2±18.8^{d}	139±14.6^{cd}	94.3±24.6^{d}	934±68.1^{a}	620±90.2^{b}	200±30.9^{cd}	65.2±17.8^{d}	207±45.2^{cd}	77.3±13.7^{d}			0.0158
13-HDoHE	134±14.8^{bc}	40.2±2.85^{e}	199±25.1^{b}	75.4±14.9^{cde}	125±14.3^{bc}	120±34.2^{bcd}	714±109^{a}	511±63.0^{b}	112±21.3^{bcd}	48.5±3.12^{de}	158±35.8^{bc}	50.8±7.97^{de}			0.0101
14-HDoHE	126±17.9^{cdef}	36.0±4.00^{h}	196±25.3^{bc}	55.1±5.23^{fgh}	132±23.9^{cde}	78.1±18.2^{defgh}	579±71.8^{a}	416±23.2^{ab}	108±23.5^{cdefg}	55.3±3.95^{efgh}	195±43.2^{bcd}	50.6±11.0^{gh}			0.0081
16-HDoHE	206±22.9^{BC}	67.6±8.11	306±44.9^{B}	83.1±7.17	189±29.5^{BC}	102±22.8	914±130^{A}	661±65.7	151±28.2^{C}	53.8±10.0	197±46.4^{BC}	81.0±13.8	<0.0001	<0.0001	
17-HDoHE	668±66.3^{B}	235±30.0	1049±134^{B}	357±44.2	645±94.6^{B}	467±119	3186±472^{A}	2403±198	556±94.1^{B}	259±22.5	666±157^{B}	279±36.2	<0.0001	<0.0001	
PDX	11.3±2.28^{cd}	4.69±0.75^{d}	15.9±1.50^{bc}	8.13±1.59^{cd}	10.6±1.49^{cd}	7.59±2.03^{cd}	47.5±3.54^{a}	27.0±2.20^{b}	15.2±3.50^{c}	8.46±1.90^{cd}	10.6±1.22^{cd}	9.04±2.56^{cd}			0.0034
15*t* PD1[†]	3.44±0.44^{cd}	1.41±0.26^{d}	5.70±0.64^{c}	2.22±0.40^{d}	3.55±0.42^{cd}	2.16±0.52^{d}	16.3±0.56^{a}	9.75±0.98^{b}	3.60±0.69^{cd}	1.87±0.50^{d}	3.20±0.32^{cd}	2.05±0.54^{d}			0.0001
RvD5[†]	2.49±0.45^{B}	0.82±0.19	3.51±0.34^{B}	1.31±0.21	2.15±0.46^{B}	1.36±0.26	8.86±0.85^{A}	7.30±1.59	3.07±0.76^{B}	0.93±0.24	1.96±0.28^{B}	1.41±0.39	<0.0001	<0.0001	
16,17-EpDPE	40.6±6.53^{B}	12.7±1.51	45.1±11.0^{BC}	8.26±1.36	34.6±9.72^{B}	18.6±4.23	143±22.9^{A}	113±19.9	13.1±2.50^{C}	7.28±2.27	34.8±10.8^{BC}	11.3±2.27	<0.0001	<0.0001	
19,20-EpDPE	4.04±0.81^{B}	1.11±0.20	4.17±1.02^{B}	0.56±0.15	2.60±0.97^{B}	1.04±0.39	9.92±1.98^{A}	7.36±1.82	2.69±1.14^{B}	0.69±0.35	4.24±1.10^{B}	0.85±0.25	<0.0001	<0.0001	
19,20-DiHDoPE	4.29±0.45^{b}	2.20±0.27^{cd}	4.09±0.41^{b}	2.13±0.18^{cd}	3.46±0.24^{bc}	2.64±0.46^{bcd}	19.9±1.39^{a}	21.6±0.98^{a}	2.45±0.36^{bcd}	2.02±0.34^{cd}	3.56±0.62^{bc}	1.85±0.25^{d}			0.0409
20-HDoHE	312±48.5^{cd}	137±10.9^{degf}	419±62.7^{bc}	118±11.1^{fg}	314±54.2^{cd}	283±54.7^{cde}	1392±189^{a}	751±84.6^{ab}	225±20.3^{cdef}	84.5±10.6^{g}	423±80.5^{bc}	127±12.5^{efg}			0.0047

Data were analyzed by 2-way ANOVA when normally distributed and by Kruskal-Wallis when not, followed by Tukey's post hoc test. Different upper-case superscripts on the female values within a row indicate significant main effects of diet (P< 0.05). Different lower-case superscripts within a row indicate simple effect differences between means (P< 0.05). Only significant P values were reported. P values for sex effects are shaded pink when levels are higher in female hearts. [#]Int. represents interaction between diet and

sex unless noted with superscript $ which denotes that P values were obtained from the Kruskal-Wallis test because the data were not normally distributed. †Denotes no primary standard, so not quantified. ¥Denotes not detected. ALA, alpha-linolenic acid; DiHDoPE, dihydroxy-docosapentaenoic acid; DiHODE, dihydroxy-octadecadienoic acid; EpDPE, epoxy-docosapentaenoic acid; EpETE, epoxy-eicosatetraenoic acid; EpODE, epoxy- octadecadienoic acid; HDoHE, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HOTrE, hydroxy-octadecatrienoic acid; LA, linoleic acid; oxoOTrE, oxo- octadecatrienoic acid; PD, protectin; Rv, resolvin.

Supplementary Table S3.4 Diet and sex effects on the heart n-6 oxylipins in rats given control, ALA, EPA, DHA, LA and LA+ALA diets for 6 weeks

Diet	Control		ALA		EPA		DHA		LA		LA+ALA		P value		
Sex	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Diet	Sex	Int.[#]
							ng/g								
LA Oxylipins															
9-HODE	2428±391	2114±392	3747±494	1651±180	3782±815	2570±383	2446±347	3247±681	5176±1266	3131±702	5275±1265	3557±873	0.07	0.0117	
9-oxoODE	945±213^{abc}	1037±413^{abc}	1120±157^{abc}	594±209^{bc}	1762±535^{abc}	1265±249^{abc}	400±88.9^{c}	904±191^{abc}	3243±1032^{a}	615±154^{abc}	2469±801^{ab}	1313±420^{abc}			0.0157
13-HODE	1076±119^{bc}	1090±201^{bc}	1547±157^{bc}	972±111^{c}	1686±213^{abc}	1326±189^{bc}	945±120^{c}	1180±94.1^{bc}	3456±497^{a}	1820±374^{abc}	2168±276^{ab}	2357±612^{ab}			0.0476
13-oxoODE	6294±672^{BC}	5921±1252	7660±475^{BC}	5947±1105	9385±2063^{ABC}	7581±963	3419±463^{C}	5982±757	18486±3433^{A}	8421±1786	12496±3214^{AB}	8712±2886	0.0004	0.0259	
9,10,13-TriHOME	1238±237^{C}	1210±241	1774±274^{BC}	2174±279	1321±206^{C}	1447±346	2121±391^{AB}	3248±474	3302±344^{A}	2513±406	3290±293^{A}	3043±669	<0.0001	0.65	
9,12,13-TriHOME	534±140^{e}	1184±138^{bcde}	1150±143^{cde}	1576±218^{abcde}	889±145de	1086±125^{cde}	1002±184^{cde}	2028±267^{abcd}	2683±277^{a}	1991±247^{abcd}	2188±263^{abc}	2468±671^{ab}			0.0313
9,10-EpOME	13.9±3.44^{b}	13.0±2.76^{b}	23.3±2.25^{b}	5.33±1.78^{b}	14.5±4.83^{b}	16.3±2.65^{b}	13.0±2.63^{b}	16.0±4.99^{b}	20.8±6.45^{b}	17.3±3.91^{b}	61.7±4.17^{a}	13.7±2.58^{b}			<0.0001
9,10-DiHOME	4.11±0.68^{B}	3.58±0.29	3.99±0.43^{B}	2.34±0.36	3.80±0.33^{B}	3.87±0.39	3.69±0.14^{B}	4.48±0.90	9.70±1.04^{A}	7.15±1.03	6.41±1.11^{A}	6.45±1.30	<0.0001	0.07	
12,13-EpOME	4.55±0.91^{bcd}	5.21±1.17^{bcd}	9.88±0.92^{b}	2.00±0.75^{d}	6.19±1.98^{bcd}	7.47±1.29^{bcd}	5.67±1.28^{bcd}	2.92±0.42^{cd}	9.71±2.32^{bc}	5.72±1.18^{bcd}	26.1±1.36^{a}	5.74±1.11^{bcd}			<0.0001
12,13-DiHOME	4.68±0.67^{BC}	3.26±0.28	4.00±0.59^{C}	2.09±0.42	4.42±0.53^{BC}	3.61±0.37	3.60±0.27^{C}	3.51±0.76	8.07±0.69^{A}	6.07±0.88	6.42±1.06^{AB}	5.51±1.11	<0.0001	0.0043	
GLA Oxylipins															
13-HOTrEγ	6.82±0.71^{BC}	5.44±0.90	6.83±0.29^{C}	3.71±0.47	3.58±0.20^{D}	2.43±0.42	3.09±0.30^{D}	3.02±0.38	14.3±2.23^{A}	8.49±1.01	11.1±1.50^{AB}	6.14±1.25	<0.0001	<0.0001	
DGLA Oxylipins															
$PGF_{1\alpha}$	0.76±0.11	0.78±0.11	0.42±0.07	0.54±0.11	0.76±0.16	0.58±0.04	0.67±0.09	0.56±0.06	0.75±0.11	0.59±0.09	0.69±0.09	0.63±0.08	0.11	0.29	
8-HETrE	17.1±1.76^{bc}	13.5±3.19^{c}	24.2±3.34^{abc}	15.5±2.83^{bc}	21.1±2.69^{abc}	17.8±3.48^{abc}	11.5±1.23^{c}	14.6±2.87^{bc}	31.7±3.10^{a}	18.1±2.61^{abc}	27.9±4.01^{ab}	15.1±2.93^{bc}			0.0493
15-HETrE	5.44±0.87^{bc}	4.07±0.29^{c}	6.39±0.82^{bc}	4.17±0.55^{c}	9.45±1.51^{ab}	4.50±1.19^{c}	3.93±0.58^{c}	4.23±0.86^{c}	7.96±1.32^{abc}	6.00±1.09^{bc}	11.8±1.87^{a}	3.88±0.35^{c}			0.0029
ARA Oxylipins															
PGD_2	11.7±0.90^{A}	9.82±1.33	6.13±0.78^{B}	4.01±0.73	6.01±1.36^{B}	5.28±0.61	1.96±0.28^{C}	2.59±0.61	10.1±1.06^{A}	8.58±1.04	7.73±1.29^{A}	10.7±0.67	<0.0001	0.40	
15d-PGD_2	1.36±0.15^{bcd}	1.00±0.15^{cd}	1.10±0.11^{cd}	0.56±0.09^{cd}	2.54±0.41^{ab}	2.66±0.50^{a}	0.37±0.10^{d}	0.53±0.09^{cd}	1.57±0.34^{abc}	0.94±0.18^{cd}	1.05±0.18^{cd}	0.57±0.08^{cd}			<0.0001[$]
PGJ_2	2.87±0.34^{ab}	1.91±0.27^{bc}	1.82±0.35^{bc}	1.97±0.30^{bc}	0.40±0.07^{c}	0.37±0.13^{c}	2.26±0.28^{ab}	3.82±0.51^{a}	2.65±0.44^{ab}	2.66±0.49^{ab}	2.09±0.26^{abc}	1.92±0.27^{bc}			0.0298
6k-$PGF_{1\alpha}$	16.6±2.09^{A}	17.0±1.82	8.84±2.29^{B}	6.29±1.30	2.52±0.55^{C}	3.35±0.62	1.99±0.53^{C}	1.51±0.27	21.4±2.74^{A}	25.1±4.71	14.8±1.73^{A}	20.8±2.81	<0.0001	0.73	
PGE_2	5.63±0.46^{A}	4.08±0.43	3.20±0.26^{B}	1.73±0.21	4.02±0.37^{A}	3.56±0.32	1.17±0.17^{C}	1.35±0.21	4.26±0.75^{A}	3.78±0.59	4.64±1.10^{A}	3.63±0.63	<0.0001	0.0159	
15k-PGE_2	4.21±0.46^{B}	2.51±0.33	2.44±0.22^{BC}	1.45±0.22	5.72±1.15^{A}	4.33±0.70	1.17±0.17^{C}	2.19±0.46	3.25±0.32^{BC}	2.68±0.48	3.12±0.64^{BC}	2.23±0.31	<0.0001	0.0159	
TxB_2	1.83±0.16^{ab}	1.99±0.12^{a}	1.13±0.05^{cde}	0.68±0.06^{de}	0.72±0.13^{de}	0.63±0.05^{e}	–¥^{b}	–^{b}	1.67±0.12^{abc}	2.27±0.07^{a}	1.28±0.14^{bcd}	2.11±0.27^{a}			<0.0001[$]
12-HHTrE	616±77.6^{A}	581±42.2	405±32.6^{B}	392±54.9	218±31.8^{C}	260±40.0	90.7±21.0^{C}	128±10.4	715±38.0^{A}	575±49.1	550±53.0^{A}	589±98.7	<0.0001	0.70	
5-HETE	662±39.8^{A}	460±74.5	516±52.1^{B}	230±36.4	365±60.2^{B}	264±51.1	119±14.5^{C}	147±34.8	782±90.5^{A}	448±73.8	583±102^{AB}	366±76.5	<0.0001	<0.0001	
5-oxoETE	367±46.3^{A}	199±29.1	231±35.0^{AB}	82.4±16.4	291±69.6^{A}	216±47.7	42.1±8.82^{B}	47.2±9.51	216±41.6^{AB}	160±35.1	242±84.5^{A}	174±53.3	0.0002	0.0035	
5,15-DiHETE	6.62±1.09^{ab}	4.46±1.05^{abc}	5.97±0.81^{ab}	1.55±0.35^{c}	4.73±1.21^{abc}	3.54±0.66^{bc}	1.92±0.39^{c}	1.69±0.65^{c}	8.49±0.58^{a}	3.24±0.65^{bc}	4.15±1.08^{abc}	5.01±1.12^{abc}			0.0050
8-HETE	1286±143^{ab}	999±179^{bcd}	1083±98.1^{bc}	554±94^{bcd}	733±121^{bcd}	591±141^{bcd}	243±37.3^{d}	306±67.5^{cd}	2035±351^{a}	1053±172^{bc}	1353±210^{ab}	858±160^{bcd}			0.0488
9-HETE	623±58.4^{ab}	363±66.4^{bc}	513±29.5^{b}	299±53.3^{bc}	349±44.7^{bc}	304±86.2^{bc}	118±18.7^{c}	154±31.9^{c}	958±109^{a}	509±99.3^{b}	620±105^{ab}	421±81.6^{bc}			0.0229
11-HETE	1578±139^{b}	1067±209^{bcde}	1404±131^{bc}	728±120^{cde}	1071±194^{bcd}	649±177^{cde}	280±50.5^{e}	381±94.7^{de}	2504±277^{a}	1141±211^{bcd}	1436±238^{bc}	972±196^{bcde}			0.0063
12-HETE	139±20.7^{AB}	87.2±12.8	91.7±7.38^{B}	47.3±6.32	89.8±18.5^{B}	61.2±14.1	26.6±3.54^{C}	29.7±7.26	144±27.6^{A}	107±17.8	94.4±23.1^{AB}	75.8±16.0	<0.0001	0.0036	
12-oxoETE	34.0±3.85^{AB}	23.0±4.11	21.3±2.05^{ABC}	22.8±2.84	14.0±3.11^{D}	15.1±4.69	6.94±1.60^{D}	6.25±1.10	42.3±8.28^{A}	24.9±6.74	16.0±2.80^{BCD}	17.4±5.53	<0.0001	0.11	
15-HETE	648±55.2^{AB}	375±43.7	541±70.3^{AB}	266±35.6	444±89.1^{BC}	269±58.1	129±22.5^{C}	184±40.0	661±119^{A}	500±85.4	551±104^{AB}	385±55.4	<0.0001	0.0001	
15-oxoETE	559±50.1^{A}	438±102	342±22.6^{BC}	232±50.5	348±74.6^{AB}	348±89.1	102±32.0^{C}	149±27.1	433±60.1^{AB}	301±65.4	366±112^{ABC}	234±80.1	<0.0001	0.07	
6t,12epi-LTB_4	10.4±1.71^{A}	5.16±0.96	8.99±1.23^{A}	3.76±0.92	7.65±1.76^{A}	4.22±1.00	1.29±0.47^{B}	2.21±0.80	10.3±2.62^{A}	6.64±1.59	5.57±1.82^{AB}	4.07±1.31	0.0002	0.0005	
LTB_4	9.91±1.40^{a}	5.73±1.00^{abc}	8.11±0.80^{ab}	3.13±0.60^{bcd}	5.09±0.85^{abc}	5.11±1.28^{abc}	1.69±0.24^{d}	2.36±0.38^{cd}	7.66±1.18^{ab}	5.76±1.38^{abc}	8.66±2.23^{ab}	4.08±1.11^{abcd}			0.0324
5,6-EpETrE	4.70±0.67^{b}	3.44±0.63^{bcd}	3.97±0.94^{bc}	0.56±0.06^{d}	2.42±0.54^{bcd}	2.44±0.51^{bcd}	1.15±0.27^{cd}	1.26±0.31^{cd}	2.83±0.72^{bcd}	1.90±0.34^{bcd}	8.17±1.43^{a}	2.83±0.50^{bcd}			0.0003
5,6-DiHETrE	13.0±1.27^{A}	9.34±0.89	7.53±0.48^{CD}	5.13±0.84	6.76±1.13^{CD}	5.66±0.80	3.66±0.42^{D}	3.86±0.40	9.65±1.04^{AB}	9.95±1.43	8.12±0.54^{BC}	8.27±1.04	<0.0001	0.05	

8,9-EpETrE	14.4±3.11^{A}	8.03±1.05	13.0±2.61^{A}	3.47±0.85	11.2±3.50^{A}	7.37±1.92	2.75±0.44^{B}	3.16±0.58	7.52±1.46^{A}	6.44±1.07	12.7±3.77^{A}	6.88±1.44	<0.0001	0.0019	
8,9-DiHETrE	1.17±0.13^{A}	0.97±0.16	0.73±0.08^{B}	0.41±0.08	0.47±0.11^{BC}	0.35±0.11	0.17±0.09^{C}	0.21±0.07	1.07±0.11^{A}	0.93±0.14	1.21±0.21^{A}	0.97±0.15	<0.0001	0.0284	
11,12-EpETrE	7.49±1.66^{A}	3.73±0.41	5.84±1.15^{AB}	1.47±0.44	4.86±1.54^{A}	3.00±0.65	1.37±0.24^{B}	1.49±0.35	5.26±1.61^{A}	3.25±0.56	6.26±1.83^{A}	3.25±0.62	<0.0001	0.0007	
11,12-DiHETrE	2.16±0.18^{A}	1.60±0.19	1.36±0.11^{BC}	0.78±0.10	1.11±0.13^{BC}	0.97±0.08	0.61±0.12^{C}	0.64±0.09	1.77±0.12^{A}	1.89±0.29	1.36±0.09^{AB}	1.64±0.30	<0.0001	0.17	
14,15-EpETrE	22.9±5.75^{a}	8.58±1.42^{cd}	12.3±2.78^{abc}	2.79±0.89^{cd}	5.81±1.40^{cd}	7.21±1.47^{cd}	2.70±0.61^{d}	3.30±0.98^{cd}	9.90±1.59^{bcd}	7.00±1.50^{cd}	19.5±3.93^{ab}	6.76±1.58^{d}			0.0007
14,15-DiHETrE	6.70±0.74^{A}	4.46±0.25	3.84±0.38^{B}	2.19±0.28	3.40±0.41^{B}	2.87±0.30	1.69±0.34^{B}	1.64±0.28	4.55±0.48^{A}	4.91±0.69	5.05±0.91^{A}	4.87±0.80	<0.0001	0.0217	
16-HETE	52.4±5.10^{A}	32.4±1.66	31.3±3.47^{BC}	24.5±2.70	23.4±3.07^{CD}	20.7±2.43	13.6±1.57^{D}	17.3±1.47	53.9±9.17^{A}	35.9±4.08	41.2±5.70^{AB}	34.4±5.84	<0.0001	0.0080	
18-HETE	1.35±0.11	1.05±0.13	1.09±0.16	1.04±0.27	1.18±0.52	1.32±0.32	0.87±0.22	1.03±0.34	1.67±0.34	1.85±0.26	1.39±0.31	1.08±0.08	0.10	0.87	

Data were analyzed by 2-way ANOVA when normally distributed and by Kruskal-Wallis when not, followed by Tukey's post hoc test. Different upper-case superscripts on the female values within a row indicate significant main effects of diet ($P < 0.05$). Different lower-case superscripts within a row indicate simple effect differences between means ($P < 0.05$). Only significant P values were reported. P values for sex effects are shaded pink when levels are higher in female hearts. $^{\#}$Int. represents interaction between diet and sex unless noted with superscript $^{\$}$ which denotes that P values were obtained from the Kruskal-Wallis test because the data were not normally distributed. $^{¥}$Denotes not detected. ALA, alpha-linolenic acid; d, deoxy; DGLA, dihomo-gamma-linolenic acid; DiHETE, dihydroxy-eicosatetraenoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; DiHOME, dihydroxy-octadecenoic acid; EpETrE, epoxy-eicosatrienoic acid; EpOME, epoxy-octadecenoic acid; GLA, gamma-linoleic acid; HETE, hydroxy-eicosatetraenoic acid; HETrE, hydroxy-eicosatrienoic acid; HHTrE, hydroxy-heptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; k, keto; LA, linoleic acid; LT, leukotriene; oxoETE, oxo-eicosatetraenoic acid; oxoODE, oxo-octadecadienoic acid; TriHOME, trihydroxy-octadecenoic acid; Tx, thromboxane; γ, gamma.

Supplementary Table S3.5 Distributions of heart oxylipins and PUFA mass in rats provided the control, ALA, EPA, DHA, LA and LA+ALA diets for 6 weeks

Diet	Sex	Oxylipin /PUFA	% of total oxylipins /PUFA						
			ALA	EPA	DHA	Other n-3	LA	ARA	Other n-6
Control	Female	Oxylipin	1.00	0.42	13.0		55.8	29.8	0.13
		PUFA-PL	0.18	0.04	19.5	2.40	34.0	38.9	5.04
		-NL	3.26		9.18	1.85	62.2	19.5	4.08
	Male	Oxylipin	1.31	0.49	6.19		66.9	25.1	0.13
		PUFA-PL	0.26	0.10	14.8	1.77	39.8	38.1	5.25
		-NL	4.60		3.31	2.16	72.3	13.5	4.14
ALA	Female	Oxylipin	11.2	5.31	13.3		53.6	16.6	0.12
		PUFA-PL	2.64	2.07	19.9	7.35	41.4	25.0	1.70
		-NL	33.1	2.33	5.79	5.05	44.9	8.01	0.88
	Male	Oxylipin	11.2	5.78	6.51		62.4	14.1	0.12
		PUFA-PL	3.19	1.76	14.1	7.11	48.2	23.9	1.74
		-NL	32.6	1.55	4.48	5.24	47.3	7.78	1.11
EPA	Female	Oxylipin	0.84	29.1	7.08		52.0	11.1	0.10
		PUFA-PL	0.37	9.89	12.3	13.8	42.9	19.5	1.29
		-NL	3.94	16.7	3.38	9.42	60.1	5.12	1.29
	Male	Oxylipin	0.83	34.4	5.89		49.1	9.82	0.08
		PUFA-PL	0.40	10.1	10.1	12.5	44.5	21.2	1.30
		-NL	3.82	17.2	4.03	6.38	60.4	6.86	1.37
DHA	Female	Oxylipin	0.97	4.77	51.2		38.6	4.47	0.07
		PUFA-PL	0.14	1.67	45.8	2.16	40.7	8.19	1.38
		-NL	2.84	2.43	39.4	3.92	48.7	1.69	1.01
	Male	Oxylipin	0.99	4.19	32.6		56.8	5.42	0.08
		PUFA-PL	0.28	1.89	38.3	2.20	45.6	9.76	1.90
		-NL	2.37	1.77	59.3	3.41	29.2	3.02	0.90
LA	Female	Oxylipin	0.93	0.24	5.18		75.7	18.0	0.12
		PUFA-PL	0.08	0.01	13.5	1.44	41.9	34.7	8.43
		-NL	2.32		2.50	0.54	81.1	9.33	4.21
	Male	Oxylipin	0.91	0.31	4.16		74.7	20.0	0.13
		PUFA-PL	0.19	0.03	8.89	1.64	45.3	35.5	8.50
		-NL	2.12		2.51	0.80	77.8	11.7	5.06
LA+ALA	Female	Oxylipin	1.81	0.40	8.41		73.7	15.7	0.14
		PUFA-PL	0.45	0.08	18.9	6.01	39.2	31.4	4.02
		-NL	4.68	0.05	4.80	1.98	74.9	10.5	3.15
	Male	Oxylipin	2.09	0.50	4.45		77.7	15.3	0.10
		PUFA-PL	0.61	0.09	11.5	3.73	46.0	33.4	4.65
		-NL	4.85		3.27	1.03	76.2	11.2	3.41

ALA, α-linolenic acid; LA, linoleic acid; NL, neutral lipid; PL, phospholipid.

Supplementary Table S3.6 Diet and sex effects on heart PL PUFA in rats given control, ALA, EPA, DHA, LA and LA+ALA diets for 6 weeks

Diet	Control		ALA		EPA		DHA		LA		LA + ALA		P Value		
Sex	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Diet	Sex	Int.[#]
								µg/g							
SFA															
14:0	9.59±4.14^{B}	11.8±2.71	17.5±3.57^{A}	20.9±2.70	20.6±3.47^{A}	20.9±2.70	13.4±1.57^{A}	25.8±4.49	9.42±1.69^{AB}	17.6±3.90	17.7±2.85^{A}	19.2±4.93	0.0006	0.0057	
16:0	1049±123	1238±136	1077±117	1340±124	1150±108	1090±136	1094±36.5	1523±265	864±90.6	1156±86.4	1377±79.8	1346±120	0.05	0.0197	
18:0	2126±280^{ab}	2380±281^{ab}	2351±293^{ab}	2807±273^{ab}	2503±300^{ab}	2053±266^{ab}	1806±72.6^{b}	2354±369^{ab}	1947±181^{b}	2275±169^{ab}	3101±172^{a}	2765±196^{ab}			0.0130$^{\$}$
20:0	38.0±6.22^{AB}	44.4±4.53	35.8±4.53^{AB}	44.9±3.62	39.1±5.74^{B}	31.3±4.58	26.4±1.47^{B}	35.4±5.98	34.8±3.93^{AB}	41.2±3.26	50.2±2.71^{A}	50.0±3.33	0.0005	0.11	
22:0	33.8±5.94^{B}	31.7±4.59	41.9±6.00^{B}	39.9±2.60	45.4±8.16^{B}	32.6±5.60	32.3±1.95^{B}	36.4±6.50	37.0±3.72^{B}	36.4±2.86	69.0±2.29^{A}	52.1±4.07	<0.0001	0.14	
24:0	18.9±1.68^{B}	23.7±4.24	34.8±4.46^{A}	35.9±4.49	27.7±1.25^{B}	25.2±1.98	28.1±1.64^{B}	25.9±1.61	23.3±2.58^{B}	26.2±2.95	38.4±1.70^{A}	34.9±3.96	0.0001	0.97	
MUFA															
16:1t	6.06±1.99^{ab}	8.69±1.08^{a}	6.73±1.15^{ab}	7.96±0.90^{ab}	5.74±0.99^{ab}	3.55±0.79^{b}	5.26±0.39^{b}	5.82±1.47^{ab}	3.63±0.56^{b}	5.30±0.54^{ab}	6.00±0.74^{ab}	6.42±0.57^{ab}			0.0061$^{\$}$
16:1n7	19.2±2.95^{abc}	46.9±8.08^{a}	26.3±3.56^{abc}	41.4±5.81^{ab}	17.8±3.23^{bc}	21.8±4.27^{abc}	13.5±3.25^{cd}	27.5±8.96^{abc}	6.21±0.85^{d}	30.5±3.14^{abc}	25.2±2.65^{abc}	32.0±5.38^{abc}			0.0054$^{\$}$
18:1n9	542±111^{A}	726±84.1	423±44.7^{A}	628.±69.9	375±54.9^{BC}	346±52.3	244±11.8^{C}	311±58.3	245±28.5^{C}	357±29.8	465±25.7^{AB}	470±38.1	<0.0001	0.0044	
18:1n7	368±30.9^{A}	505±47.5	277±29.7^{ABC}	424±41.1	287±31.0^{BC}	318±35.7	207±7.57^{C}	334±59.2	233±23.0^{BC}	400±27.6	353±29.2^{AB}	463±42.9	<0.0001	<0.0001	
20:1n9	8.57±2.86^{abc}	12.3±2.63^{a}	6.49±1.37^{abc}	10.1±1.90^{ab}	5.80±1.93^{abc}	4.04±1.08^{bc}	2.19±0.26^{c}	5.06±1.77^{abc}	4.12±0.57^{bc}	6.61±0.50^{abc}	8.64±1.23^{abc}	8.92±1.64^{abc}			0.0005$^{\$}$
24:1n9	6.15±1.76^{bc}	15.8±2.74^{a}	7.52±1.59^{abc}	12.8±2.61^{ab}	5.11±1.72^{bc}	4.00±0.88^{c}	4.17±0.21^{c}	9.69±2.80^{abc}	6.14±0.76^{bc}	9.51±0.71^{abc}	7.75±0.79^{abc}	8.44±1.60^{abc}			0.0002$^{\$}$
N-3 PUFA															
18:3n3	9.42±4.14^{D}	15.8±4.00	151±23.6^{A}	232±28.1	22.9±5.78^{C}	21.7±5.13	6.67±1.11^{D}	16.3±5.25	3.47±0.37^{D}	10.2±4.05	35.7±2.76^{B}	41.9±4.04	<0.0001	0.0020	
20:3n3	2.82±0.85^{b}	4.07±1.22^{b}	16.8±2.98^{a}	19.2±2.96^{a}	6.02±1.86^{b}	2.27±0.53^{b}	2.73±0.30^{b}	3.55±0.65^{b}	2.65±0.26^{b}	2.46±0.47^{b}	7.26±1.04^{b}	6.36±1.48^{b}			<0.0001$^{\$}$
20:5n3	2.34±0.51^{b}	5.98±1.44^{b}	118.5±21.4^{b}	128±16.3^{b}	611±82.7^{a}	538±71.8^{a}	80.9±8.46^{b}	110±24.4^{b}	0.50±0.24^{b}	1.37±0.38^{b}	6.66±1.06^{b}	5.95±1.12^{b}			<0.0001$^{\$}$
22:5n3	123±60.5^{cd}	105±11.5^{cd}	404±78.0^{bcd}	498±145^{abc}	847±108^{a}	667±103^{ab}	102±3.25^{cd}	124±23.6^{cd}	60.2±5.10^{d}	87.3±8.44^{cd}	467±173^{abcd}	250±72.4^{bcd}			<0.0001$^{\$}$
22:6n3	1021±128^{B}	915±205	1138±130^{B}	1029±99.5	763±94.9^{C}	538±63.6	2213±108^{A}	2225±143	587±47.9^{C}	487±54.0	1494±171^{B}	790±85.2	<0.0001	0.0006	
N-6 PUFA															
18:2n6	1778±289^{B}	2456±220	2371±299^{AB}	3505±415	2651±312^{AB}	2378±268	1966±117^{B}	2647±405	1822±207^{B}	2478±164	3089±233^{A}	3164±280	0.0009	0.0015	
18:3n6	2.30±1.29^{ab}	4.56±2.43^{ab}	3.25±1.30^{ab}	7.67±2.16^{ab}	6.51±2.06^{ab}	6.52±1.05^{ab}	0.82±0.54^{b}	8.69±1.48^{ab}	2.19±0.72^{ab}	4.30±1.50^{ab}	5.07±1.91^{ab}	10.0±2.99^{a}			0.0128$^{\$}$
20:2n6	6.31±0.98^{B}	10.8±2.03	10.0±1.63^{B}	13.7±1.98	13.2±2.70^{B}	12.3±2.90	7.71±0.93^{B}	14.8±2.78	19.7±2.47^{A}	25.4±2.62	27.3±1.69^{A}	30.2±4.02	<0.0001	0.0095	
20:3n6	34.2±4.58^{AB}	55.2±7.75	47.5±6.57^{A}	69.2±7.47	36.3±5.05^{BC}	36.9±5.76	32.3±1.52^{BC}	44.6±6.90	25.4±2.71^{C}	33.5±2.25	49.3±3.03^{AB}	42.3±6.29	<0.0001	0.0055	
20:4n6	2036±252^{ab}	2351±252^{a}	1431±195^{bcd}	1740±163^{abcd}	1203±180^{cde}	1134±144^{def}	396±14.2^{f}	566±91.2^{ef}	1509±118^{bcd}	1943±145^{abcd}	2474±125^{a}	2300±183^{a}			<0.0001$^{\$}$
22:2n6	3.09±1.41^{ab}	24.2±9.19^{ab}	13.5±7.26^{ab}	10.1±2.21^{ab}	10.9±5.86^{ab}	5.53±1.69^{ab}	0.72±0.33^{b}	4.45±1.92^{ab}	5.56±1.49^{ab}	15.2±8.40^{ab}	18.3±10.4^{ab}	29.2±4.48^{a}			0.0216$^{\$}$
22:4n6	97.8±18.1^{b}	101±12.0^{b}	18.1±3.11^{c}	20.3±3.63^{c}	9.41±2.22^{c}	5.53±1.27^{c}	2.04±0.48^{c}	3.40±0.73^{c}	134±11.3^{ab}	151±9.53^{a}	122±10.0^{ab}	129±9.07^{ab}			<0.0001$^{\$}$
22:5n6	120±19.4^{bc}	131±21.2^{bc}	4.83±1.53^{f}	5.43±1.43^{ef}	3.11±0.69^{f}	2.82±0.64^{f}	23.0±1.28^{def}	34.4±5.80^{def}	180±26.3^{ab}	236±32.9^{a}	95.5±8.90^{cd}	78.8±9.14^{cde}			<0.0001$^{\$}$

Data were analyzed by 2-way ANOVA when normally distributed and by Kruskal-Wallis when not, followed by Tukey's post hoc test. Different upper-case superscripts on the female values within a row indicate significant main effects of diet ($P < 0.05$). Different lower-case superscripts within a row indicate (simple effect) differences between means ($P < 0.05$). Only significant P values were reported. P values for main sex effects are shaded blue and pink when oxylipins are higher in male and female hearts, respectively. [#]Int. represents interaction between diet and sex unless noted with superscript $ which denotes that P values were obtained from the Kruskal-Wallis test because data were not normally distributed. ALA, alpha-linolenic acid; LA, linoleic acid; SFA, saturated fatty acid.

Supplementary Table S3.7 Diet and sex effects on heart NL PUFA in rats given control, ALA, EPA, DHA, LA and LA+ALA diets for 6 weeks

Diet	Control		ALA		EPA		DHA		LA		LA + ALA		P Value		
Sex	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Diet	Sex	Int.[#]
							µg/g								
SFA															
16:0	_[¥d]	482±83.0[b]	_[d]	278±83.0[bcd]	234±96.8[bc]	_[d]	_[d]	_[d]	_[d]	_[d]	603±77.6[a]	349±65.4[b]			0.0003[$]
20:0	_[d]	3.06±1.27[b]	_[d]	1.45±0.72[c]	_[d]	_[d]	_[d]	_[d]	_[d]	_[d]	5.53±0.81[a]	2.94±0.81[b]			<0.0001[$]
22:0	0.21±0.21[b]	0.58±0.49[b]	0.44±0.35[b]	0.77±0.34[b]	2.07±0.94[ab]	_[b]	0.64±0.41[b]	0.14±0.14[b]	1.42±0.88[ab]	0.70±0.39[b]	3.77±0.86[a]	1.50±0.46[ab]			0.0069[4]
MUFA															
16:1t	6.04±1.10[A]	8.84±1.27	6.85±1.85[AB]	4.95±0.54	4.70±0.79[B]	2.52±0.60	4.36±0.40[B]	2.95±1.70	4.36±1.19[AB]	4.82±0.63	6.75±0.58[AB]	6.58±1.40	0.0044	0.55	
16:1n7	13.59±3.83[B]	27.4±4.64	26.8±5.19[B]	35.7±6.57	21.4±4.57[B]	23.9±8.24	22.0±5.27[B]	9.28±4.65	21.5±8.61[B]	32.2±10.1	36.4±5.95[A]	70.8±14.3	<0.0001	0.0284	
18:1n9	327±98.1[ab]	238±143[ab]	403±86.3[ab]	378±66.5[ab]	276±62.1[ab]	176±69.1[b]	236±86.6[ab]	159±80.7[b]	201±80.1[ab]	251±51.7[ab]	552±61.1[a]	423±79.7[ab]			0.0216[$]
18:1n7	30.3±13.1[b]	53.4±9.38[a]	_[c]	15.1±9.13[bc]	_[c]	_[c]	_[c]	_[c]	15.2±8.86[bc]	15.4±2.95[bc]	19.6±5.25[b]	20.0±8.44[b]			0.0002[$]
20:1n9	4.33±1.16[abc]	5.47±1.52[ab]	3.36±0.94[abc]	4.80±1.18[ab]	2.22±0.61[bc]	1.89±1.09[bc]	0.65±0.21[c]	1.66±1.13[bc]	1.54±0.39[bc]	3.36±0.34[abc]	6.82±0.74[a]	5.41±0.34[ab]			0.0001[$]
N-3 PUFA															
18:3n3	6.67±2.27[b]	13.7±2.31[b]	148±22.6[a]	133±21.3[a]	19.8±3.61[b]	10.0±1.47[b]	11.2±2.95[b]	9.22±3.61[b]	12.1±2.79[b]	8.77±0.84[b]	37.3±3.39[b]	24.5±3.22[b]			<0.0001[$]
20:3n3	0.11±0.07[d]	0.22±0.05[d]	1.51±0.09[b]	3.35±0.13[a]	0.20±0.07[d]	0.16±0.06[d]	0.27±0.07[d]	_[d]	0.14±0.06[d]	0.19±0.06[d]	0.80±0.04[c]	0.62±0.10[c]			<0.0001
20:5n3	018±0.18[c]	0.29±0.26[c]	8.02±1.39[c]	8.38±1.26[c]	89.9±7.60[a]	58.5±2.35[b]	9.60±1.00[c]	11.0±4.40[c]	1.44±0.78[c]	0.12±0.12[c]	0.53±0.15[c]	0.17±0.13[c]			<0.0001[$]
22:5n3	3.67±1.66[b]	4.19±2.00[b]	19.7±3.73[ab]	25.7±4.93[ab]	47.1±7.60[a]	46.6±16.3[a]	15.2±0.65[b]	21.1±7.34[ab]	2.58±0.61[b]	3.85±1.59[b]	15.0±3.42[b]	5.73±1.74[b]			0.0026[$]
22:6n3	18.8±3.06[bc]	12.0±2.91[c]	25.9±5.08[bc]	24.2±3.64[bc]	17.0±1.91[bc]	13.7±4.54[c]	155±5.47[a]	367±66.7[a]	13.0±0.96[c]	12.7±2.40[c]	38.2±1.43[b]	19.7±4.42[bc]			0.0023[$]
N-6 PUFA															
18:2n6	127±39.4[d]	261±40.6[bcd]	201±29.1[cd]	256±60.0[bcd]	302±61.2[bcd]	206±41.6[cd]	192±17.9[cd]	181±43.7[cd]	421±86.8[abc]	393±56.2[abcd]	597±43.3[a]	460±74.4[ab]			<0.0001[$]
18:3n6	_[b]	_[b]	_[b]	_[b]	_[b]	_[b]	_[b]	_[b]	_[b]	_[b]	2.78±0.90[a]	2.74±1.10[a]			<0.0001[$]
20:2n6	1.02±0.26[C]	2.50±0.74	1.65±0.62[C]	1.58±0.37	2.65±0.71[C]	1.62±0.58	1.17±0.26[C]	1.43±0.49	3.75±0.98[B]	4.61±0.70	6.39±0.38[A]	5.66±0.78	<0.0001	0.72	
20:3n6	1.64±0.57	3.58±1.41	2.06±0.96	4.17±1.24	3.33±0.96	2.76±1.05	1.43±0.33	2.26±0.91	3.83±1.14	3.57±0.67	6.53±0.79	4.10±0.86			0.0514[$]
20:4n6	39.8±8.89[bcde]	48.6±10.0[abcd]	35.9±6.50[bcde]	42.1±9.67[bcde]	25.7±4.59[cde]	23.4±8.69[cde]	6.65±2.26[e]	18.7±9.48[de]	48.5±6.66[abcd]	59.2±11.1[abc]	83.6±4.66[a]	67.9±10.6[ab]			<0.0001[$]
22:4n6	3.43±0.80[de]	4.32±0.98[cd]	0.23±0.09[f]	0.28±0.10[f]	0.50±0.30[ef]	0.28±0.19[f]	0.20±0.03[f]	0.08±0.05[f]	9.80±1.03[a]	9.93±0.76[a]	7.70±0.76[ab]	6.52±0.81[bc]			<0.0001[$]
22:5n6	2.25±0.27[bc]	3.45±1.37[bc]	_[c]	_[c]	_[c]	_[c]	1.20±0.40[bc]	1.80±0.88[bc]	4.51±1.37[ab]	7.43±1.15[a]	1.69±0.51[bc]	1.55±0.45[bc]			<0.0001[$]

Data were analyzed by 2-way ANOVA when normally distributed and by Kruskal-Wallis when not, followed by Tukey's post hoc test. Different upper-case superscripts on the female values within a row indicate significant main effects of diet ($P < 0.05$). Different lower-case superscripts within a row indicate simple effect differences between means ($P < 0.05$). Only significant P values were reported. P values for main sex effects are shaded blue when oxylipins are higher in male hearts. [#]Int. represents interaction between diet and sex unless noted with superscript [$] which denotes that P values were obtained from the Kruskal-Wallis test because data were not normally distributed. [¥]Not detected. ALA, alpha-linolenic acid; LA, linoleic acid; SFA, saturated fatty acid.

Supplementary Table S3.8 Heart oxylipin to PUFA ratios for enzymes in rats provided control, ALA, EPA, DHA, LA and LA+ALA diets for 6 weeks

a. 5-Lipoxygenase

Ratio	5-HETE/ARA		9-HODE/LA		9-HOTrE/ALA		5-HEPE/EPA		7-HDoHE/DHA		P Value	
Sex	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Ratio	Sex
Diet	nmol/mmol											
Control	281±43.2^{C}	193±31.7	1264±189^{B}	889±201	7550±1128^{A}	4308±1588	10407±2844^{A}	5572±1240	265±40.7^{C}	185±36.4	<0.0001	0.0009
ALA	379±61.8^{C}	133±28.2	1750±434^{B}	483±87.9	5541±935^{A}	2874±570	3534±340^{A}	2489±307	406±69.0^{C}	154±24.1	<0.0001	<0.0001
EPA	311±65.6^{D}	231±47.6	1412±386^{C}	1092±225	3827±1140^{B}	2349±402	6414±805^{A}	4612±771	360±55.1^{D}	335±72.1	<0.0001	0.0440
DHA	284±31.3^{D}	302±97.1	1213±191^{C}	1381±411	16110±2918^{A}	6890±2134	4499±491^{B}	3441±635	675±72.3^{C}	762±301	<0.0001	0.0316
LA	530±116^{c}	224±39.4^{c}	2679±580^{c}	1204±277^{c}	29931±4426^{a}	10749±3025^{b}	–¥	15019±2965^{b}	577±117^{c}	253±54.7^{c}	<0.0001$	
LA+ALA	235±49.1^{C}	211±53.2	1754±480^{B}	1102±254	5734±1441^{A}	3558±746	6352±1024^{A}	8923±1774	254±54.8^{C}	261±79.8	<0.0001	

b. 12-Lipoxygenase

Ratio	12-HETE/ARA		12-HEPE/EPA		14-HDoHE/DHA		P value	
Sex	Female	Male	Female	Male	Female	Male	Ratio	Sex
Diet	nmol/mmol							
Control	56.2±4.12^{C}	36.1±3.93	2503±304^{A}	1494±166	103±17.6^{B}	59.4±2.05	<0.0001	<0.0001
ALA	79.6±15.8^{C}	26.7±4.29	1129±142^{A}	745±83.0	181±31.9^{B}	67.3±14.1	<0.0001	<0.0001
EPA	76.3±18.9^{C}	53.2±12.6	1252±160^{A}	995±202	176±37.1^{B}	144±49.8	<0.0001	
DHA	63.6±7.66^{C}	56.9±19.5	1391±157^{A}	956±159	253±34.3^{B}	128±24.2	<0.0001	0.0066
LA	129±39.4^{C}	53.6±9.78	9079±2851^{A}	3792±843	257±77.8^{B}	122±26.8	<0.0001	0.0024
LA+ALA	59.4±16.6^{B}	42.7±10.2	2170±483^{A}	2121±326	102±32.6^{B}	104±36.3	<0.0001	

c. 15-Lipoxygenase

Ratio	15-HETE/ARA		13-HODE/LA		15-HETrE/DGLA		13-HOTrE/ALA		15-HEPE/EPA		17-HDoHE/DHA		P value	
Sex	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Ratio	Sex
Diet							nmol/mmol							
Control	277±46.9^{D}	189±29.2	574±33.4^{C}	445±88.0	159±25.5^{D}	92.1±15.2	7406±1497^{A}	4671±1505	2190±708^{B}	1152±232	560±85.3^{C}	480±100	<0.0001	0.0009
ALA	394±67.9^{D}	152±26.6	714±167^{C}	277±42.7	170±36.0^{E}	59.0±8.53	7493±1507^{A}	3285±658	1041±164^{B}	703±103	954±158^{BC}	343±46.0	<0.0001	<0.0001
EPA	381±96.0^{E}	305±86.7	626±100^{CD}	569±119	236±51.5^{E}	167±51.1	4913±1308^{A}	2200±383	1231±126^{B}	1548±572	864±152^{BC}	713±187	<0.0001	0.0347
DHA	306±47.8^{C}	377±115	471±69.4^{C}	482±90.0	117±17.9^{D}	147±52.0	11575±1934^{A}	7651±2378	1146±150^{B}	1163±281	1381±199^{B}	1822±748	<0.0001	
LA	591±176^{D}	248±43.3	1577±315^{C}	696±147	382±80.6^{D}	177±38.9	38330±7955^{A}	22658±2457	8304±3333^{B}	4126±441	1243±303^{C}	582±126	<0.0001	<0.0001
LA+ALA	266±66.1^{E}	215±46.7	705±126^{C}	641±166	181±51.1^{E}	130±32.4	5231±881^{A}	4960±1378	1882±410^{B}	1920±358	439±130^{CD}	384±64.3	<0.0001	

d. Cytochrome P450-hydroxylase

Ratio	18-HETE/ARA		18-HEPE/EPA		20-HDoHE/DHA		P value	
Sex	Female	Male	Female	Male	Female	Male	Ratio	Sex
Diet				nmol/mmol				
Control	0.66±0.08^{C}	0.52±0.04	1364±92.7^{A}	1086±128	214±19.5^{B}	237±30.8	<0.0001	
ALA	0.78±0.17^{C}	0.74±0.18	945±166^{A}	378±40.8	384±76.9^{B}	122±18.9	<0.0001	0.0004
EPA	1.41±0.56^{C}	1.40±0.27	1600±215^{A}	1664±475	417±77.8^{B}	436±94.8	<0.0001	
DHA	2.07±0.52^{C}	2.11±0.89	914±141^{A}	746±152	555±93.1^{B}	375±147	<0.0001	
LA	1.11±0.25^{C}	0.90±0.11	4791±954^{A}	798±250	408±82.9^{B}	199±49.4	<0.0001	0.0004
LA+ALA	0.67±0.09^{C}	0.46±0.05	607±142^{A}	773±48.3	220±68.4^{B}	184±39.5	<0.0001	

e. Cytochrome P450-epoxygenase

Ratio	14,15-EpETrE/ARA		12,13-EpOME/LA		12,13-EpODE/ALA		14,15-EpETE/EPA		16,17-EpDPE/DHA		P value	
Sex	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Ratio	Sex
Diet	nmol/mmol											
Control	9.34±2.44^{c}	5.18±0.34^{c}	4.81±1.44^{c}	2.27±0.62^{c}	325±50.0^{a}	176±74.0^{b}	29.5±5.28^{bc}	–	28.6±5.13^{bc}	21.9±1.76^{bc}	<0.0001$^{\$}$	
ALA	8.96±2.34^{C}	1.78±0.72	4.97±1.63^{D}	0.69±0.35	239±38.1^{A}	64.0±11.7	19.5±4.81^{BC}	3.76±1.26	41.2±11.0^{B}	8.55±2.19	<0.0001	<0.0001
EPA	10.3±3.78^{C}	6.42±1.45	2.93±0.91^{D}	3.16±0.72	172±50.7^{A}	177±36.1	41.3±14.8^{B}	23.0±8.94	44.8±10.9^{B}	36.0±9.78	<0.0001	
DHA	7.29±1.29^{C}	3.40±1.40	3.43±0.62^{D}	1.11±0.33	887±86.5^{A}	475±124	15.3±3.23^{C}	7.80±3.06	62.2±10.1^{B}	42.5±13.6	<0.0001	<0.0001
LA	4.66±1.40^{C}	3.48±0.73	5.32±1.40^{C}	2.83±0.73	1126±247^{A}	398±140	–	–	19.5±5.36^{B}	8.85±1.22	<0.0001	0.0087
LA+ALA	6.83±1.97^{b}	3.32±0.81^{b}	7.58±1.57^{b}	2.70±0.58^{b}	276±86.0^{a}	145±40.0^{ab}	8.18±2.57^{b}	–	10.8±3.24^{b}	11.1±1.27^{b}	<0.0001$^{\$}$	

Data were analyzed by 2-way ANOVA when normally distributed and by Kruskal-Wallis when not, followed by Tukey's post hoc test. Different upper-case superscripts on the female values within a row indicate significant main effects of ratio ($P < 0.05$). Different lower-case superscripts within a row indicate simple effect differences between means ($P < 0.05$). Only significant P values were reported. P values for sex effects are shaded pink when ratios are higher in female hearts. P values centred between the ratio and sex columns denote interaction effects. $^{\$}$Denotes that P values were obtained from the Kruskal-Wallis test because data were not normally distributed. $^{¥}$Indicates zero value (not detected) for the numerator or denominator, therefore the ratio could not be calculated. ARA, arachidonic acid; ALA, α-linolenic acid; EpDPE, epoxy-docosapentaenoic acid; EpETE, epoxy-eicosatetraenoic acid; EpETrE, epoxy-eicosatrienoic acid; EpODE, epoxy- octadecadienoic acid; EpOME, epoxy-octadecenoic acid; HDoHE, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HETrE, hydroxy-eicosatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; LA, linoleic acid.

3.5 Discussion

The data reported herein provide fundamental data on the rat heart oxylipin profile and demonstrate how it is affected by sex and by dietary oils enriched in ALA, EPA, DHA and LA. Compared to kidney and liver in these rats, the distribution of n-6 and n-3 PUFA derived oxylipins was generally similar, with the exception of 11 detectable epoxygenated fatty acids (i.e. epoxy-octadecenoic acid, EpODE, EpETrE, epoxy-eicosatetraenoic acid, EpDPE) in the heart, compared to one in the kidney and three in the liver in these same rats [23]. These CYP derived oxylipins also have been reported in a study of mouse heart oxylipins [15]. CYP epoxygenase enzymes are highly expressed in heart [33, 34], and their epoxygenated products appear to be cardioprotective, especially in post-ischemic states, with roles such as vasodilation via activation of Ca^{2+}-sensitive K^{+} channels, mitochondrial protection of cardiomyocytes, anti-apoptotic and pro-survival effects, and decreased cardiac fibrosis and inflammation effects [35-37].

Altering the dietary oil content markedly changed the heart oxylipin profiles, as would be expected from the effects on their fatty acid precursors, and from what has been observed in other tissues in these rats [21, 23, 24]. The effects of individual n-3 PUFA could be evaluated because each diet was only enriched in one specific n-3 PUFA compared to the control diet. As in the other tissues in these rats, n-3 oxylipins were higher in rats provided the diets rich in their own individual PUFA precursor fatty acid. The heart compared to other tissues, however, was much more resistant to changes in oxylipins derived from other n-3 PUFA. EPA oxylipins were higher in hearts from rats provided the EPA, as well as the ALA and DHA diets, while ALA and DHA oxylipins were only elevated when rats were provided their direct precursor PUFA. In comparison, in kidney and some adipose depots, DHA oxylipins were higher in rats given diets enriched either in ALA, EPA or DHA. Heart oxylipins also changed less in response to dietary

PUFA than the liver [24], in which some ALA oxylipins were higher in rats given EPA and DHA diets, and in kidneys [23] and some adipose depots, in which some ALA oxylipins were lower in rats given EPA and/or DHA diets [21]. The apparent lack of effect of ALA or EPA on DHA oxylipins in the heart is supported by findings that the rat heart lacks elongase-2 [38].

These findings may indicate that EPA oxylipins are key n-3 derived oxylipins in the heart, and a number of EPA oxylipins have beneficial effects in this regard. For example, 18-HEPE can prevent cardiac remodeling in the pressure overload-induced mouse model [39] and epoxygenated EPAs such as 17,18-EpETrE have antiarrhythmic effects in neonatal cardiomyocytes [40] and vasodilatory effects in coronary smooth muscle cells [11]. Other EPA derived eicosanoids such as PGD_3, PGE_3 and TxB_3 have lesser or no effects on inducing arrhythmias in cultured neonatal rat cardiac myocytes, when compared to their ARA derived counterparts [41], and EPA derived resolvin E_1 can limit infarct size and ischemia-reperfusion injury in male rats [42]. Nevertheless, DHA oxylipins such as 13,14-EpDPE, 13,14-dihydroxy-docosahexaenoic acid and 17,18-EpDPE have vasodilatory effects in coronary smooth muscle cells [11, 43] and 19,20-EpDPE has antiarrhythmic effects in neonatal cardiomyocytes [44], indicating that these also have potential roles in the heart. To our knowledge, no effects of ALA oxylipins on heart function have been reported.

In addition to these effects on n-3 oxylipins, dietary n-3 PUFA may also protect the heart by reducing the levels of n-6 oxylipins, particularly those derived from ARA. DHA was more potent in this regard, compared to both EPA and ALA, suggesting that DHA may have been more responsible for the reduction in the ARA oxylipins that has previously been observed in rat myocardium, as well as other tissues, with fish oil feeding [22, 45, 46]. ARA oxylipins are associated with several functional effects in the heart. For example, increased $PGF_{2\alpha}$ and PGE_2

are associated with hypertrophic growth and production of arrhythmias in neonatal cardiac myocytes [41, 47, 48], the lipoxygenase generated HETEs are increased in mitochondria from failing human hearts [49] and 20-HETE induces vasoconstriction in small porcine coronary arteries [50]. Some effects of ARA oxylipins are protective, however, such as the vasodilatory EpETrEs, which may prevent obesity induced cardiomyopathy and reduce post-ischemic ventricular polarization in an isolated heart model [51, 52], and PGI_2, which displays protective effects against the arrhythmias induced by other prostanoids in rat cardiac myocytes [41]. However, ARA oxylipins are more often associated with detrimental effects, while the EPA and DHA oxylipins are associated with cardio protective effects, as described above. Thus, the combination of higher levels of EPA (and DHA, and possibly ALA) oxylipins and lower levels of ARA oxylipins with ALA, EPA and DHA feeding would be predicted to have an overall protective effect on rat heart functions.

Increased dietary LA also has been promoted as heart healthy, due to improvements in the blood lipoprotein profile [53]. However, dietary LA increases renal and hepatic n-6 oxylipins derived from n-6 PUFA, including ARA [19, 23], suggesting that there may be effects on oxylipins that are not evident based on tissue fatty acid composition alone. In contrast to these tissues, dietary LA effects in the heart are restricted to increases in LA, gamma-linolenic acid and DGLA oxylipins, further indicating greater resistance to changes in oxylipin composition by dietary oils in the heart compared to other tissues. Whether these oxylipins have significant effects on heart health, however, remains to be elucidated. On the one hand, the LA oxylipin 9,10-EpOME can induce heart failure in dogs [54], and its metabolite, 9,10-dihydroxy-octadecenoic acid, has been reported to decrease left ventricular-developed pressure recovery in the ischemic/reperfused

mouse heart [55]. On the other hand, the LA oxylipin, 13-hydroxy-octadecadienoic acid, and several DGLA oxylipins have been demonstrated to have anti-platelet effects [56-58].

Interestingly, there also were several exceptions to these generalized patterns. For example, ARA oxylipins such as 15-deoxy-PGD_2 in EPA male, PGJ_2 in DHA male and 15-k-PGE_2 in (both male and female) EPA rats were increased while most of the other ARA oxylipins were decreased. Exceptions similar to this were observed in the kidney, liver and serum of these rats [23], as well as in human plasma after dietary intervention with individual EPA or DPAn-3 [59]. These PG metabolites are formed by hydroxyPG dehydrogenase (PGDH) activity [60] and often have been considered to be inactive metabolites of their parent compounds. Although their functions are not well characterized, several studies report physiological effects. For example, 15-k-PGE_2 can inhibit inflammatory cytokine production in acute liver injury [61] and 15-PGDH activity suppresses colon tumorigenesis *in vivo* [62]. How dietary n-3 PUFA affects PGDH activity or whether its oxylipins play functional roles in the heart by increasing these PG metabolites remains to be elucidated.

These data also illustrated other differences between fatty acid and oxylipin levels. For example, oxylipin product to PUFA precursor ratios are higher for shorter compared to longer chain PUFA and for n-3 compared to n-6 PUFA. This is similar to findings in other rat tissues [19, 23, 24], and in studies of AA compared to LA, and to EPA and DHA metabolism by 15-LOX [63, 64], and AA compared to EPA and DHA metabolism by CYP enzymes *in vitro* [40, 65]. These findings may suggest that dietary 18-carbon and n-3 PUFA may have greater effects on oxylipins in the heart. Thus, along with the greatest sex difference in oxylipins in ALA fed rats, dietary ALA may have greater impact on heart physiology than suggested by fatty acid data alone.

To our knowledge, sex effects on the heart oxylipin profile have not been examined. We have examined oxylipin sex differences in other tissues in these rats, and found that males generally have higher levels of oxylipins in kidney and liver [23]. In several adipose depots, oxylipin sex effects were observed in fewer oxylipins, with many having interaction effects. Interestingly, in those with interactions, they were higher in females given the control, ALA and EPA diets, but higher in males given the DHA diet [21]. This pattern is similar to the current findings in the heart where oxylipins in DHA rats were similar in females and males, but oxylipins in rats given the ALA diet were higher in females. The mechanism by which dietary PUFA interact with sex effects on heart oxylipins is not known, as this is a novel finding. In one study, a high fat diet reduced renal cortical 20-HETE and EpETrE production in male but not female rats [66], indicating that dietary fat may exert differential sex effects. However, the mechanisms by which sex alone, or in combination with diet, affects oxylipin levels remains largely unexplored in any tissue. There are examples of estrogen increasing individual oxylipins and/or the enzymes that produce them [25, 26, 67] or testosterone reducing these [68]. However, there are also studies which demonstrate higher levels of oxylipins in males or ovariectomized females [23, 69, 70], so much remains to be elucidated in this regard. Interestingly, sex differences in oxylipins are exhibited to the greatest extent in oxylipins derived from DHA, compared with all other PUFA. This may be due to the higher levels of DHA in female heart PL, but this also remains to be investigated.

3.6 *Conclusion*

In conclusion, this study provides novel data on the rat heart oxylipin profile and on the differential effects of dietary n-3 and n-6 PUFA and sex on this profile. We hypothesized that

oxylipins derived from ARA, DHA, EPA, LA and ALA are at quantifiable levels and we quantified 75 oxylipins derived from these PUFA in the rat heart. The dietary PUFAs have the greatest effect on their own oxylipins, more so in the heart than other tissues. In addition, ARA oxylipins are markedly reduced by dietary n-3 PUFA with the effect being much greater with DHA compared to EPA and ALA. LA oxylipins, however, were largely unaffected by dietary n-3 PUFA, but were increased with higher dietary LA intake, even in the absence of increased LA in heart PL. Other discrepancies between oxylipin and fatty acid levels were observed for several PGDH derived ARA oxylipin metabolites in EPA rats, for differences in oxylipin to parent PUFA ratios and for sex differences, further demonstrating that oxylipins do not necessarily reflect fatty acid compositions. Notably, these data show that sex differences in heart oxylipins interact with dietary PUFA effects, with dietary ALA compared to DHA resulting in higher levels of oxylipins in females. These fundamental data on heart oxylipins with unique dietary PUFA and sex effects will help guide further investigations on the functions of oxylipins in the heart.

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Transition to next chapter

Chapter 3 provides novel data on the rat heart oxylipin profile and on the differential effects of dietary n-3 and n-6 PUFA and sex on this profile. Although several oxylipins have been reported to have functional effects in neuroinflammation and neuroprotection, a more complete brain oxylipin profile derived from many PUFA is still lacking. Also, DHA is the major PUFA in brain, but it is not known whether DHA derived oxylipins are the most abundant in this tissue. As observed in the heart, whether brain oxylipins are also modulated by dietary changes and affected by sex remains to be elucidated. Therefore, the next chapter will examine the dietary PUFA and sex differences on the comprehensive oxylipin profile in rat brain, and will test hypotheses 2, 4 and part of 5 in section 2.6.

Chapter 4

4. The brain oxylipin profile is resistant to modulation by dietary n-6 and n-3 polyunsaturated fatty acids in male and female rats

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Running Head Brain oxylipins are resistant to modulation by dietary n-6 and n-3 polyunsaturated fatty acids in male and female rats

4.1 Abstract

Oxylipins are bioactive lipids formed by the monooxygenation of polyunsaturated fatty acids (PUFA). Eicosanoids derived from arachidonic acid (ARA) are the most well-studied class of oxylipins that influence brain functions in normal health and in disease. However, comprehensive profiling of brain oxylipins from other PUFA with differing functions, and the examination of the effects of dietary PUFA and sex differences in oxylipins are warranted. Therefore, female and male Sprague-Dawley rats were provided standard rodent diets that provided additional levels of the individual n-3 PUFA α-linolenic acid (ALA), eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), or the n-6 PUFA linoleic acid (LA) alone or with ALA (LA+ALA) compared to essential fatty acid sufficient control diets. Oxylipins and PUFA were quantified in whole brains by HPLC-MS/MS and GC, respectively. Eighty-seven oxylipins were present at quantifiable levels: 51 and 17% of these were derived from ARA and DHA, respectively. At the mass level, ARA and DHA oxylipins comprised 81-90% and 6-12% of total oxylipins, while phospholipid ARA and DHA represented 25-35% and 49-62% of PUFA mass, respectively. Increasing dietary n-3 PUFA resulted in higher levels of oxylipins derived from their precursor PUFA; otherwise, the brain oxylipin profile was largely resistant to modulation by diet. Approximately 25% of oxylipins were higher in males, and this was largely unaffected by diet, further revealing a tight regulation of brain oxylipin levels. This fundamental data on brain oxylipin composition, diet effects, and sex differences, will help guide future studies examining the functions of oxylipins in the brain.

4.2 Introduction

Oxylipins are bioactive lipid mediators synthesized from polyunsaturated fatty acids (PUFA) by cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) monooxygenase activities [1]. They are major regulators of physiological processes and inflammatory responses in tissues but are relatively less characterized in the brain. The most well studied class of oxylipins are the eicosanoids generated from arachidonic acid (ARA), and they play important roles in regulating neural functions [2, 3]. ARA oxylipins such as prostaglandin D_2 (PGD_2), PGE_2 and lipoxin A_4 display neuroprotective characteristics in both *in vitro* and *in vivo* studies [4-6], but PGE_2 also has inflammatory effects and is associated with pathogenesis of Alzheimer's disease [7], and PGD_2, PGE_2 and leukotrienes play a role in inducing ischemic brain edema [8-10]. In addition, docosahexaenoic acid (DHA) is the major PUFA in the brain, and generates oxylipins that have largely neuroprotective and anti-inflammatory functions in the brain [11, 12]. DHA oxylipins such as 7-, 10- and 17-hydroxydocosapentaenoic acid (HDoHE), protectin D1 (PD)1, resolvin D1 (RvD1) and RvD2 are negatively associated with age-related memory decline and inhibit neuronal cell death through reduction in caspase activation [11, 13]. Other oxylipins such as eicosapentaenoic acid (EPA) derived RvE1 have anti-inflammatory effects in microglial cells [14], and linoleic acid (LA) derived 9-hydroxy-octadecadienoic acid (HODE), 13-HODE and 9,10-DiHOME are associated with increased brain oxidative stress in the aging brain [15]. However, while there are reports of selected ARA, EPA and DHA oxylipins in the brain [16-22], a comprehensive profile of whole brain oxylipins is needed to identify the oxylipins present and to guide further studies on the potential functions of brain oxylipins.

Some brain oxylipins have been shown to be amenable to alterations in dietary lipid content. For example, n-3 PUFA (EPA and DHA) supplementation increases some EPA and

DHA derived oxylipins and decreases some ARA oxylipins in parts of or in whole brain compared to controls in animal models [13, 23]. An n-3 PUFA rich diet also reduced epoxy-PUFA and the ratio of epoxy- to dihydroxy-PUFA, an indication of higher soluble epoxide hydrolase activity [24] and promoted synthesis of pro-resolving oxylipins in the murine brain [25]. Increased dietary n-6 PUFA (LA) feeding results in increased LA and ARA oxylipins and decreased EPA oxylipins in the cerebral cortex of rat brain [26], while limiting dietary LA reduces lipopolysaccharide (LPS)-induced increases in brain PGE_2 concentration [27]. However, while the effects of dietary PUFA on brain fatty acid composition have been well characterized, oxylipin profiles do not necessarily reflect tissue PUFA composition [28-30]. Thus, while DHA is the predominant PUFA in the brain [31, 32], for example, whether DHA oxylipins constitute the majority of brain oxylipins is not clear.

Further, few studies examining sex differences in a small number of brain oxylipins have been reported [33, 34]. For example, 17-β-estradiol treatment in ovariectomized rats increases basal prostacyclin synthesis in cerebral blood vessels compared to untreated control rats [34]. There are also few studies of sex differences on the oxylipin profile in other tissues, but these indicate that oxylipins are generally higher in males in kidney, liver and serum [28, 29], and higher in females in heart (See Chapter 3) and adipose tissues [30]. Further, in these latter tissues, diet can influence the sex differences – for example, adipose oxylipins are higher in male rats provided a DHA rich diet but higher in female rats provided diets enriched in either α-linolenic acid (ALA) or EPA [30].

Therefore, the objectives of the current study were (1) to provide fundamental data on the rat brain oxylipin profile, (2) to investigate the effects of dietary n-3 and n-6 PUFA on this profile, and (3) to examine sex differences in this profile.

4.3 Materials and Methods

4.3.1 *Animals and Diets*

A total of 72 Sprague-Dawley weanling rats at 3 weeks of age were provided six different diets, resulting in 6 females and 6 males in each diet group. The diets were based on the standard AIN93G diet [35], as described in detail in [28-30] and in appendix A. These publications also describe the diet and sex effects on oxylipins in kidney, liver, adipose and serum in these same rats. The control diet contained adequate levels of the essential fatty acids (ALA and LA) and high levels of monounsaturated fatty acids. In four other diets, the oil levels were manipulated so that monounsaturated fatty acids were replaced with higher levels of ALA, EPA, DHA and LA. These diets contained 3g (per 100g diet) more of each of these PUFA, respectively, compared to the control diet. The last diet (LA+ALA) contained 3g more LA per 100g diet similar to the LA diet, plus it had additional ALA (0.43 g per 100 g diet) so that the LA/ALA ratio remained similar to the control diet. The oil blends were designed in such a way that each diet had similar levels of unsaturated and saturated fatty acids.

The dietary intervention was carried out for 6 weeks and body weight was measured weekly. At termination, rats were anesthetized using isoflurane and decapitated. Harvested brain tissues were weighed, immediately frozen in liquid nitrogen and stored at -80°C until further analysis. All animal procedures performed were approved by the University of Manitoba Animal Care Committee and followed the Canadian Council for Animal Care guidelines.

4.3.2 *Oxylipin analysis*

Whole brains were homogenized (appendices B and H) in a ratio of 17.5 mg brain per 100 µL of ice-cold Tyrode's salt (pH 7.6) solution. Preliminary analyses were used to determine the optimum amount of brain homogenate extract (400 µL) for oxylipin quantitation (appendix E). Methanol-formic acid (100:1), pH 3 water, 100% ethanol, antioxidants (20 mg butylated hydroxytoluene, 20 mg ethylenediaminetetraacetic acid, 200 mg tripolyphosphate and 200 mg in 2:1 methanol-ethanol solution) and deuterated internal standards (Cayman Chemicals, MI) were added to the samples, and pH was adjusted to <3 prior to loading on solid phase columns (Strata-X columns, Phenomenex, CA) that had been preconditioned with methanol followed by pH 3 water. The sample was centrifuged, and the supernatant was loaded on the column. After column washing with 2 mL pH 3 water followed by 1 mL hexane, oxylipins were eluted with 100% methanol, dried down under nitrogen and re-constituted in the mobile phase (water/acetonitrile/acetic acid, 70/30/0.02 v/v/v) for oxylipin analysis by HPLC/MS/MS (QTRAP 6500; Sciex, ON) as described [36, 37]. For details oxylipin extraction protocol see, appendix C. Quantification was carried out using the stable isotope dilution method [38]. Oxylipins screened but below the level of detection (<3 times above baseline) and those detected but below the level of quantification (>3 to <5 times above baseline) are presented in Supplementary Tables S4.1 and 2, respectively. Details of the total oxylipins scanned, mass transitions, retention times, internal standards and standard curve slopes are available in [28, 36, 37] (appendix F).

4.3.3 *Fatty acid analysis*

Total lipids were extracted from 250 µL of the brain homogenate obtained as described above and fatty acids were analyzed as described [39, 40] (appendix G). Briefly, after solvent-solvent extraction of total lipids, total phospholipid (PL) and neutral lipid (NL) fractions were separated by thin layer chromatography (heptane/isopropyl ether/acetic acid,60/40/3/,v/v/v) [41]. Fatty acids were methylated using methanolic H_2S0_4 and extracted in hexane prior to analysis by gas chromatography [42, 43].

4.3.4 *Statistical analysis*

All data analyses were carried out using SAS Software Version 9.3 (SAS Institute Inc, NC) and data are reported as mean ± SEM with significance set at $p<0.05$. The test for normality was performed using the Shapiro-Wilk Test. If data were distributed normally, analysis was performed using 2-way analysis of variance to test the main and interaction effects. The Kruskal-Wallis test was performed when data could not be normalized even after logarithmic transformation. Post hoc analysis was performed using Tukey's test. Observations outside of the mean ± 3 SD were considered to be outliers and removed from the analysis.

4.4 Results

4.4.1 *General Findings*

All rats were healthy throughout the study and at termination there were no effects of dietary PUFA on body [28, 29] or brain weights (Supplementary Table S4.3). However, brain and body weights were higher in males, and the differences were greater for body weight, so the brain to body weight ratios were higher in females.

Out of 164 oxylipins scanned, 87 were present in rat brain at quantifiable levels and 9 others were detected but below the level of quantitation (Fig. 4.1(a) and (b), and Supplementary Table S4.2). Half (51%) of all oxylipins were derived from ARA, followed by oxylipins derived from DHA (17%), EPA (9%), LA (9%), (DGLA (6%), adrenic acid (AdA) (5%) and ALA (3%). On a mass basis, ARA oxylipins made up an even greater proportion of the total, comprising 81-90% of the total oxylipin mass; this was followed by oxylipins formed from DHA (6-12%), LA (2.8-5.2%) and EPA (0.1-2.9%); the remaining oxylipins made up <1% of the total oxylipin mass (Supplementary Table S4.4).

This oxylipin mass distribution differed markedly from the PUFA composition of the brain. While ARA oxylipins predominated in the brain, DHA was the PUFA present at the highest levels. For example, in the control group (male and female average), the percentage of ARA and DHA oxylipin mass was approximately 89 and 7%, respectively; in comparison, the percent distribution of their PUFA precursors 33 and 50% in the PL fraction (Fig. 4.2(a)). Interestingly, the oxylipin distribution was more similar to the distribution of their PUFA precursors in the NL, but NLs made up <3% of the total PUFA precursors. These patterns were

similar in rats provided all diets; representative charts for control, DHA and LA diets are shown in Fig. 4.2 and values for all diets can be found in Supplementary Table S4.4.

4.4.2 *Effects of dietary PUFA on n-3 derived oxylipins and PUFA*

The main effect of dietary PUFA on n-3 derived oxylipins was mediated by diets enriched in n-3 PUFA. These diets increased oxylipins derived from each of their precursor n-3 PUFA, with the number of EPA oxylipins being changed the most. Compared to the control, 2 of 3 ALA oxylipins were higher in rats provided the ALA diet, 8 of 8 EPA oxylipins were elevated by the EPA diet, and 5 of 15 DHA oxylipins were elevated by the DHA diet (Fig. 4.1(a); complete data including means, standard errors and statistics are presented in Supplementary Table S4.5). The mass of EPA oxylipins also changed the most in response to dietary intervention: several EPA oxylipins increased by over 20-fold in rats provided the EPA compared to the control diet, while ALA oxylipins increased up to 4-fold with ALA feeding and DHA oxylipins increased up to 2-fold with DHA feeding. In addition to more EPA oxylipins being increased and to a greater extent by dietary EPA, they also were altered by dietary ALA and DHA. i.e. 3 and 7 EPA oxylipins out of 8 were elevated in the brains of rats given the ALA and DHA diets, respectively, although these oxylipins were not increased as much as when the rats were provided the EPA diet. It should be noted, however, that EPA oxylipins represented < 0.2% of total oxylipin mass in rats not provided additional dietary n-3 PUFA (Supplementary Table S4.4). Diets enriched in LA had no effect on n-3 oxylipins; the LA+ALA diet which included ALA at a similar ratio as the control diet also did not alter n-3 oxylipins.

The source of PUFA for oxylipin synthesis is thought to be primarily membrane PL [(44)], but there is also evidence that NL stores could be a source [(45)], so PUFA in both fractions were

analyzed and compared with the oxylipin levels. Similar to the oxylipin patterns, ALA, EPA and DHA in the brain PL and NL were higher in the rats provided their specific precursor PUFA, and EPA also was higher in brains from rats provided ALA and DHA compared to control diets (Fig. 4.1(c); complete data including means, standard errors and statistics are presented in Supplementary Tables S4.7 and 8). Similar to their lack of effect on n-3 oxylipins, the LA and LA+ALA diets did not alter n-3 PUFA levels in either the PL or NL fraction (Fig. 4.1(c), Supplementary Tables S4.7 and 8). Docosapentaenoic acid (DPAn-3) was increased in all three n-3 PUFA diets in both the PL and NL fractions but 17-keto DPA (17k-DPA), the only DPAn-3 oxylipin scanned for was not detected in the rat brain.

4.4.3 *Effects of dietary PUFA on n-6 derived oxylipins and PUFA*

In contrast to the n-3 oxylipins, the oxylipins derived from n-6 PUFA were minimally affected by either the n-3 or n-6 PUFA diets. Those altered included 3 out of the 5 DGLA oxylipins that were higher in DHA fed rats, 2 of 4 epoxy-eicosatrienoic acid (EpETrE) derived from ARA that were higher in EPA fed female rats, and 2 of 4 AdA oxylipins that were lower in EPA and DHA fed rats. This minimal effect on n-6 oxylipins also contrasted to the effects on n-6 PUFA, where several were altered when compared to the control group in either the PL or NL fraction. However, only the higher DGLA in DHA fed rats and lower AdA in EPA and DHA compared to control fed rats appeared to be similar to their oxylipin patterns (Fig. 4.1(b and c); complete data including means, standard errors and statistics are presented in Supplementary Tables S4.6, 4.7 and 4.8).

The distinctly higher EpETrE levels in females provided the EPA diet were an anomaly from the otherwise general lack of effects of EPA or other n-3 PUFA on ARA oxylipins in the

brain (Fig 4.1(b)), and also from the lowering effect of EPA and other n-3 PUFA on ARA oxylipins in all other tissues in these rats examined [28, 30]. Therefore, all the ARA epoxygenated metabolites and their analogs from all other PUFA were examined together. This revealed a consistent pattern of higher total epoxy-ARA oxylipins and total epoxy-PUFA oxylipins in the EPA female group only (Fig. 4.3, Supplementary Table S4.9). In contrast, their individual or total soluble epoxide hydrolase metabolites (dihydroxy-PUFA) did not display this pattern (Supplementary Tables S4.5, 4.6 and 4.9).

4.4.4 *Sex differences in oxylipin and PUFA levels*

Previous studies with these rats demonstrated that oxylipins were generally higher in males in kidney, liver and serum [28, 29], and generally higher in females in heart (Chapter 3) and adipose tissue [30]. Hence, sex differences in oxylipins were examined in the rat brain. These analyses revealed that 15 out of 17 oxylipins with main sex effects were higher in males and only two (15-k-PGE_2 and 15-k-$PGF_{2\alpha}$) oxylipins were higher in females. This pattern was similar among the few oxylipins with sex by diet interactions – 6 were higher in males in at least one group, and 2 were higher in females in a least one group (Fig. 4.4; Supplementary Tables S4.5 and 4.6). Similar to the diet effects, the effects of sex on oxylipins were not necessarily reflective of sex effects on their PUFA precursors in either the NL or PL fractions. Most PUFA sex differences were dissimilar (e.g. LA, ARA, AdA, ALA, EPA) from their oxylipin patterns; the only PUFA with sex differences that resembled the sex differences in their oxylipins were higher DGLA in males in the PL fraction and higher DHA in females in the NL fraction (Supplementary Table S4.7 and 4.8).

4.4.5 *Oxylipin to PUFA ratios*

Oxylipin to PUFA ratios were used to compare the relative levels of oxylipins to their precursor PUFA in the PL fraction as >97% of brain PUFA was in the PL fraction. Out of the 36 sets of ratios calculated, the ratio was higher 8 times in males, consistent with the ~20% of oxylipins being higher in males. The one exception was higher ratios in females for the CYP epoxygenase ratios in EPA fed rats, consistent with the EPA diet effect on epoxy-PUFA noted above and in Fig. 4.3. In all cases, n-3 oxylipin to PUFA ratios were higher than or equal to n-6 oxylipin to PUFA ratios, and the order of oxylipin to PUFA ratios by chain length was 18-carbon ≥ 20-carbon ≥ 22-carbon PUFA. Fig. 4.5 is a representative figure of these data; all ratios are provided in Supplementary Table S4.10.

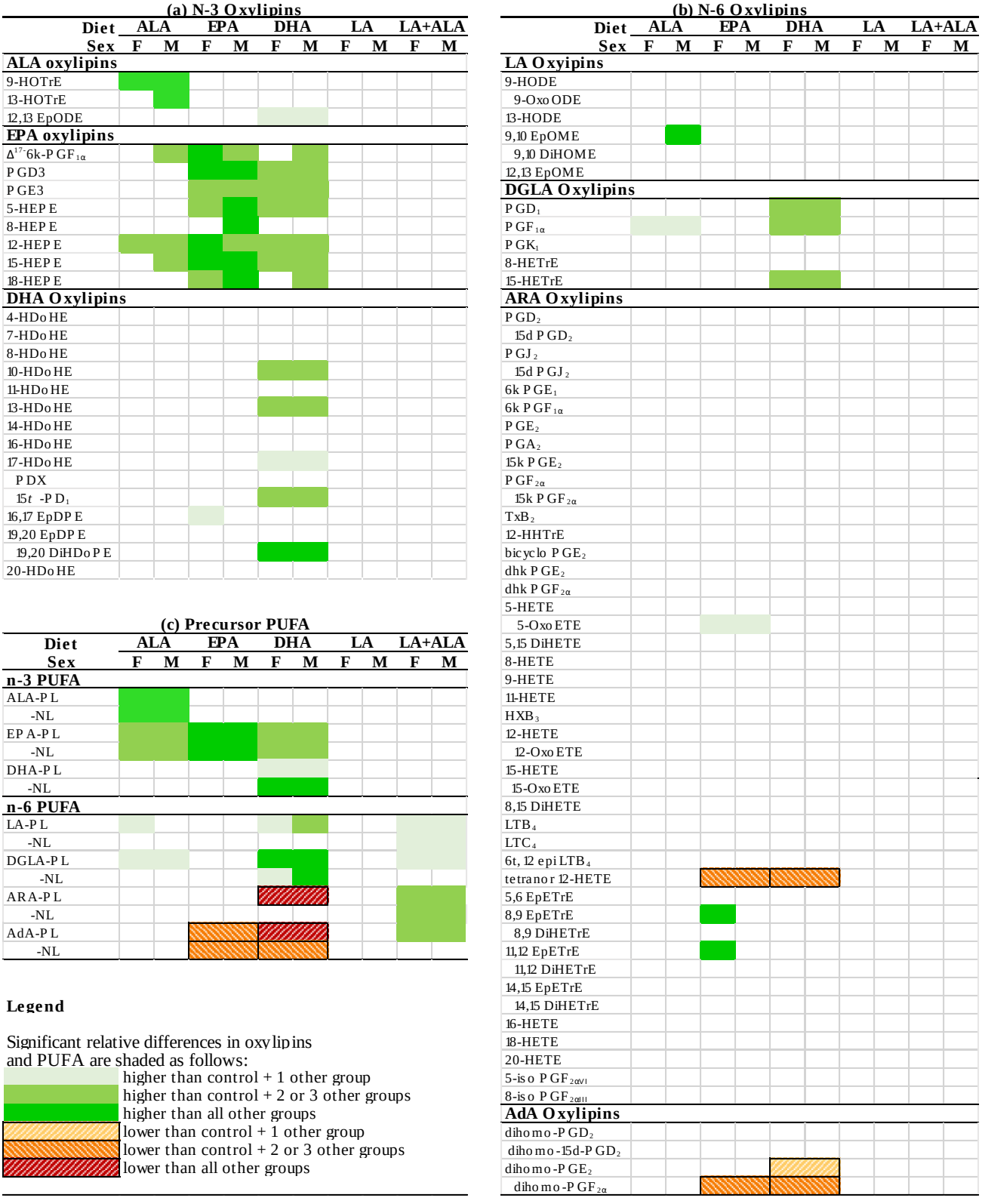

Fig. 4.1 Brain n-3 oxylipins (a) and n-6 oxylipins (b), and their precursor PUFA (c) in rats provided ALA, EPA, DHA, LA and LA+ALA diets compared to control diets. d, deoxy; dhk, dihydroketo; DiHDoPE, dihydroxy-docosapentaenoic acid; DiHETE, dihydroxy-eicosatetraenoic

acid, DiHETrE, dihydroxy-eicosatrienoic acid; DiHOME, dihydroxy-octadecenoic acid; EpDoPE, epoxy-docosapentaenoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETrE, hydroxy-eicosatrienoic acid; HHTrE, hydroxyheptadecatrienoic acid; HOTrE, hydroxy-octadecatrienoic acid; HX, hepoxilin; LT, leukotriene; *t*, trans; Tx, thromboxane.

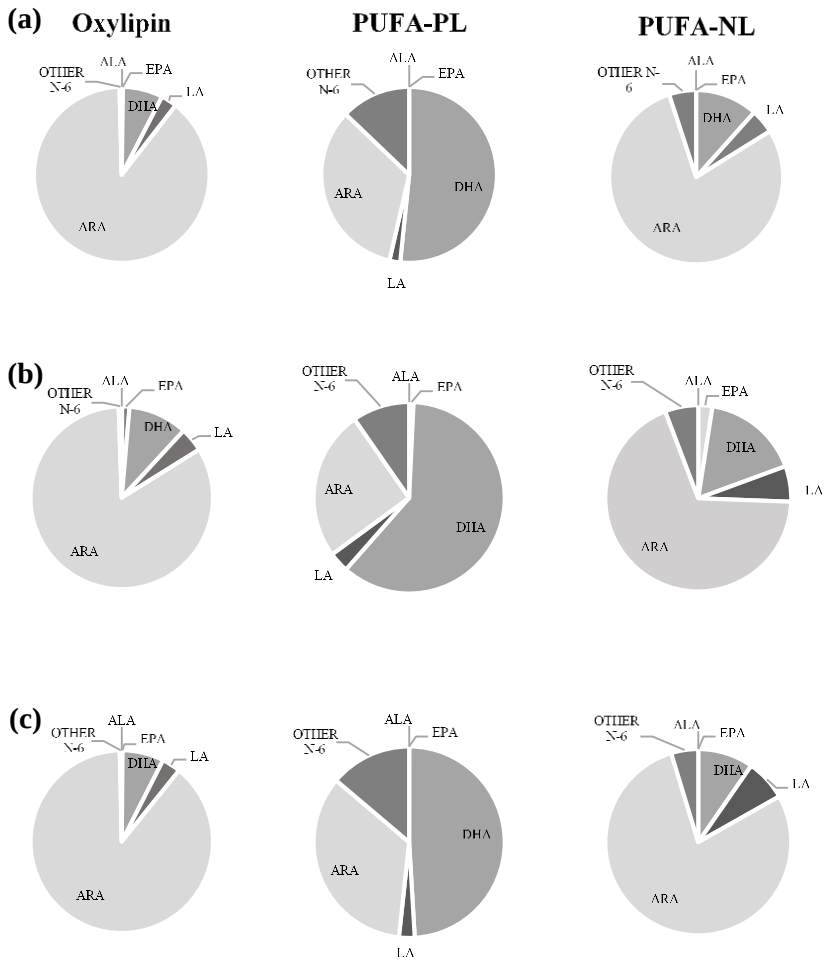

Fig. 4.2 Distribution of brain oxylipin and PUFA mass in rats provided the control (a), DHA (b) and LA (c) diets. Data shown are for combined data from female and male rats. Separate female and male data for all diet groups are provided in Supplementary Table S4.4.

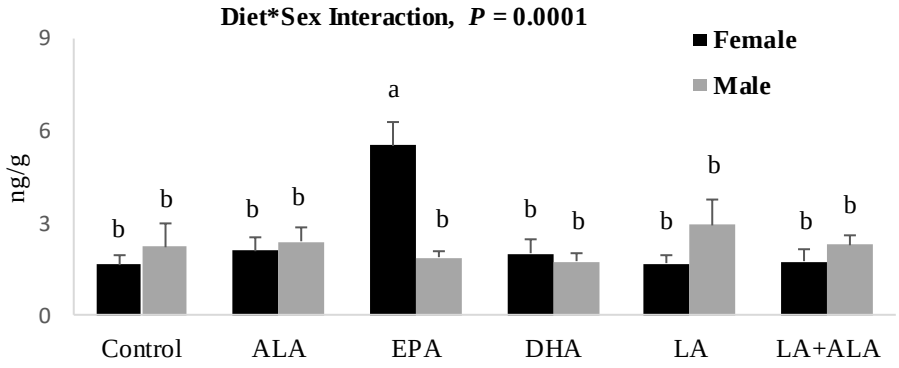

Fig. 4.3 Total epoxy-PUFA in rats given Control, ALA, EPA, DHA, LA and LA+ALA diets for six weeks. Differing letters above male and female bars are significantly different from each other. Data for total epoxy-ARA, dihydroxy-ARA and dihydroxy-PUFA are provided in Supplementary Table S4.9.

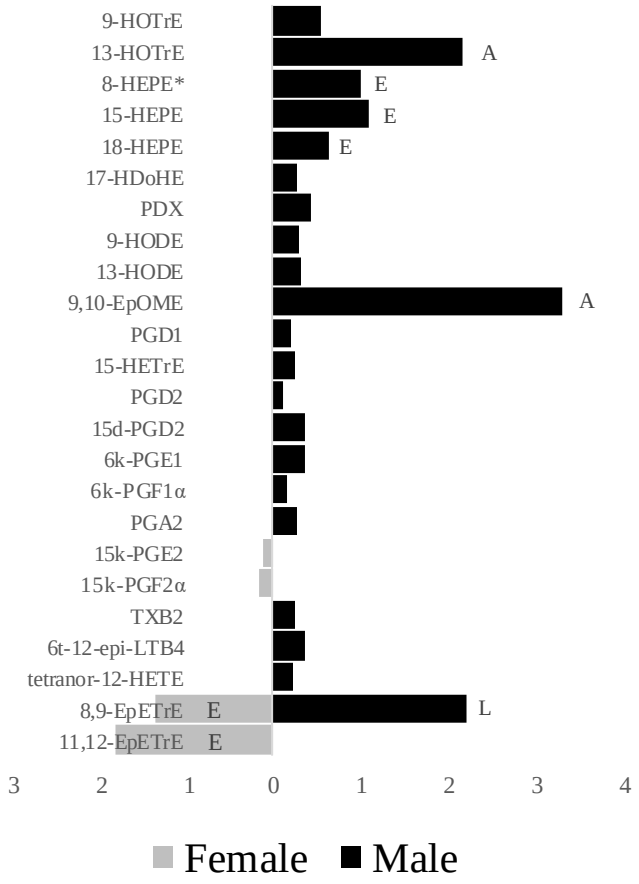

Fig. 4.4 Oxylipins with significant sex differences in rat brain. Data are presented as fold differences by comparing the mean oxylipin level in the sex that is higher compared to the other. Oxylipins higher in males are presented as black bars to the right; those higher in females are presented as grey bars to the left. Bars without labels indicate main sex effects. Bars with labels A, E and L indicate sex effects that were significant for only ALA, EPA and LA diets, respectively, due to interactions with diet effects. *8-HEPE was not detected in the EPA female group so a ratio could not be calculated; a value of 1 was arbitrarily assigned to this ratio. Details of the statistics including mean, SEM and *p* values are provided in Supplementary Tables S4.5 and 6.

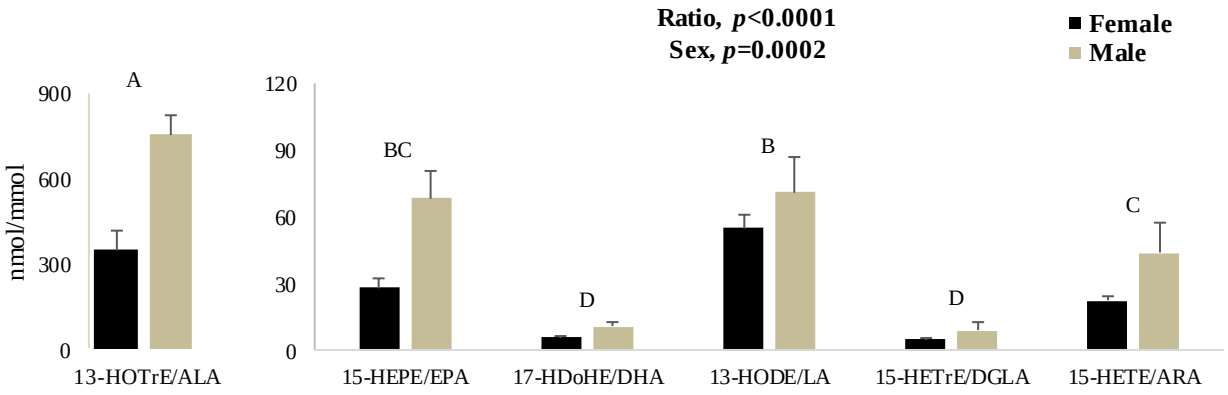

Fig. 4.5 Brain oxylipin to PUFA-PL ratios for the 15-lipoxygenase enzyme in rats provided the ALA diet. Ratios with differing letters are significantly different from each other. All ratios for the cyclooxygenase/PGD synthase, 5-, 12-, 15-lipoxygenase, cytochrome P450 epoxygenase and hydroxylase enzymes for all diets are provided in Supplementary Table S4.10. HDoHE, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETrE, hydroxy-eicosatrienoic acid; HOTrE, hydroxy-octadecatrienoic acid.

Supplementary Table S4.1 Oxylipins scanned but below the level of detection (0 to <3 times baseline)

ARA Oxylipins	Lower Limit of Detection(ng/g)	Lower Limit of Quantification (ng/g)
2,3-dinor-TxB_2	18.8	31.4
5, 6-DiHETrE	249	416
6t-LTB_4	5.43	9.05
11β-PGE_2	36.5	60.8
11β-$PGF_{2\alpha}$	0.55	0.91
11β-dhk-$PGF_{2\alpha}$	12.6	21.0
11d-TxB_2	8.29	13.8
12-epi-LTB_4	289	482
12-oxo-LTB_4	1.26	2.09
14, 15-LTE_4 (EXE_4)	0.03	0.05
15d-PGA_2	2.76	4.60
15k-PGD_2	3.03	5.05
15k-$PGF_{1\alpha}$	11.0	18.4
15R-LXA_4	6.39	10.7
17-HETE	2.69	4.49
19-HETE	114	191
19oh-PGE_2	4.12	6.87
19oh-$PGF_{2\alpha}$	4.73	7.88
20cooh-AA	5.43	9.04
20cooh-LTB_4	25.8	42.9
20oh-PGE_2	2.49	4.15
20oh-$PGF_{2\alpha}$	6.06	10.1
dh-$PGF_{2\alpha}$	3.90	6.50
dhk-PGD_2	21.0	35.1
HXA_3	0.06	0.09
LXA_4	69.0	115
LXB_4	59.2	98.7
PGB_2	14.7	24.5
$PGF_{3\alpha}$	39.9	66.5
PGK_2	39.7	66.2
tetranor-PGDM	0.06	0.11
tetranor-PGEM	10.0	16.7
tetranor-PGFM	143	239
Other Oxylipins		
2,3-dinor-6k-$PGF_{1\alpha}$	11.3	18.8
2,3-dinor-8-iso $PGF_{2\alpha}$	1.39	2.31
2,3-dinor-11β-$PGF_{2\alpha}$	4.56	7.60
5,6-DiHETE	0.43	0.72
5-HETrE	15.9	26.6
6,15-dk-,dh-$PGF_{1\alpha}$	51.8	86.3
7R-Maresin-1	14.0	23.3
8-iso-15k-$PGF_{2\beta}$	164	273
8-iso-$PGF_{3\alpha}$	58.6	97.6
9,10-EpODE	114	190
9-Nitrooleate	12.5	20.8
9-oxoOTrE	0.01	0.01

9,10,13-TriHOME	359	598
11-HEPE	8.99	15.0
12,13-DiHODE	43.0	71.7
13-oxoODE	251	418
13-oxoOTrE	3.91	6.51
14,15-EpETE	6.60	11.0
15,16-DiHODE	8.23	13.7
15,16-EpODE	1.43	2.38
15-oxoEDE	53.4	89.0
15-t-PD_1	0.46	0.76
17,18-EpETE	12.6	21.0
17k-DHA	0.44	0.73
17k-DPA	1.30	2.16
Dihomo-PGJ_2	1.98	3.31
PGE_1	0.03	0.05
RvD_1	81.5	136
RvD_2	5.69	9.48
RvD_5	95.5	159
RvE_1	1.42	2.36
TxB_1	3.95	6.58
TxB_3	121	202

Supplementary Table S4.2 Oxylipins detected but below the level of quantification (>3 to <5 times baseline)

Oxylipins	Lower Limit of Detection(ng/g)	Lower Limit of Quantification (ng/g)
9-HEPE	6.93	11.5
9, 10-DiHODE	1.99	3.31
13-HOTrE-γ	57.5	95.8
14, 15-LTC_4 (EXC_4)	28.0	46.7
14, 15-LTD_4 (EXD_4)	5.76	9.60
20oh-LTB_4	25.8	42.9
LTD_4	8.39	14.0
LTE_4	62.5	104
LXA_5	6.49	10.8

Supplementary Table S4.3 Brain and brain/body (Br/B) weight at termination in rats provided control, ALA, EPA, DHA, LA and LA+ALA diets for 6 weeks

	Control		ALA		EPA		DHA		LA		LA+ALA		P Value	
Weight	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Diet	Sex
Brain,g	1.94±0.31	2.09±0.35	1.92±0.44	2.09±0.48	1.88±0.35	2.13±0.34	1.92±0.41	2.04±0.23	1.88±0.23	2.13±0.47	1.90±0.24	2.04±0.58		<0.0001
Br/B,mg/g	6.15±0.25	4.10±0.10	6.43±0.34	4.24±0.16	6.18±0.26	4.15±0.08	5.68±0.27	4.42±0.10	6.50±0.11	4.33±0.12	5.92±0.06	4.14±0.12		<0.0001

Values are mean ± SE (n=6 for each group)
Only significant *P* values were reported
P values shaded blue or pink indicate main sex effects with higher levels in males and females, respectively
Superscript letters within a row indicate differences between means
Body weights of these rats are reported in references 28 and 29

Supplementary Table S4.4 Mass distributions of brain oxylipins and precursor PUFA in PL and NL fractions in rats provided control, ALA, EPA, DHA, LA and LA+ALA diets for 6 weeks

			% of total oxylipins/PUFA					
Diet	Sex	Oxylipin /PUFA	ALA	EPA	DHA	LA	ARA	Other n-6
Control	Female	Oxylipin	0.09	0.12	8.42	2.96	88.0	0.43
		PUFA-PL		0.04	52.1	1.79	33.6	12.5
		-NL			11.7	4.32	78.6	5.37
	Male	Oxylipin	0.11	0.12	6.32	2.87	90.2	0.40
		PUFA-PL			51.4	2.17	33.6	12.8
		-NL			11.6	4.70	79.1	4.59
ALA	Female	Oxylipin	0.15	0.60	7.15	3.15	88.5	0.40
		PUFA-PL	0.08	0.35	54.4	2.70	31.1	11.4
		-NL	1.16	1.49	12.6	5.87	73.4	5.52
	Male	Oxylipin	0.37	0.60	7.49	4.75	86.4	0.40
		PUFA-PL	0.06	0.29	53.3	3.06	31.1	12.2
		-NL	2.20	0.84	10.6	6.41	76.0	4.04
EPA	Female	Oxylipin	0.05	2.27	10.1	2.75	84.4	0.46
		PUFA-PL		1.00	56.1	2.30	30.1	10.5
		-NL		3.91	12.7	5.80	72.6	5.00
	Male	Oxylipin	0.10	2.90	9.72	3.59	83.2	0.46
		PUFA-PL		1.04	55.2	2.53	30.6	10.7
		-NL		3.56	9.99	5.63	77.5	3.33
DHA	Female	Oxylipin	0.05	1.08	9.51	3.38	85.5	0.48
		PUFA-PL		0.71	60.4	3.23	26.0	9.66
		-NL		2.18	16.8	5.76	69.7	5.56
	Male	Oxylipin	0.10	1.51	11.5	5.16	81.1	0.60
		PUFA-PL		0.78	61.5	3.64	24.9	9.18
		-NL		2.74	17.1	6.62	67.7	5.83
LA	Female	Oxylipin	0.06	0.12	7.04	3.47	89.0	0.36
		PUFA-PL		0.03	49.4	2.44	34.9	13.2
		-NL			10.8	6.94	77.2	5.07
	Male	Oxylipin	0.05	0.07	7.65	3.18	88.7	0.34
		PUFA-PL		0.03	48.9	2.89	34.2	13.9
		-NL			8.51	7.39	79.9	4.24
LA+ALA	Female	Oxylipin	0.10	0.16	6.14	3.72	89.5	0.33
		PUFA-PL		0.03	51.7	2.67	33.0	12.6
		-NL		0.06	11.2	8.06	74.8	5.86
	Male	Oxylipin	0.11	0.12	6.90	3.76	88.7	0.43
		PUFA-PL		0.04	49.9	2.79	33.7	13.6
		-NL		0.23	7.88	8.39	79.3	4.18

Supplementary Table S4.5 Brain n-3 oxylipins in rats provided control, ALA, EPA, DHA, LA and LA+ALA diets for 6 weeks

Diet	Control		ALA		EPA		DHA		LA		LA+ALA		P Value	
Sex	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Diet**	**Sex**
ALA Oxylipins (ng/g)														
9-HOTrE	0.33±0.06^{B}	0.32±0.05	0.59±0.07^{A}	1.38±0.32	0.26±0.04^{B}	0.32±0.03	0.22±0.06^{B}	0.35±0.05	0.23±0.06^{B}	0.24±0.04	0.30±0.06^{B}	0.39±0.07	<0.0001	0.0046
13-HOTrE	0.85±0.18^{b}	1.26±0.23^{b}	1.53±0.30^{b}	4.85±0.92^{a}	0.37±0.07^{b}	1.04±0.13^{b}	0.36±0.09^{b}	0.85±0.23^{b}	0.48±0.05^{b}	0.58±0.10^{b}	1.20±0.32^{b}	1.15±0.24^{b}	<0.0001$^{\$}$	
12,13-EpODE	0.09±0.03^{B}	0.07±0.03	0.12±0.02^{AB}	0.19±0.06	0.04±0.01^{B}	0.11±0.02	0.18±0.05^{A}	0.21±0.05	0.12±0.04^{AB}	0.09±0.03	0.07±0.03^{AB}	0.14±0.04	0.0073	
EPA Oxylipins (ng/g)														
Δ^{17}-6k-PGF$_{1\alpha}$	_$^{\#e}$	_^{e}	0.03±0.01^{de}	0.08±0.01^{cd}	0.17±0.02^{a}	0.18±0.02^{a}	0.08±0.01^{bc}	0.13±0.01^{ab}	_^{e}	_^{e}	_^{e}	_^{e}	<0.0001$^{\$}$	
PGD$_3$	0.12±0.04^{e}	0.08±0.02^{e}	0.56±0.08^{e}	0.67±0.11^{de}	2.04±0.26^{ab}	2.12±0.11^{a}	1.20±0.15^{cd}	1.53±0.14^{bc}	0.08±0.02^{e}	0.09±0.04^{e}	0.10±0.04^{e}	0.09±0.02^{e}	<0.0001	
PGE$_3$	_^{e}	0.09±0.09^{de}	0.19±0.05^{de}	0.29±0.09^{cde}	0.68±0.09^{ab}	0.85±0.13^{a}	0.40±0.05^{bcd}	0.61±0.13^{abc}	_^{e}	_^{e}	_^{e}	0.02±0.02^{e}	<0.0001$^{\$}$	
5-HEPE	0.77±0.10^{e}	0.78±0.35^{de}	2.10±0.26^{cde}	2.61±0.62^{cde}	8.94±1.46^{ab}	11.8±2.17^{a}	5.14±0.46^{bcd}	6.47±1.50^{bc}	0.48±0.12^{e}	0.44±0.14^{e}	1.13±0.15^{de}	0.93±0.29^{de}	<0.0001$^{\$}$	
8-HEPE	_^{b}	_^{b}	_^{b}	_^{b}	_^{b}	1.01±0.32^{a}	_^{b}	_^{b}	_^{b}	_^{b}	_^{b}	_^{b}	<0.0001$^{\$}$	
12-HEPE	0.50±0.07^{d}	0.52±0.09^{d}	4.11±0.25^{bc}	4.04±0.51^{c}	11.78±2.21^{a}	14.8±1.14^{a}	5.41±0.64^{bc}	8.14±1.15^{ab}	0.44±0.08^{d}	0.35±0.04^{d}	0.61±0.05^{d}	0.34±0.03^{d}	0.0136	
15-HEPE	0.07±0.04^{e}	0.06±0.03^{e}	0.62±0.12^{de}	1.41±0.27^{bcd}	2.35±0.44^{b}	4.92±0.40^{a}	1.28±0.19^{cd}	2.18±0.19^{bc}	0.10±0.05^{e}	0.03±0.03^{e}	0.09±0.04^{e}	0.08±0.02^{e}	<0.0001$^{\$}$	
18-HEPE	0.27±0.10^{d}	0.34±0.17^{d}	1.17±0.34^{cd}	1.29±0.46^{cd}	4.67±0.33^{b}	7.63±1.66^{a}	2.56±0.25^{bcd}	3.28±0.81^{bc}	0.67±0.24^{cd}	0.23±0.08^{d}	0.54±0.15^{cd}	0.42±0.11^{cd}	<0.0001$^{\$}$	
DHA Oxylipins (ng/g)														
4-HDoHE	18.2±2.93	16.4±2.35	17.3±2.24	17.1±3.31	23.7±1.95	23.0±3.27	19.6±2.23	25.0±3.89	15.6±3.00	21.8±1.95	14.7±2.06	17.5±2.85	0.0497	
7-HDoHE	4.14±0.93	6.10±1.91	5.84±1.37	5.55±0.84	6.20±0.94	5.56±0.47	6.74±0.88	5.20±0.69	4.90±1.22	5.55±1.49	5.17±0.75	4.70±0.54		
8-HDoHE	5.48±1.01	5.28±1.56	5.65±1.09	5.79±0.85	6.55±1.22	7.02±1.10	6.53±0.51	9.62±1.11	5.25±0.64	7.29±1.54	5.83±0.84	6.20±1.12		
10-HDoHE	5.75±0.95^{B}	4.99±0.88	4.95±0.36^{B}	6.03±1.24	6.13±0.63^{AB}	6.74±0.61	8.03±0.72^{A}	8.15±1.25	5.41±1.15^{AB}	7.04±0.71	4.76±0.66^{B}	5.36±0.42	0.0078	
11-HDoHE	4.16±0.66^{AB}	3.61±1.03	3.92±0.62^{AB}	4.63±0.71	4.15±0.43^{AB}	4.99±0.88	5.39±0.48^{A}	6.24±0.83	4.68±0.82^{AB}	5.77±0.83	3.46±0.85^{B}	3.63±0.39	0.0272	
13-HDoHE	8.72±1.33^{BC}	6.86±0.33	8.85±0.68^{ABC}	11.1±1.93	11.3±1.25^{AB}	11.7±1.05	12.7±1.50^{A}	14.5±1.84	7.91±1.34^{BC}	8.80±1.01	6.92±0.91^{C}	7.75±0.49	0.0004	
14-HDoHE	11.1±2.93^{b}	6.92±0.72^{b}	7.60±1.12^{b}	14.6±3.38^{ab}	9.06±1.44^{b}	15.5±2.32^{ab}	13.6±2.10^{ab}	23.1±2.25^{b}	7.96±1.66^{b}	10.5±1.81^{b}	7.77±1.69^{b}	8.88±1.05^{b}	0.0225	
16-HDoHE	5.96±0.91	4.89±0.70	5.59±0.48	6.10±0.98	7.25±0.87	7.24±0.84	7.08±0.57	7.83±1.00	5.62±0.93	6.55±0.84	5.25±0.68	5.29±0.43	0.0261	
17-HDoHE	26.5±4.44^{BC}	20.3±2.33	22.3±1.64^{ABC}	36.7±6.87	31.7±4.31^{AB}	38.4±5.10	29.9±3.23^{A}	43.4±3.91	21.78±4.05^{ABC}	30.4±4.55	20.4±2.73^{C}	24.2±1.24	0.0018	0.0050
PDX	0.42±0.06^{AB}	0.40±0.12	0.45±0.10^{AB}	0.58±0.07	0.29±0.06^{AB}	0.53±0.09	0.41±0.05^{A}	0.88±0.17	0.33±0.02^{AB}	0.48±0.04	0.37±0.06^{B}	0.37±0.04	0.0144	0.0014
***15*-PD$_1$**	0.28±0.04^{B}	0.21±0.02	0.28±0.05^{AB}	0.32±0.07	0.27±0.01^{AB}	0.29±0.02	0.35±0.05^{A}	0.37±0.05	0.23±0.01^{B}	0.25±0.03	0.25±0.02^{B}	0.25±0.02	0.0099	
16,17-EpDoPE	0.34±0.16^{b}	1.38±0.55^{ab}	0.85±0.23^{ab}	0.63±0.07^{ab}	1.83±0.35^{a}	0.49±0.14^{ab}	1.16±0.32^{ab}	0.44±0.20^{b}	0.31±0.15^{b}	1.12±0.33^{ab}	0.99±0.34^{ab}	0.63±0.11^{ab}	0.0010	
19,20-EpDoPE	0.08±0.03	0.06±0.02	0.00±0.00	0.02±0.01	0.08±0.03	0.07±0.01	0.06±0.02	0.05±0.02	0.05±0.02	0.08±0.03	0.06±0.02	0.03±0.01		
19,20 DiHDoPE	0.53±0.04^{B}	0.44±0.06	0.51±0.02^{B}	0.51±0.05	0.51±0.03^{B}	0.47±0.03	0.72±0.03^{A}	0.72±0.06	0.46±0.03^{B}	0.50±0.06	0.52±0.04^{B}	0.41±0.02	<0.0001	
20-HDoHE	26.5±5.71	18.5±2.06	20.6±2.63	20.6±3.83	26.4±3.56	23.2±3.23	28.8±4.26	24.4±3.54	25.6±5.51	26.3±4.16	16.0±2.27	23.8±2.38		

Values are mean ± SEM (n=5 to 6 for each group)

Uppercase superscript letters on the female values within a row indicate significant differences between diets

Lowercase superscript letters within a row indicate differences between means

Only significant *P* values were reported

P values shaded blue indicate main sex effects with higher levels of oxylipins in males
P values in between diet and sex columns indicate interaction between diet and sex unless noted with superscript $
[$]Indicates Kruskal-Wallis test when data is not normally distributed
[#]Indicates oxylipin not detected
*Denotes no primary standard, so not quantified

Higher than control + 1 group	Lower than control + 1 group
Higher than control and 2 or 3 other groups	Lower than control and 2 or 3 other groups
Higher than all other groups	Lower than all other groups

Supplementary Table S4.6 Brain n-6 oxylipins in rats provided control, ALA, EPA, DHA, LA and LA+ALA diets for 6 weeks

Diet	Control		ALA		EPA		DHA		LA		LA+ALA		P Value	
Sex	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Diet**	**Sex**
LA Oxylipins (ng/g)														
9-HODE	7.86±1.06	11.0±2.97	10.7±1.46	13.2±3.24	8.43±0.42	11.2±2.39	10.8±2.17	15.3±2.49	10.9±0.76	12.7±1.94	10.0±3.08	12.8±1.45		0.0389
9-OxoODE	19.9±8.33	16.1±3.03	24.1±4.33	48.8±15.5	13.5±3.90	23.4±4.63	24.8±8.92	36.2±8.87	29.7±5.50	15.4±6.16	26.0±11.0	25.4±7.39		
13-HODE	8.03±1.02	9.00±2.08	9.87±0.86	11.0±1.38	8.81±0.40	11.5±1.80	9.77±1.83	15.3±2.16	11.2±0.78	13.1±1.63	7.93±0.85	13.3±1.12		0.0009
9,10-EpOME	0.15±0.06^{b}	0.15±0.08^{b}	0.17±0.08^{b}	0.73±0.14^{a}	0.13±0.04^{b}	0.08±0.02^{b}	0.19±0.07^{b}	0.31±0.07^{b}	0.18±0.07^{b}	0.11±0.05^{b}	0.07±0.04^{b}	0.21±0.06^{b}	0.0006	
9,10-DiHOME	0.05±0.03	0.05±0.02	0.09±0.04	0.03±0.02	0.05±0.02	0.07±0.02	0.11±0.04	0.07±0.02	0.07±0.03	0.08±0.03	0.08±0.05	0.06±0.03		
12,13-EpOME	0.11±0.05	0.06±0.01	0.14±0.07	0.14±0.09	0.09±0.03	0.05±0.01	0.14±0.05	0.22±0.04	0.14±0.06	0.07±0.03	0.06±0.03	0.16±0.05		
12,13-DiHOME	0.01±0.01	0.01±0.01	0.13±0.07	0.21±0.14	0.04±0.02	0.06±0.02	0.16±0.08	0.05±0.03	0.03±0.03	0.08±0.04	0.14±0.09	0.18±0.15		
9,12,13-TriHOME	5.45±2.77	7.36±4.67	0.81±0.81	8.25±5.22	5.86±4.04	7.27±3.54	4.12±2.18	8.80±4.13	0.00±0.00	13.4±7.58	11.7±10.0	7.28±3.19		
DGLA Oxylipins (ng/g)														
PGD$_1$	1.19±0.11^{B}	1.36±0.21	1.37±0.05^{AB}	1.87±0.37	1.35±0.07^{AB}	1.42±0.13	1.76±0.20^{A}	2.15±0.28	1.19±0.07^{B}	1.40±0.18	1.17±0.06^{B}	1.52±0.20	0.0019	0.0170
PGF$_{1\alpha}$	0.61±0.04^{C}	0.58±0.08	0.79±0.06^{AB}	0.87±0.10	0.71±0.05^{BC}	0.66±0.10	0.93±0.03^{A}	0.94±0.06	0.74±0.05^{BC}	0.65±0.07	0.67±0.07^{BC}	0.69±0.08	<0.0001	
PGK$_1$	0.13±0.01	0.13±0.02	0.13±0.01	0.15±0.02	0.12±0.01	0.12±0.01	0.10±0.01	0.11±0.01	0.14±0.02	0.13±0.02	0.10±0.01	0.15±0.01		
8-HETrE	1.12±0.40	1.41±0.51	0.71±0.31	1.42±0.42	1.13±0.25	1.72±0.54	1.38±0.11	2.72±0.62	0.44±0.19	0.70±0.30	0.53±0.17	0.92±0.40		
15-HETrE	0.50±0.07^{B}	0.53±0.10	0.59±0.03^{AB}	0.77±0.21	0.59±0.10^{AB}	0.81±0.10	0.89±0.10^{A}	1.06±0.16	0.49±0.04^{B}	0.48±0.16	0.37±0.04^{B}	0.62±0.08	<0.0001	0.0324
ARA Oxylipins (ng/g)														
PGD$_2$	51.7±4.33	46.3±3.82	48.5±2.33	58.9±8.09	47.9±3.43	51.8±4.23	42.6±2.97	47.7±4.66	48.8±3.41	56.5±4.49	46.0±3.22	56.8±3.31		0.0176
15d-PGD$_2$	3.32±0.88	3.58±0.86	3.93±0.47	4.66±0.94	2.83±0.59	5.59±0.87	3.16±0.74	2.87±0.68	3.09±0.56	4.95±1.06	3.64±0.83	5.82±0.36		0.0061
PGJ$_2$	6.54±1.45	6.13±1.20	6.10±0.77	8.46±1.71	5.99±1.09	7.89±1.10	5.98±0.92	4.96±0.96	4.97±0.88	8.74±1.12	6.53±1.02	8.52±1.01		
15d-PGJ$_2$	5.79±2.21	3.11±1.20	6.27±2.00	4.68±1.89	2.69±1.29	6.66±1.91	2.12±1.03	2.40±1.26	2.63±0.83	2.95±1.72	3.20±1.66	6.50±1.35		
6k-PGE$_1$	0.08±0.02	0.10±0.02	0.10±0.03	0.15±0.04	0.11±0.01	0.20±0.05	0.09±0.02	0.12±0.02	0.09±0.01	0.06±0.03	0.12±0.02	0.17±0.02	0.0453	0.0303
6k-PGF$_{1\alpha}$	5.12±0.64^{AB}	5.00±0.66	5.20±0.51^{A}	6.70±0.85	5.88±0.64^{AB}	5.48±0.45	4.08±0.43^{B}	4.68±0.41	5.04±0.29^{A}	7.06±0.69	4.56±0.43^{AB}	5.93±0.54	0.0212	0.0114
PGE$_2$	6.45±0.61	6.68±0.89	6.40±0.47	8.02±1.15	6.90±0.61	6.82±0.53	5.78±0.43	6.31±0.75	6.19±0.57	7.70±0.69	5.84±0.31	6.75±0.63		
PGA$_2$	1.72±0.41	1.50±0.31	1.69±0.41	1.94±0.43	1.12±0.27	1.82±0.28	1.30±0.43	0.93±0.28	0.79±0.12	2.39±0.58	1.44±0.41	1.71±0.19		0.0429
15k-PGE$_2$	1.12±0.05	1.07±0.12	1.28±0.10	1.03±0.07	1.06±0.06	1.08±0.16	1.26±0.05	0.99±0.11	1.17±0.11	1.03±0.11	1.23±0.14	1.14±0.10		0.0323
PGF$_{2\alpha}$	23.4±1.14	24.8±1.33	27.6±1.38	27.6±2.36	24.8±2.24	23.1±1.90	27.6±1.77	25.2±1.45	26.6±1.73	26.1±2.12	26.1±2.14	26.5±2.43		
15k-PGF$_{2\alpha}$	10.7±1.13	10.9±1.50	14.6±1.90	11.6±1.38	8.87±1.61	10.7±2.44	15.6±0.86	11.9±1.14	13.6±1.16	10.0±1.22	14.8±1.68	12.3±1.13		0.0408
TxB$_2$	3.57±0.30^{AB}	4.45±0.75	3.11±0.23^{AB}	4.29±0.42	2.66±0.21^{B}	2.79±0.24	2.39±0.22^{B}	2.81±0.39	3.87±0.48^{A}	5.55±0.86	4.08±0.60^{A}	4.67±0.49	<0.0001	0.0046
12-HHTrE	646±31.7^{AB}	831±80.5	744±39.0^{A}	841±53.6	566±58.9^{B}	617±82.8	757±49.1^{AB}	667±57.1	766±78.4^{A}	831±94.5	810±70.5^{A}	766±91.5	0.0123	
bicyclo-PGE$_2$	0.27±0.04	0.24±0.05	0.24±0.03	0.31±0.04	0.26±0.04	0.26±0.03	0.22±0.02	0.21±0.06	0.19±0.03	0.23±0.04	0.27±0.03	0.29±0.06		
dhk-PGE$_2$	3.26±1.03	2.93±1.01	2.86±0.55	3.47±1.22	2.58±0.47	4.25±1.39	3.78±0.43	4.97±1.17	2.75±0.58	3.33±0.59	2.42±0.71	3.39±0.94		
dhk-PGF$_{2\alpha}$	0.01±0.01	0.01±0.01	0.02±0.01	0.01±0.01	0.02±0.01	0.01±0.01	0.00±0.00	0.02±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01		
5-HETE	38.1±7.19	35.2±8.99	31.9±4.66	39.4±9.28	36.5±4.16	40.4±7.74	28.1±3.44	29.3±6.19	34.2±6.13	47.3±6.94	32.2±4.92	42.7±7.80		
5-OxoETE	7.16±1.26^{B}	4.41±0.47	8.04±2.03^{AB}	7.61±0.68	11.7±2.06^{A}	8.96±1.82	5.75±0.62^{B}	4.51±0.47	6.75±1.49^{AB}	9.90±1.56	5.57±0.90^{AB}	7.60±1.61	0.0059	

5,15-DiHETE	0.89±0.18	0.81±0.17	0.74±0.13	0.86±0.25	0.73±0.10	1.17±0.31	0.57±0.09	0.93±0.22	0.80±0.11	1.09±0.24	0.92±0.19	1.04±0.22		
8-HETE	28.8±2.74	27.6±7.18	26.7±4.00	33.2±5.26	33.6±5.20	33.9±4.05	22.0±2.06	25.8±3.63	27.5±4.14	47.7±8.64	27.2±2.85	29.8±2.99		
9-HETE	18.9±2.33	12.9±3.46	17.2±3.75	19.5±2.80	20.5±2.97	24.5±4.91	14.4±1.45	18.1±3.43	18.2±2.87	27.6±4.92	17.6±2.66	19.5±3.07		
11-HETE	236±27.2	233±29.0	227±8.04	289±51.0	227±21.1	239±19.8	222±19.2	216±33.0	248±28.6	287±25.5	218±21.2	259±21.9		
HXB$_3$	0.12±0.02	0.10±0.02	0.13±0.03	0.15±0.04	0.14±0.02	0.21±0.05	0.08±0.01	0.16±0.03	0.12±0.02	0.21±0.03	0.13±0.03	0.15±0.02		
12-HETE	16.5±2.39	11.9±3.34	11.9±0.99	17.8±2.52	11.4±1.31	14.6±2.06	9.99±0.93	11.8±0.99	15.6±3.23	20.3±4.40	14.6±1.77	14.3±0.93		
12-OxoETE	2.36±0.54	2.86±1.01	2.81±0.70	3.44±0.60	2.74±0.35	3.15±0.77	1.68±0.33	1.96±0.30	2.03±0.75	2.67±0.90	2.53±0.61	2.22±0.13		
15-HETE	61.6±13.7	48.5±6.69	49.2±5.20	52.0±10.7	57.3±7.32	75.1±14.1	46.0±7.17	59.1±12.1	51.0±8.91	64.5±4.58	49.6±6.01	66.1±11.2		
15-OxoETE	23.8±1.05	20.7±4.27	19.5±3.50	25.7±3.29	24.8±3.81	25.5±3.30	19.3±1.37	17.9±1.33	20.6±2.14	25.0±3.09	20.0±3.12	19.1±1.41		
8,15-DiHETE	14.3±2.44	12.0±2.89	11.5±2.01	11.6±1.86	10.2±1.21	13.6±3.22	10.5±1.18	13.7±2.76	12.6±1.27	15.3±2.74	13.5±2.12	14.9±2.84		
LTB$_4$	0.40±0.04	0.38±0.06	0.45±0.06	0.40±0.05	0.33±0.04	0.33±0.03	0.30±0.02	0.38±0.04	0.37±0.06	0.31±0.02	0.39±0.02	0.38±0.03		
LTC$_4$	0.21±0.14	0.17±0.08	0.11±0.07	0.43±0.20	0.05±0.05	0.38±0.16	0.18±0.08	0.13±0.10	0.23±0.11	0.34±0.26	0.19±0.11	0.26±0.12		
6t-12-*epi*-LTB$_4$	0.31±0.03	0.24±0.07	0.30±0.07	0.34±0.05	0.24±0.05	0.38±0.08	0.21±0.06	0.30±0.05	0.21±0.05	0.40±0.09	0.23±0.04	0.39±0.10		0.0210
tetranor-12-HETE	0.31±0.07^{AB}	0.32±0.07	0.15±0.02^{BC}	0.19±0.02	0.14±0.02^{C}	0.18±0.02	0.10±0.01^{C}	0.20±0.05	0.26±0.05^{AB}	0.31±0.06	0.31±0.03^{A}	0.36±0.02	<0.0001	0.0093
5,6-EpETrE	0.25±0.04^{ab}	0.49±0.09^{ab}	0.27±0.05^{ab}	0.15±0.02^{b}	0.78±0.17^{a}	0.30±0.07^{ab}	0.24±0.03^{ab}	0.25±0.04^{b}	0.29±0.04^{ab}	0.47±0.10^{ab}	0.27±0.09^{ab}	0.32±0.05^{ab}	<0.0001	
8,9-EpETrE	0.23±0.07^{bc}	0.33±0.10^{bc}	0.32±0.09^{bc}	0.18±0.03^{c}	0.96±0.22^{a}	0.41±0.10^{bc}	0.24±0.06^{c}	0.25±0.05^{c}	0.23±0.06^{c}	0.74±0.18^{ab}	0.19±0.07^{c}	0.31±0.06^{bc}	<0.0001$^{\$}$	
8,9-DiHETrE	0.17±0.03	0.15±0.05	0.17±0.03	0.21±0.05	0.17±0.03	0.16±0.02	0.18±0.03	0.18±0.03	0.20±0.03	0.19±0.03	0.19±0.03	0.17±0.03		
11,12-EpETrE	0.13±0.03^{b}	0.16±0.06^{b}	0.12±0.03^{b}	0.08±0.00^{b}	0.42±0.07^{a}	0.15±0.04^{b}	0.11±0.02^{b}	0.10±0.01^{b}	0.13±0.02^{b}	0.22±0.07^{b}	0.07±0.01^{b}	0.15±0.03^{b}	0.0001	
11,12-DiHETrE	0.18±0.02	0.14±0.03	0.17±0.02	0.17±0.02	0.17±0.02	0.20±0.04	0.15±0.01	0.17±0.04	0.18±0.01	0.25±0.04	0.17±0.03	0.21±0.03		
14,15-EpETrE	0.31±0.10	0.19±0.06	0.26±0.08	0.17±0.06	0.59±0.15	0.37±0.13	0.16±0.04	0.19±0.06	0.20±0.02	0.51±0.13	0.19±0.07	0.24±0.03		
14,15-DiHETrE	0.37±0.06	0.37±0.08	0.34±0.03	0.38±0.04	0.37±0.04	0.38±0.07	0.35±0.03	0.30±0.06	0.38±0.03	0.50±0.06	0.39±0.06	0.38±0.04		
16-HETE	4.14±1.07	3.30±0.39	3.53±0.64	5.47±1.78	3.53±0.78	4.48±0.92	2.79±0.70	4.42±0.95	3.40±0.83	4.27±0.61	3.48±0.46	4.50±1.09		
18-HETE	0.31±0.01	0.25±0.03	0.22±0.05	0.35±0.09	0.33±0.05	0.31±0.07	0.32±0.09	0.24±0.09	0.32±0.09	0.33±0.03	0.27±0.07	0.37±0.05		
20-HETE	0.66±0.47	0.59±0.35	0.74±0.17	1.51±0.62	0.87±0.40	0.12±0.12	0.31±0.15	0.19±0.12	0.48±0.30	0.09±0.06	0.57±0.37	0.51±0.32		
5-iso-PGF$_{2\alpha VI}$	1.36±0.29	1.05±0.22	1.30±0.21	1.32±0.45	1.08±0.16	1.76±0.49	1.09±0.19	1.49±0.38	1.35±0.23	1.87±0.41	1.15±0.22	1.46±0.33		
8-iso-PGF$_{2\alpha III}$	7.68±0.43	7.97±0.51	8.59±0.29	7.63±0.30	7.50±0.44	8.43±0.99	7.80±0.39	8.27±0.91	8.42±0.62	9.31±1.07	8.24±0.30	8.97±0.87		
AdA Oxylipins (ng/g)														
dihomo-PGD$_2$	0.19±0.04^{AB}	0.15±0.02	0.13±0.01^{AB}	0.17±0.05	0.11±0.01^{AB}	0.15±0.04	0.08±0.01^{B}	0.11±0.02	0.16±0.02^{A}	0.23±0.04	0.14±0.03^{A}	0.20±0.04	0.0053	
dihomo-15d-PGD$_2$	1.30±0.31	0.71±0.14	1.31±0.37	0.69±0.11	1.43±0.39	1.23±0.27	1.31±0.20	1.19±0.15	1.09±0.05	1.00±0.43	0.95±0.29	1.34±0.28		
dihomo-PGE$_2$	0.07±0.02^{A}	0.05±0.00	0.04±0.01^{AB}	0.05±0.02	0.03±0.01^{AB}	0.05±0.01	0.03±0.01^{B}	0.02±0.01	0.05±0.01^{AB}	0.07±0.00	0.04±0.01^{AB}	0.07±0.01	0.0237	
dihomo-PGF$_{2\alpha}$	0.98±0.09^{A}	1.17±0.23	0.84±0.06^{AB}	0.97±0.13	0.74±0.06^{BC}	0.71±0.12	0.62±0.06^{C}	0.58±0.06	1.06±0.08^{A}	1.17±0.12	0.95±0.06^{A}	1.21±0.19	<0.0001	

Values are mean ± SEM (n=5 to 6 for each group)
Uppercase superscript letters on the female values within a row indicate significant differences between diets
Lowercase superscript letters within a row indicate differences between means
Only significant *P* values were reported
P values shaded blue or pink indicate main sex effects with higher levels of oxylipins in males and females, respectively
P values in between diet and sex columns indicate interaction between diet and sex unless noted with superscript $

[$]Indicates Kruskal-Wallis test when data is not normally distributed

Higher than control + 1 group	Lower than control + 1 group
Higher than control and 2 or 3 other groups	Lower than control and 2 or 3 other groups
Higher than all other groups	Lower than all other groups

Supplementary Table S4.7 PUFA levels in the phospholipid (PL) fraction in brains of rats provided control, ALA, EPA, DHA, LA and LA+ALA diets for 6 weeks

Diet	Control		ALA		EPA		DHA		LA		LA+ALA		P value	
Sex	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Diet	Sex
							µg/g brain homogenate							
Saturated Fatty acids														
12:0	0.17±0.10	0.08±0.08	0.61±0.30	0.91±0.24	0.80±0.47	1.62±0.65	0.44±0.14	0.54±0.31	1.00±0.60	0.51±0.22	0.87±0.74	2.14±1.17		
14:0	275±89.2	209±63.5	187±70.1	198±56.9	168±63.3	183±51.9	270±64.6	183±66.0	288±62.2	216±54.0	316±57.4	148±50.3		
16:0	4969±225	4928±212	5511±255	5208±269	5385±171	5237±182	5482±224	5083±154	5350±91.4	5158±173	5790±230	5577±242		
18:0	4940±130	5073±221	5384±249	4919±263	5431±176	5363±319	5247±193	4951±215	5340±244	5181±280	5766±208	5829±336	0.0510	
20:0	208±10.5^{AB}	219±10.1	201±11.4^{B}	203±13.4	194±8.51^{B}	224±13.1	217±9.88^{B}	193±10.2	213±9.15^{AB}	231±8.09	230±15.2^{A}	253±14.7	0.0097	
22:0	210±18.2	235±13.9	239±6.68	233±25.2	217±8.30	262±9.94	241±8.63	222±14.3	223±11.2	238±11.3	164±52.7	239±26.7		
24:0	419±18.1	422±25.4	435±21.4	390±39.7	399±20.8	474±31.9	440±19.6	413±19.8	392±27.3	434±28.9	421±24.9	440±40.0		
Monounsaturated Fatty acids														
14:1n5	105±66.3	51.7±51.7	175±78.5	103±65.3	114±72.2	103±65.6	172±77.0	108±68.3	112±70.8	134±63.7	60.1±60.1	91.2±60.0		
16:1t	53.5±13.1	91.4±37.7	65.7±19.4	85.0±28.0	116±45.6	117±47.2	163±48.8	86.8±43.5	186±74.0	113±50.9	35.6±9.34	44.4±12.6		
16:1n7	295±76.9	232±45.6	347±87.5	187±40.9	174±31.8	146±13.9	215±33.5	307±61.5	336±86.0	204±32.2	358±97.6	185±42.1		
17:1	2.16±1.32	5.65±3.59	5.73±3.66	8.59±3.85	8.34±3.90	6.60±4.18	10.6±4.73	6.71±4.12	6.31±3.90	8.27±4.51	–$^{\#}$	8.23±3.79		
18:1n9	4401±84.8	4555±201	4887±178	4752±203	5018±172	5096±309	5098±138	4678±189	4654±212	4547±165	4947±172	5026±306	0.0445	
18:1n7	855±15.7	914±30.1	928±51.0	928±86.4	964±24.5	1033±56.2	903±32.4	826±44.8	976±53.7	884±17.9	920±72.3	1075±63.2	0.0330	
20:1n9	499±15.9	554±27.5	516±25.0	518±45.1	512±22.0	561±28.4	555±15.7	499±21.2	463±138	545±22.7	470±102	567±50.6		
22:1n9	53.9±13.6	72.4±4.25	65.3±3.05	69.1±6.12	63.2±1.95	70.5±4.01	69.0±2.90	64.7±3.62	65.1±4.44	77.3±3.50	74.6±6.00	70.7±10.3		
24:1n9	705±24.8	766±49.7	691±42.8	757±78.5	676±26.6	788±39.2	745±18.9	732±44.0	672±53.2	777±38.3	741±35.6	783±77.2		0.0278
N-3 Polyunsaturated Fatty Acids														
18:3n3	–^{c}	–^{c}	5.24±0.56^{a}	3.39±0.92^{b}	–^{c}	–^{c}	–^{c}	–^{c}	–^{c}	–^{c}	–^{c}	–^{c}	<0.0001$^{\$}$	
20:3n3	–^{b}	–^{b}	5.45±0.52^{a}	5.9±0.71^{a}	–^{b}	–^{b}	–^{b}	–^{b}	–^{b}	–^{b}	–^{b}	–^{b}	0.0001$^{\$}$	
20:5n3	2.54±0.58^{d}	–^{d}	23.6±1.83^{c}	17.8±1.50^{c}	64.6±4.89^{a}	64.6±4.66^{a}	46.3±3.40^{b}	48.5±2.98^{b}	2.02±0.56^{d}	1.78±0.57^{d}	2.51±0.67^{d}	2.58±0.59^{d}	<0.0001$^{\$}$	
22:5n3	18.1±5.36^{c}	28.7±1.29^{c}	104±6.33^{b}	98.5±5.49^{b}	261±16.6^{a}	304±24.2^{a}	97.9±4.10^{b}	100±6.60^{b}	18.1±6.01^{c}	23.8±2.62^{c}	35.6±2.27^{c}	36.4±3.63^{c}	<0.0001$^{\$}$	
22:6n3	3249±132^{B}	3276±195	3632±127^{AB}	3253±178	3628±80.6^{AB}	3443±181	3924±117^{A}	3823±185	3245±183^{B}	3163±166	3713±110^{AB}	3643±223	0.0009	
N-6 Polyunsaturated Fatty Acids														
18:2n6	112±1.50^{d}	138±7.26^{cd}	180±5.12^{abc}	187±12.0^{abc}	149±6.64^{d}	158±8.53^{bcd}	210±6.75^{ab}	226±8.54^{a}	160±13.0^{bcd}	187±5.22^{abc}	192±12.3^{abc}	204±24.0^{ab}	<0.0001$^{\$}$	
18:3n6	0.78±0.38	4.24±2.44	1.01±0.51	0.85±0.38	1.31±0.62	0.95±0.61	5.18±1.47	1.04±0.49	1.13±0.58	1.00±0.48	0.56±0.36	1.35±0.43		
20:2n6	35.7±4.09^{bc}	40.7±2.84^{bc}	36.8±1.83^{bc}	46.2±3.32^{b}	30.1±1.10^{c}	36±2.47^{bc}	40.5±1.92^{bc}	41.2±1.00^{bc}	45.4±3.84^{b}	61.1±2.19^{a}	45.9±2.12^{b}	68.1±1.76^{a}	0.0046$^{\$}$	
20:3n6	86.9±5.51^{C}	101±5.09	112±3.47^{B}	122±9.88	103±3.92^{BC}	105±7.01	159±2.71^{A}	158±3.31	88.2±6.11^{C}	103±3.33	109±4.88^{B}	120±8.63	<0.0001	0.0149
20:4n6	2096±94.4^{BC}	2141±103	2073±76.1^{BC}	1899±114	1950±56.2^{C}	1907±104	1693±75.7^{D}	1545±69.3	2289±95.4^{AB}	2213±106	2371±105^{A}	2464±157	<0.0001	
22:2n6	14.6±1.48^{ABC}	20.4±1.73	17.7±1.58^{ABC}	21.5±2.45	12.3±3.11^{C}	17.3±2.24	16.8±2.04^{BC}	17.2±2.21	19.9±0.94^{AB}	24.3±2.06	22.1±1.40^{A}	26.7±4.32	0.0015	0.0045
22:4n6	694±27.0^{BC}	713±41.5	649±26.7^{CD}	621±44.9	574±14.8^{D}	561±29.3	469±21.6^{E}	412±17.7	778±49.6^{AB}	798±48.8	798±52.7^{A}	871±58.5	<0.0001	
22:5n6	34.6±12.7	61.2±19.6	28.4±5.88	30.1±7.18	38.9±3.08	39.9±2.11	42.9±4.52	28.0±9.29	40.5±20.2	60.8±32.6	43.9±13.8	51.5±22.6		

Values are mean ± SEM (n=5 to 6 for each group)
Uppercase superscript letters on the female values within a row indicate significant differences between diets
Lowercase superscript letters within a row indicate differences between means

Only significant *P* values were reported

P values shaded blue indicate main sex effects with higher levels of oxylipins in males

P values in between column diet and sex indicates interaction between diet and sex unless noted with superscript $

[$] Indicates Kruskal-Wallis test when data is not normally distributed

[#] Indicates fatty acids not detected

Higher than control + 1 group	Lower than control + 1 group
Higher than control and 2 or 3 other groups	Lower than control and 2 or 3 other groups
Higher than all other groups	Lower than all other groups

Supplementary Table S4.8 PUFA levels in the neutral lipid (NL) fraction in brains of rats provided control, ALA, EPA, DHA, LA and LA+ALA diets for 6 weeks

Diet	Control		ALA		EPA		DHA		LA		LA+ALA		P value	
Sex	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Diet	Sex
							µg/g brain homogenate							
Saturated Fatty Acids														
12:0	3.00±1.33	5.09±2.07	3.96±1.56	7.81±1.70	1.98±0.81	6.32±2.26	3.96±1.61	6.59±1.73	5.93±2.68	5.46±2.25	3.84±1.33	4.58±1.39		0.0375
14:0	7.20±3.57	5.59±2.11	10.8±3.85	6.80±2.91	10.5±2.41	10.2±1.92	8.28±2.05	8.81±3.07	8.80±3.67	6.65±1.77	4.02±1.92	6.35±1.72		
16:0	71.0±22.1	91.9±14.1	81.9±12.3	112±29.9	103±20.4	114±23.1	82.8±18.4	97.1±27.9	95.3±26.8	100±25.9	59.1±5.87	93.5±22.0		
18:0	127±27.3	135±12.3	197±55.6	163±35.5	155±40.3	160±41.1	132.±33.3	148±31.5	157±31.3	148±32.7	122±14.9	152±28.0		
20:0	0.56±0.55	1.70±0.70	2.05±0.68	0.85±0.21	2.06±0.73	0.75±0.33	1.56±0.62	1.69±0.30	0.97±0.40	1.20±0.53	0.67±0.32	–[#]		
22:0	1.32±0.53	1.98±0.65	2.16±0.31	2.12±0.32	1.89±0.38	2.08±0.25	1.89±0.41	1.89±0.25	1.83±0.31	1.77±0.33	2.30±0.23	1.95±0.63		
24:0	2.17±0.72	2.82±0.92	3.46±0.39	2.62±0.42	3.08±0.25	2.92±0.29	2.92±0.29	3.77±0.62	3.30±1.05	2.09±0.43	3.58±0.40	3.09±0.59		
Monounsaturated Fatty Acids														
16:1t	0.61±0.19	0.57±0.15	1.68±0.54	0.90±0.36	0.88±0.24	0.64±0.11	0.72±0.18	0.52±0.18	0.66±0.25	0.64±0.26	0.99±0.36	0.31±0.15		
16:1n7	4.33±2.57	5.10±2.03	5.34±1.60	5.70±1.79	6.28±1.52	4.84±1.47	5.54±1.52	5.89±1.57	4.56±1.66	3.70±1.39	6.48±2.15	5.54±2.02		
17:1	4.67±2.93	–	2.34±1.69	6.90±3.24	1.74±0.92	2.06±0.88	2.23±1.00	4.07±2.56	3.00±1.38	1.65±1.54	2.37±1.71	3.69±1.59		
18:1n9	98.2±13.0	69.9±17.9	128±29.1	118±22.1	103±11.1	115±18.5	105±15.3	73.5±25.3	101±20.5	105±23.9	125±26.2	125±24.4		
18:1n7	18.1±2.66	13.8±3.52	17.6±1.64	17.1±2.55	21.7±2.73	19.9±1.56	19.5±2.05	46.6±33.0	19.4±2.72	16.2±1.67	21.1±2.56	18.1±2.45		
20:1n9	6.04±0.90	6.57±1.93	7.19±0.47	7.04±1.30	7.44±0.34	7.56±0.97	6.14±1.29	7.32±0.92	6.66±0.49	6.53±1.45	6.88±0.34	6.95±1.11		
22:1n9	1.09±0.76	1.04±0.66	1.73±0.63	1.39±0.79	1.51±0.80	2.38±0.93	1.42±0.47	2.14±0.99	1.31±0.55	0.95±0.57	1.60±0.41	1.37±0.76		
24:1n9	1.49±0.53	1.29±0.57	3.42±1.18	2.92±0.95	2.66±0.34	2.58±0.65	2.55±0.40	2.69±0.85	2.98±1.16	1.76±0.52	3.09±0.57	2.28±0.57		
N-3 Polyunsaturated Fatty Acids														
18:3n3	–[c]	–[c]	2.47±0.60[b]	3.92±0.36[a]	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	<0.0001[$]	
20:3n3	–	–	–	0.06±0.04	0.14±0.09	0.01±0.01	0.06±0.03	0.05±0.03	0.05±0.04	0.15±0.13	0.06±0.06	0.05±0.03		
20:5n3	–[f]	–[f]	3.16±0.70[d]	1.50±0.35[e]	7.23±0.75[a]	6.29±0.77[ab]	4.24±0.57[cd]	4.89±0.86[bc]	–[f]	–[f]	0.12±0.09e[f]	0.49±0.37[ef]	<0.0001[$]	
22:5n3	0.11±0.07[C]	0.18±0.07	2.82±0.33[B]	1.99±0.36	4.92±0.52[A]	3.86±0.44	2.29±0.22[B]	2.01±0.35	0.50±0.14[C]	0.21±0.06	0.98±0.31[C]	0.53±0.17	<0.0001	0.0103
22:6n3	21.0±3.01[B]	21.3±1.67	26.7±2.40[B]	18.9±1.10	23.5±1.57[B]	17.6±1.09	32.7±3.19[A]	30.6±3.25	22.1±2.50[B]	15.6±1.50	25.0±2.58[B]	16.8±1.20	<0.0001	0.0003
N-6 Polyunsaturated Fatty Acids														
18:2n6	7.76±0.97[B]	8.62±3.31	12.5±2.38[AB]	11.4±2.91	10.7±1.64[B]	9.94±2.76	11.2±1.02[AB]	11.8±1.22	14.2±2.34[AB]	13.5±0.76	17.9±3.23[A]	17.9±4.19	0.0065	
20:2n6	0.42±0.13	0.33±0.14	0.54±0.14	0.26±0.12	0.54±0.05	0.25±0.11	0.54±0.13	0.39±0.13	0.55±0.19	0.38±0.18	0.74±0.16	0.72±0.17		0.0492
20:3n6	2.40±1.33[bcd]	1.69±1.17[abc]	5.01±1.02[abc]	2.23±0.30[bcd]	3.61±0.88[abcd]	1.44±0.36[cd]	6.35±1.01[a]	6.34±0.99[a]	2.03±0.64[bcd]	1.00±0.59[d]	5.06±1.74[ab]	1.60±0.45[bcd]	0.0014[$]	
20:4n6	141±8.60[BC]	145±10.7	156±8.57[BC]	136±6.09	134±3.90[BC]	137±7.36	136±4.50[C]	121±7.91	157±5.81[AB]	146±3.94	166±7.05[A]	169±13.1	<0.0001	
22:2n6	0.56±0.29	0.40±0.20	0.72±0.24	0.20±0.12	0.42±0.21	0.48±0.26	0.47±0.10	0.45±0.23	1.07±0.37	0.40±0.15	0.86±0.30	0.21±0.10		
22:4n6	7.25±0.81[AB]	6.73±0.92	6.70±0.28[BC]	4.98±0.50	5.62±0.33[C]	4.44±0.27	4.47±0.23[C]	4.08±0.49	8.32±0.62[A]	6.76±0.60	7.97±0.50[A]	7.33±0.79	<0.0001	0.0027
22:5n6	1.03±0.42	0.48±0.22	0.70±0.31	–	0.45±0.23	0.03±0.03	0.43±0.22	0.33±0.29	1.30±0.46	0.57±0.24	0.58±0.28	0.34±0.23		

Values are mean ± SEM (n=5 to 6 for each group)
Uppercase superscript letters on the female values within a row indicate significant differences between diets
Lowercase superscript letters within a row indicate differences between means
Only significant *P* values were reported

P values shaded blue or pink indicate main sex effects with higher levels of oxylipins in males and females, respectively
P values in between diet and sex columns indicate interaction between diet and sex unless noted with superscript $
[$]Indicates Kruskal-Wallis test when data is not normally distributed
[#]Indicates fatty acids not detected

Higher than control + 1 group	Lower than control + 1 group
Higher than control and 2 or 3 other groups	Lower than control and 2 or 3 other groups
Higher than all other groups	Lower than all other groups

Supplementary Table S4.9 Total epoxy and dihydroxy-fatty acids in brain from rats provided control, ALA, EPA, DHA, LA and LA+ALA diets for 6 weeks

Oxylipin	Control		ALA		EPA		DHA		LA		LA+ALA		P value	
	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Diet	Sex
						ng/g								
Epoxy-ARA	0.83±0.16^{bc}	1.04±0.37^{bc}	0.89±0.20^{bc}	0.51±0.05^{c}	3.11±0.48^{a}	1.12±0.29^{bc}	0.59±0.03^{c}	0.65±0.10^{c}	0.85±0.11^{bc}	1.55±0.46^{b}	0.64±0.10^{c}	1.01±0.16^{bc}	0.0039$^{\$}$	
Dihydroxy-ARA	0.72±0.09	0.55±0.11	0.68±0.07	0.76±0.06	0.71±0.08	0.74±0.12	0.67±0.06	0.65±0.13	0.76±0.04	0.94±0.14	0.72±0.10	0.75±0.08		
Total Epoxy-PUFA	1.66±0.30^{b}	2.23±0.75^{b}	2.11±0.42^{b}	2.40±0.49^{b}	5.55±0.72^{a}	1.87±0.25^{b}	2.02±0.43^{b}	1.75±0.26^{b}	1.70±0.26^{b}	2.96±0.82^{b}	1.75±0.38^{b}	2.30±0.28^{b}	0.0001$^{\$}$	
Total Dihydroxy-PUFA	18.1±2.63	10.8±0.95	13.6±2.63	13.6±2.11	12.3±1.33	15.7±3.58	12.3±1.49	15.5±3.69	14.7±1.36	17.7±3.09	13.8±2.18	17.4±2.99		

Values are mean ± SEM (n=5 to 6 for each group)
Superscript letters within a row indicate differences between means
$^{\$}$Indicate Kruskal-Wallis test when data is not normally distributed
Values shaded green indicate higher compared to all other values within a row
Only significant *P* values were reported

Supplementary Table S4.10 Oxylipin/PUFA (in PL) ratios for enzymes in brains from rats provided control, ALA, EPA, DHA, LA and LA+ALA diets for 6 weeks

a. Cyclooxygenase/PGD Synthase

	PGD_1/DGLA		PGD_2/ARA		PGD_3/EPA			
	Female	Male	Female	Male	Female	Male	P value	
Diet	nmol/mmol						PUFA	Sex
Control	11.4±1.52	12.1±2.44	18.9±3.31	22.2±3.80	18.6±10.3	-[#]		
ALA	10.6±0.55[B]	14.1±3.48	20.3±1.07[A]	26.3±4.47	20.9±2.87[A]	32.0±3.86	0.0002	0.0102
EPA	11.5±0.78[C]	10.6±0.99	21.3±1.57[B]	21.4±1.91	27.0±2.29[A]	32.2±3.58	<0.0001	
DHA	9.56±1.00[B]	10.2±2.46	21.7±1.14[A]	27.9±3.78	22.4±2.43[A]	28.8±3.57	<0.0001	0.0384
LA	9.94±2.19	11.9±1.69	15.9±3.13	22.6±2.66	23.3±7.66	18.3±3.78	0.0358	
LA+ALA	9.49±0.91[B]	13.2±2.65	16.1±1.18[A]	23.1±3.09	37.0±13.2[A]	31.5±10.7	0.0015	

b. 5-Lipoxygenase

	9-HODE/LA		5-HETE/ARA		9-HOTrE/ALA		5-HEPE/EPA		7-HDoHE/DHA		P Value	
	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male		
Diet	nmol/mmol										PUFA	Sex
Control	54.1±11.6[ab]	79.5±24.9[ab]	15.9±3.96[b]	31.7±16.0[b]	-	-	173±49.8[a]	-	2.14±0.69[c]	1.91±0.66[c]	<0.0001[$]	
ALA	59.1±8.44[c]	66.8±16.2[c]	14.5±1.86[d]	29.5±11.5[d]	149±33.5[b]	274±62.3[a]	96.9±11.7[bc]	190±50.3[b]	1.52±0.33[e]	1.60±0.31[e]	0.0448	
EPA	53.9±3.05[B]	55.0±12.7	17.9±2.05[C]	19.8±4.51	-	-	133±23.9[A]	165±31.0	1.84±0.18[D]	1.56±0.14	<0.0001	
DHA	49.4±10.8[B]	69.7±13.7	15.8±1.81[C]	17.9±5.34	-	-	121±14.9[A]	130±38.7	1.63±0.19[D]	1.19±0.23	<0.0001	
LA	66.9±10.6[B]	64.9±10.3	14.7±2.90[C]	25.0±5.57	-	-	222±20.4[A]	137±0.75	1.45±0.33[D]	2.02±0.41	<0.0001	
LA+ALA	34.9±5.24[B]	63.6±9.27	12.2±2.38[C]	20.6±5.87	-	-	275±61.7[A]	231±111	1.38±0.26[D]	1.50±0.37	<0.0001	

c. 12- Lipoxygenase

	12-HETE/ARA		12-HEPE/EPA		14-HDoHE/DHA			
	Female	Male	Female	Male	Female	Male	P Value	
Diet	nmol/mmol						PUFA	Sex
Control	6.83±1.55^{b}	7.68±2.67^{b}	150±29.0^{a}	-	4.14±1.11^{b}	3.39±1.40^{b}	0.0118$^{\$}$	
ALA	5.52±0.55^{B}	8.68±1.51	144±19.9^{A}	216±21.1	1.99±0.29^{C}	4.22±1.05	<0.0001	0.0014
EPA	5.58±0.63^{B}	7.38±1.09	169±27.5^{A}	223±27.1	2.37±0.37^{C}	4.48±0.84	<0.0001	0.0046
DHA	5.65±0.54^{B}	7.10±0.70	112±12.5^{A}	172±30.7	3.33±0.53^{C}	5.33±0.90	<0.0001	0.0068
LA	7.08±1.34^{B}	8.92±2.00	170±27.0^{A}	110±19.7	2.28±0.40^{C}	3.26±0.66	<0.0001	
LA+ALA	5.58±0.63^{b}	6.42±0.92^{b}	146±12.6^{a}	104±15.4^{a}	1.61±0.25^{c}	2.62±0.31^{c}	0.0192	

d. 15- Lipoxygenase

	13-HODE/LA		15-HETrE/DGLA		15-HETE/ARA		13-HOTrE/ALA		15-HEPE/EPA		17-HDoHE/DHA			
	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	P Value	
Diet	nmol/mmol												PUFA	Sex
Control	64.7±5.97^{A}	46.2±7.25	5.23±0.95^{C}	7.08±2.27	24.9±7.04^{B}	21.9±4.07	-	-	-	-	10.1±2.36^{C}	10.2±4.38	<0.0001	
ALA	54.9±5.73^{B}	70.8±16.3	4.97±0.23^{D}	8.95±3.58	22.3±1.68^{C}	43.6±13.8	349±65.3^{A}	755±138	28.3±4.00B^{C}	68.1±12.4	5.85±0.35^{D}	10.7±2.42	<0.0001	0.0002
EPA	56.4±3.37^{A}	72.6±15.4	5.41±0.75^{C}	7.56±1.20	27.8±3.11^{B}	39.7±9.91	-	-	33.2±4.32^{AB}	66.8±9.73	8.32±1.10^{C}	11.0±1.80	<0.0001	0.0036
DHA	44.2±8.77^{A}	66.4±12.6	5.31±0.60^{C}	5.95±1.02	25.8±3.85^{B}	40.0±10.8	-	-	26.7±4.38^{AB}	39.5±4.75	7.27±0.79^{C}	9.96±1.65	<0.0001	0.0083
LA	67.1±7.73^{A}	66.9±9.19	4.22±0.84^{C}	7.06±1.87	22.1±3.78^{B}	35.0±7.84	-	-	-	-	6.28±0.88^{C}	9.40±1.72	<0.0001	0.0202
LA+ALA	39.1±3.64^{ab}	64.9±6.23^{a}	3.33±0.45^{d}	5.63±0.83^{cd}	18.7±2.73^{b}	30.7±7.18^{b}	-	-	104±31.5^{a}	40.4±3.19^{ab}	4.67±0.49^{cd}	7.38±1.02^{c}	0.0060	

e. Cytochrome P450 Epoxygenase

	12,13-EpOME/LA		14,15-EpETrE/ARA		12,13-EpODE/ALA		16,17-EpDPE/DHA			
	Female	Male	Female	Male	Female	Male	Female	Male	P value	
Diet	nmol/mmol								PUFA	Sex
Control	0.70±0.26^{A}	0.38±0.06	0.13±0.05^{B}	0.16±0.08	-	-	0.38±0.15^{AB}	0.52±0.20	0.0225	
ALA	2.01±0.84^{b}	2.60±0.69^{b}	0.12±0.04^{cd}	0.07±0.02^{d}	21.9±2.96^{a}	64.5±9.77^{a}	0.30±0.09^{c}	0.17±0.02^{cd}	0.0279	
EPA	0.66±0.15^{A}	0.36±0.04	0.30±0.08^{B}	0.18±0.06	-	-	0.49±0.10^{AB}	0.18±0.03	0.0152	0.0024
DHA	0.84±0.24^{A}	0.88±0.21	0.09±0.02^{C}	0.07±0.02	-	-	0.28±0.07^{B}	0.21±0.03	<0.0001	
LA	0.43±0.09^{A}	0.43±0.16	0.07±0.01^{B}	0.21±0.05	-	-	0.17±0.03^{AB}	0.34±0.10	0.0057	
LA+ALA	0.44±0.20^{A}	0.70±0.25	0.21±0.14^{B}	0.11±0.03	-	-	0.62±0.28^{A}	0.37±0.20	0.0177	

f. Cytochrome P450 Hydroxylase

	18-HETE/ARA		18-HEPE/EPA		20-HDoHE/DHA			
	Female	Male	Female	Male	Female	Male	P Value	
Diet	nmol/mmol						PUFA	Sex
Control	0.20±0.09^{B}	0.17±0.06	-	-	9.79±2.29^{A}	5.48±0.97	<0.0001	
ALA	0.12±0.01^{C}	0.18±0.05	56.9±9.35^{A}	75.1±9.48	5.33±0.54^{B}	10.6±3.39	<0.0001	
EPA	0.16±0.03^{C}	0.20±0.04	62.7±4.84^{A}	124±36.3	6.98±1.00^{B}	10.9±3.29	<0.0001	
DHA	0.18±0.05^{C}	0.18±0.08	59.1±6.24^{A}	67.1±19.8	7.02±1.07^{B}	9.12±2.92	<0.0001	
LA	0.11±0.02^{c}	0.14±0.01^{c}	-	103±12.9^{a}	7.32±1.28^{b}	8.23±1.65^{b}	0.0004$^{\$}$	
LA+ALA	0.12±0.02^{c}	0.14±0.02^{c}	144±5.43^{a}	99.8±10.5^{a}	4.06±0.49^{b}	7.41±1.42^{b}	0.0308	

Values are mean ± SEM (n=5 to 6 for each group)
Uppercase superscript letters on the female values within a row indicate significant differences between diets
Lowercase superscript letters within a row indicate differences between means
Only significant *P* values were reported

P values shaded blue or pink indicate main sex effects with higher levels in males and females, respectively
P values in between column PUFA and sex indicates interaction unless noted with superscript $
[$]Indicates Kruskal-Wallis test when data is not normally distributed
[#]Indicates ratios for which the numerator or denominator was zero (not detected)

4.5 Discussion

The current study provides fundamental data on the rat whole brain oxylipin profile. These data show that while DHA is the predominant PUFA, the oxylipins in the brain are primarily derived from ARA. This is not simply a function of more ARA oxylipins being screened for, since the mass of each ARA oxylipin was always higher than the mass of its DHA analog (e.g. 15-hydroxy-eicosatetraenoic acid (15-HETE) vs 17-HDoHE). This preponderance (>80% of total) of ARA oxylipin mass in the brain differs from the pattern observed in other tissues in these rats. In kidney, adipose [28, 30] and heart (Chapter 3), oxylipins derived from LA have the highest mass, and in liver the ARA oxylipins have the greatest mass, but only make up approximately 40% of the total [28]. Interestingly, serum has similarly high levels of ARA oxylipins, but very low levels of DHA oxylipins [28, 29]. The rat whole brain oxylipin profile reported herein was similar to the female mouse whole brain profile [13, 23]. In all tissues, including the brain, the oxylipin levels do not necessarily reflect PUFA levels, demonstrating that PUFA levels cannot be used to predict oxylipin levels and need to be measured directly.

Compared to other tissues, brain oxylipins exhibit much more resistance to diet-induced changes in composition, as illustrated when comparing the brain to other tissues in these rats: 1) dietary n-3 PUFA had minimal effects on ARA oxylipins in the brain, but reduced ARA oxylipins to varying extents in all other sites examined (kidney, liver, adipose, serum) [28-30] and heart (Chapter 3) in these rats, 2) dietary ALA did not alter the levels of DHA oxylipins in the brain, but in the kidney, serum and some adipose sites many DHA oxylipins were increased with higher dietary ALA, even when DHA levels were not altered [29, 30], 3) dietary LA or LA+ALA did not alter any n-6 derived oxylipins in the brain, but in all other sites examined (kidney, liver, adipose, heart, serum) in these rats, these high LA diets increased LA oxylipins, and in the

kidney and liver it also increased ARA oxylipins, even when tissue ARA levels did not change (Chapter 3) [28, 30], and 4) dietary PUFA had little effect on the sex differences in brain oxylipins, but providing DHA compared to the other diets resulted in fewer oxylipins that were higher in female hearts (Chapter 3) and adipose tissue [30] in these rats. This tighter regulation of oxylipin levels in the brain relative to other tissues suggests that these oxylipins may be critical for proper brain functioning.

This relative resistance of brain oxylipins to change in response to dietary PUFA is consistent with the brain's ability to maintain ARA and DHA levels when an n-6 or n-3 PUFA deficient diet is provided to rats [46-49]. In these studies, it also was shown that the levels of oxylipin synthesizing enzymes (Phospholipase A_2 and COX) specific for ARA and DHA were modulated accordingly, and it was hypothesized that this helped maintain their levels. It is not clear whether opposite adaptations occur with the added dietary n-3 and n-6 PUFA in the current study. In addition, the levels of oxylipins in brain, as in other tissues, are 2-3 orders of magnitude lower than PUFA levels, so other metabolic adaptations are also likely to occur to conserve oxylipin levels. Increased LA compared to very low dietary LA can increase LA and ARA oxylipins and reduce EPA oxylipins in the rat cerebral cortex [26]. This was not observed in the current study, possibly because the control rats had adequate levels of dietary LA and the differences in dietary LA between diets were not as great, or because regional brain effects were masked by our whole brain analyses.

As the main group of oxylipins in the brain, ARA oxylipins function as regulators and protectors of normal brain homeostasis [2]. For example, a lipoxin A_4 analogue is neuroprotective and reduces inflammation in a rat model of cerebral ischemia reperfusion injury [50],15-deoxy-PGJ_2 has antipyretic effects associated with reduction in LPS-induced COX-2 expression in the

hypothalamus [51], 8,9- and 11,12-EpETrE from astrocytes have angiogenic and mitogenic effects in cultured cerebral capillary endothelial cells [52], 11,12-EpETrE attenuates interleukin-1β (IL-1β) induced fever in the anterior hypothalamus [53], 11,12- and 14,15-EpETrE have vasodilatory effects in the cerebral microcirculation [54] and astrocyte 14,15-EpETrE enhances cell viability in oxidation induced ischemic injury [55, 56]. On the other hand, abnormal levels of ARA oxylipins are also associated with brain dysfunction, such as PGE_2, TxB_2 and leukotriene B_4, which increase production of Aβ peptides that contributes to the formation of amyloid plaques in the development of Alzheimer's disease (57), 12-HETE, a marker of brain injury [58] and 20-HETE, which contributes to vasoconstriction and vasospasm in brain, which has detrimental effects in ischemic stroke [59].

Evidence for brain and CNS roles for the next largest group of oxylipins in the brain, namely DHA oxylipins, also have been reported. For example, EpDPEs have strong anti-hyperalgesic (pain-relieving) effects in spinal cords of rats [22], PDX inhibits leukocyte infiltration, nuclear factor-κB and COX-2 induction in a mouse model of ischemic stroke [60], PD1 inhibits apoptosis and suppresses Aβ42-induced neurotoxicity [61] and RvD1 promotes the resolution of inflammation in microglial cells *in vitro* by decreasing LPS-induced expression of tumor necrosis factor- α, IL-6 and IL-1β [14].

Functions for LA oxylipins in the brain, which made up 3-5% of oxylipin mass, have also been reported in a few studies. Reduced chronic headaches have been associated with reduced levels 9- and 13-HODE in plasma [62]; 9-HODE, 13-HODE, 9-oxoODE and 13-oxo-ODE released from depolarized rat spinal cord induce pain by activating transient receptor potential vanilloid 1 [63]; and oxidative stress in the aging brain may be associated with higher level of HODEs and dihydroxy-octadecenoic acids [15]. Minor brain oxylipins that were also affected by

diet were the ALA, EPA, DGLA and AdA derived oxylipins, but these oxylipins represent <1% of oxylipins [except for slightly higher levels of EPA oxylipins in rats given the EPA (<3%) and DHA diets (<2%)]. Roles for EPA oxylipins in the brain have been suggested in a few studies: 18-HEPE in remyelination after toxic injury to CNS oligodendrocytes [64] and RvE1 in the resolution of inflammation in microglial cells *in vitro* [14]. To our knowledge, no effects of DGLA, AdA or ALA derived oxylipins have been reported in mammalian brain.

One exception to the resistance to change in ARA oxylipins with dietary n-3 PUFA was that EpETrE were elevated in females provided the EPA diet. Other epoxy-PUFA oxylipins also were elevated in females provided the EPA diet, resulting in higher levels of total epoxy-PUFA oxylipins in this group. We also have observed unique effects of dietary EPA on other specific n-6 PUFA in other tissues. For example: all ARA oxylipins except specific hydroxyPG dehydrogenase metabolites in kidney, liver and serum were lower in rats provided the EPA diets [28, 29]. Similarly, higher levels of the hydroxyPG dehydrogenase metabolite of PGD_2, namely 13,14-dihydro-15-k-PGD_2, has been reported in human plasma after dietary intervention with individual EPA or DPA [65]. This would suggest that there are some enzymes in some tissues that are uniquely affected by dietary EPA in particular, but at this time there is no explanation for these apparent exceptions. However, these apparent anomalies are worth noting, in case these patterns are confirmed in other situations and an explanation becomes apparent in the future.

Data on sex differences in the brain oxylipin profile have not been reported to date. Similar data in other tissues in these rats reveals unique tissue and diet effects on oxylipin sex differences – oxylipins are generally higher in males for serum, kidney and liver [28, 29], and higher in females in several adipose tissues [30] and in the heart (Chapter 3). In adipose and heart, however, these differences depended on which diet the rat was consuming. In the brain,

oxylipins that did display sex differences were mostly higher in male rats and this was not altered by diet. As is the case with the other tissues, the levels of precursor PUFA do not explain these differences. As is also the case with the other tissues, these findings of specific sex differences in oxylipin profiles in the brain are novel, so the mechanisms by which this occurs still need to be explored.

Differences between PUFA and oxylipin patterns suggest that the synthesis or turnover of oxylipins varies depending on the PUFA precursor. As such, the oxylipin/precursor ratios were higher for n-3 vs n-6 ratios, and were higher for ratios derived from PUFA with 18C compared to 20C and 22C PUFA. This pattern also was observed in all other tissues examined for these ratios in kidney, liver, serum [28, 29] and heart (Chapter 3) in these rats, as well as in other rats, and *in vitro* enzymes studies [40, 66-68]. This suggests that dietary n-3 and 18C PUFA could have proportionately greater effects on tissue oxylipins, compared to their n-6 and longer-chain counterparts.

There are several important limitations for this data. At termination, brains were quickly removed from the rats and immediately frozen in liquid N_2 and stored at -80 ºC. However, the time between death and freezing would have resulted in changes in oxylipins compared to a procedure such as microwave fixation, an effective method to prevent postmortem oxylipin changes in the brain [69, 70]. Second, whole brains were analyzed, so unique oxylipin profiles in specific regions could not be detected. Third, over 160 oxylipins were screened, but there may be many other oxylipins present in the brain that have yet to be identified and for which no standards exist. This could significantly affect the total and relative number of n-3 and n-6 oxylipins quantified. Fourth, the protocol used in this study is for the analysis of free oxylipins. These are presumably the most active form but esterified oxylipins can be a major component of

the total oxylipins [71]. However, esterified oxylipin extraction requires a base hydrolysis step which potentially degrades a large number of oxylipins including PGs and Txs [72], and changes in the ratio of free versus esterified oxylipins in blood did not change after n-3 PUFA supplementation [71]. Finally, different solid phase extraction methods vary in their efficacy of oxylipin extraction; however, the method used in this study is the most efficient in general, although some of the ARA derived CYP oxylipins may not be extracted as well as by other procedures [23, 73, 74].

4.6 Conclusion

In conclusion, this study provides novel fundamental data on the rat brain oxylipin profile and the modulation of oxylipin levels in male and female rats provided increased levels of dietary n-3 and n-6 PUFA. We hypothesized that oxylipins derived from ARA, DHA, EPA, LA and ALA are present at quantifiable level and we quantified 87 oxylipins derived from these PUFA in the rat brain. This provides impetus for further studies on the many oxylipins detected, but for which no function in the brain has yet been reported. Compared to other tissues, the brain has a much higher proportion of ARA oxylipins despite not being the predominant PUFA present. The brain also is much more resistant to dietary modulation of its oxylipins. Oxylipins displaying sex differences were mostly higher in male rats, and diet also did not alter these differences, further revealing tight control of oxylipin levels in the rat brain.

4.7 References for chapter 4

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Chapter 5

5. General discussion

5.1 Introduction

The data presented herein examined the effects of dietary PUFA and sex on the heart and brain oxylipin profile in healthy rats. This study provided novel data on the comprehensive oxylipin profile in these tissues and revealed dietary effects of individual n-3 and n-6 PUFA on these profiles. N-3 PUFA derived oxylipins in the heart and brain are modulated not only by their direct precursor PUFA in the diet but also by the other n-3 PUFA that are not their direct precursors. In the heart, dietary n-3 PUFA (DHA>EPA>ALA) also reduced n-6 oxylipins especially those derived from ARA. On the other hand, dietary n-6 PUFA (LA) have little effect on modulating both n-6 and n-3 derived oxylipins in these tissues. This study also revealed sex differences in the heart and brain oxylipin profiles. Oxylipins with main sex effects generally have higher levels of oxylipins in the female rats in the heart but higher levels in males in the case of brain. Sometimes oxylipin sex differences also interact with diets as observed in the heart where rats given the DHA diet had similar levels of oxylipins in female and male rats. However, oxylipins in the brain show few dietary interactions with sex. Thus, the current study provides fundamental data on the heart and brain oxylipin profile in the rat with unique and interactive effects of dietary PUFA and sex on these profiles.

5.2 Effects of dietary PUFA on heart and brain oxylipins

Analysis of heart and brain oxylipins revealed the distinct effects of EPA, DHA and ALA rich oils on oxylipins synthesized from n-3 PUFA. N-3 oxylipins were higher in rats provided the diets rich in their own precursor PUFA, but these changes were not as great as has been observed in other tissues such as the kidney, liver, serum and several adipose sites examined in these rats [1, 2]. N-3 oxylipins in the heart and brain were also modulated by other dietary n-3 PUFA that are not their direct precursors. For example, EPA oxylipins in the heart and brain were elevated in all three n-3 PUFA rich diets, with greater effects being observed in the heart than in the brain. Similar but greater, effects also have been observed in other tissues examined in these rats [1, 2]. For example, DHA oxylipins in the heart and brain are only elevated in the presence of DHA, unlike the dietary effects observed in kidney and some adipose (subcutaneous, mesenteric and perirenal adipose) sites where DHA oxylipins were elevated in rats provided the ALA diets also. Also, unlike the dietary effects observed in liver, where some ALA oxylipins were higher in rats provided dietary EPA and DHA; and in kidneys and some adipose (gonadal and mesenteric) depots, where some ALA oxylipins were lower in rats provided dietary EPA and/or DHA, heart and brain ALA oxylipins did not change in the presence of other n-3 PUFAs [1, 2].

Dietary n-3 PUFAs also could modulate heart n-6 oxylipins by reducing n-6 oxylipins, especially those derived from ARA. The order of effectiveness of ARA oxylipin lowering in the heart by dietary n-3 PUFAs was in the order: DHA>EPA>ALA diets, consistent with what has been observed in studies with fish oil feeding in diseased models [3-5]. These effects of dietary n-3 PUFA in reducing ARA oxylipins could be beneficial, as some ARA oxylipins have detrimental effects such as vasoconstrictive and arrhythmic effects in animal cardiac myocytes [6-9]. This pattern was similar to what has been observed in other tissues in these rats including the kidney, liver, serum and several adipose (gonadal, mesenteric and perirenal) depots [1, 2].

However, in contrast to the heart, LA oxylipins were also decreased by n-3 PUFA in several adipose (gonadal, mesenteric, subcutaneous and perirenal) depots in the same rats [1].
On the other hand, dietary n-3 PUFA had negligible effects on brain n-6 oxylipins including those derived from ARA (the major oxylipin class in the brain).

Dietary LA (the only n-6 PUFA provided in this feeding trial) and LA+ALA diets, elevated only LA oxylipins, and had minimal effects on other n-6 oxylipins including ARA oxylipins in the rat heart. In the brain, dietary LA has no effects on ARA oxylipins but few effects on DGLA and AdA derived oxylipins (their functions are not well characterized yet). In comparison, in kidney and liver, dietary LA increased LA as well as ARA oxylipins to varying extents [10, 11]. These findings suggest that brain and heart ARA oxylipins are more resistant to changes by dietary n-6 PUFA.

Dietary n-6 PUFA (LA) also did not affect either heart or brain n-3 oxylipins. Thus, maintaining steady levels of oxylipins even in the presence/absence of dietary LA is possibly critical for optimum heart and brain function.

5.3 Exceptions to dietary PUFA effects on heart and brain oxylipins

While dietary n-3 PUFA reduced tissue ARA levels in the kidney, liver, serum, some adipose (gonadal, mesenteric and perirenal) sites [1, 2, 10] and in human plasma [12], certain PG metabolites of ARA such as bicyclo-PGE_2 in liver and 15-k-PGE_2 in serum were increased. Our study also observed similar types of exceptions in the heart. For example, 15-d-PGD_2 in EPA male, PGJ_2 in DHA male and 15-k-PGE_2 in (both male and female) EPA rats were increased in the rat heart of our study. PGDH is the enzyme that forms these metabolites from PGs [13] and their functions are not characterized in any of the above tissues, including in the heart. In the case of brain, such exceptions for PG metabolites were not observed, but two EpETrEs were elevated in EPA

female rats. As well, epoxy-oxylipins from other PUFAs in the brain were also elevated in EPA females. However, if this exception is due to selectivity of CYP-epoxygenase enzymes towards EPA in females remained to be elucidated.

5.4 Sex effects on heart and brain oxylipin profile

Sex effects were observed in different tissues in these rats: males usually had higher levels of oxylipins in kidney, liver and serum [2, 10]. Thus, sex effects were also investigated in the rat heart and brain in this study. Oxylipins with sex effects were usually higher in the female rats in the heart but were higher in male rats in the brain. In several adipose sites, sex effects interacted with diet effects, where oxylipins were higher in females in gonadal and subcutaneous adipose sites when provided ALA or EPA diets but higher in males when provided DHA diets [1]. In the heart, similar to adipose, sometimes sex interacted with diet where oxylipins were higher in females when provided with EPA or ALA diets but higher in males when provided with DHA diets. In the case of brain, oxylipins with sex effects were resistant to changes by diet. These are considered to be novel findings as sex effects have not been examined before in these tissues, to our knowledge. However, the mechanism by which sex itself or along with diet affects oxylipin levels largely remains unexplored in any tissues.

5.5 Apparent PUFA selectivity for conversion into oxylipins

Similar to sex effects, apparent selectivity in the conversion of PUFA to oxylipins was also observed in heart and brain tissues. Turnover of oxylipins was predicted based on oxylipin/PUFA ratios in heart and brain tissues. Considering PUFA type, the ratio was higher for n-3 compared to n-6 PUFA, and considering chain length, it was higher for 18C compared with their longer chain counterparts. This result is consistent with other tissues examined in these rats

[2, 10] and in other rats [11]. Although changes in the oxylipin/PUFA ratio are not sufficient to indicate whether it is due to changes in conversion of PUFA to its respective oxylipins or due to lower degradation of the oxylipins, studies that have reported selectivity for oxylipin synthesizing enzymes have shown a higher selectivity for CYP-epoxygenase enzymes for EPA and DHA compared to ARA [14, 15], and better enzyme-substrate compatibility for LA than ARA for 15-LOX enzyme [16].

5.6 Conclusions

In conclusion, the current studies provide novel fundamental data on the heart and brain oxylipin profiles and the effects of higher levels of individual dietary n-3 and n-6 PUFA in male and female rats in heart and brain. This study quantified 75 and 87 oxylipins at quantifiable levels in the rat heart and brain, respectively for which no functional effects have been reported yet. This study also revealed that heart has more epoxy-oxylipins compared to other tissues examined in these rats, and that brain has more ARA than DHA oxylipins, despite DHA being the most abundant PUFA in this tissue. While most of the ARA oxylipins in the heart were reduced by n-3 diets, n-6 oxylipins in the brain were minimally affected by dietary PUFA. Oxylipins with sex effects are predominantly higher in female rats in the heart, while in the brain they are mostly higher in male rats. In the heart, sometimes higher level of oxylipins in one sex is dependent on the which PUFA is provided in the diet, while in the brain such interactions were absent, indicating tighter control of oxylipin levels in the brain than in the heart. Thus, dietary PUFA and sex effects in these comprehensive oxylipin profiles are unique for each tissue, provides fundamental data on which oxylipin to target for functional effects and how dietary PUFA modulates their levels in female and male rats.

5.7 Future directions

The findings revealed from this study requires further investigation to pinpoint its implications. The regional distribution of heart (atria and ventricle) and brain (cerebral cortex, hippocampus and cerebellum) oxylipins could be analyzed in rats under the same dietary conditions and could be compared with the current findings from whole tissues. Data on the regional distribution of oxylipins in the heart and brain will be helpful since individual (atria, ventricle, cerebral cortex, hippocampus and cerebellum) regions in these tissues play important functional roles in physiology. Thus, (novel) oxylipin changes in these regions can be noted for any physiological changes. Since oxylipin levels in heart and brain were different in male and female rats and were not explainable from the existing literature, ovariectomized, orchidectomized and normal healthy male and female rats can be included in future studies to examine the influence of sex hormones on rat oxylipins.

5.8 Strengths and limitations

This fundamental study allowed us to understand and compare the effects of varying levels of ALA, EPA, DHA and LA in the rat heart and brain oxylipin profile without changing other PUFA in the diet. It also allowed us to evaluate unique sex differences in these oxylipin profiles. It has provided a side by side comparison of effects of individual EPA and DHA enriched fish oils on oxylipin profiles in these rat tissues. The study was conducted on growing rats, which allowed us to observe the highest effects of individual dietary PUFA since at this stage changes are rapid. This study was conducted on Sprague Dawley rats for which research findings are easily comparable with the existing, but insufficient oxylipin profiling studies where most of the times rats were used as experimental animals. The experimental diets we used were isocaloric to AIN93G diet suitable for growing rats but with 3% higher oil content in each diet (replacing isocaloric amount of carbohydrates from the AIN93G diets) and allowed us to examine and

compare individual dietary PUFA effects on the oxylipin profile in these rats. This study also allowed us to compare oxylipin levels in the male and female rats at the same age, under the same dietary treatment and experimental conditions which was largely unexplored in the existing literature.

However, this study also has some potential limitations. This study used whole heart and brain tissues which potentially could affect the precise oxylipin composition in individual compartments of heart and brain tissues since evidence suggests heart has uneven fatty acid distribution, and both fatty acids and oxylipin distributions in different brain compartments are different. Rat heart and brain tissues were frozen immediately after termination using liquid N_2 and stored at -80 ºC to avoid post mortem changes. However, extra-cardiac blood from veins and arteries remained inside the heart as blood was not pumped out, and the time between death and freezing would have resulted in changes in brain oxylipins due to oxidative stress. Although before homogenization each heart was rinsed out with Tyrode's salt (pH 7.6), still remaining blood might contribute to the level and number of oxylipins detected and quantified. Over 160 oxylipins were screened in these tissues, but it is likely that other oxylipins are present in these tissues for which no standards exist; therefore these could not be identified yet, but would significantly affect the total and relative number of n-3 and n-6 oxylipins detected and quantified. Also, only the free form of oxylipins was analyzed in this study and not the esterified oxylipins. However, there is no evidence on how they are correlated to each other although it was reported that dietary modulation affects both forms of oxylipins in a similar manner [17]. For oxylipin extraction, different solid phase extraction methods may vary in their efficacy [18, 19], but the method used in this study is usually the most efficient [19], although some of the CYP pathway derived ARA oxylipins may not be extracted as efficiently using this method [18, 19].

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Appendices

A: Ingredient, oil and fatty acid composition of the diets

	Control	ALA	EPA	DHA	LA	LA+ALA
Diet ingredients	g/100g diet					
Cornstarch	34.9	34.9	34.9	34.9	34.9	34.9
Casein (87%) (Protein)	20.7	20.7	20.7	20.7	20.7	20.7
Dextrinized cornstarch	13.7	13.7	13.7	13.7	13.7	13.7
Sucrose	10.3	10.3	10.3	10.3	10.3	10.3
Fiber	5.2	5.2	5.2	5.2	5.2	5.2
Mineral mix (AIN93G)	3.6	3.6	3.6	3.6	3.6	3.6
Vitamin mix (AIN 93)	1	1	1	1	1	1
L-Cystine	0.3	0.3	0.3	0.3	0.3	0.3
Choline	0.3	0.3	0.3	0.3	0.3	0.3
TBHQ	0.002	0.002	0.002	0.002	0.002	0.002
Olive oil	7	-		-	-	-
Flax oil	0.15	6.4		0	-	-
EPA oil	-	-	3.3	-	-	-
DHA oil	-	-		3.3	-	-
Safflower oil	-	-	-	-	4.3	-
Soy oil	2.2	1.3	3.8	3.8	3.8	10
Coconut oil	0.65	2.3	2.9	2.9	1.9	-
Total Diet	100	100	100	100	100	100
Fatty acids in the diet	g/100g					
ALA	0.27	3.43	0.27	0.27	0.28	0.71
EPA	0	0	3.14	0.01	-	-
DHA	0	0	0.01	3.14	-	-
LA	2.13	1.77	2.09	2.09	5.21	5.35
Saturated Fatty Acid	1.93	2.39	2.64	2.65	2.37	1.56
Unsaturated Fatty Acid	6.63	6.75	6.62	6.55	7.16	8.15
MUFA	4.22	1.53	0.99	0.98	1.66	2.07
PUFA	2.41	5.22	5.63	5.58	5.5	6.08
LA/ALA	7.76	0.52	7.63	7.66	18.31	7.5
n6/n3 Ratio	7.74	0.52	0.64	0.63	18.11	7.45

B: Protocol for heart and brain homogenization

Tyrode's (pH 7.6) salt solution was used to homogenize wet heart and brain tissues. For every ~ 350 mg wet tissue, 2000 uL of Tyrode's (pH 7.6) salt solution was added. Before homogenization, Tyrode's (pH 7.6) salt solution was also used to rinse out extra-cardiac blood from heart. Before starting homogenization, the following solutions were prepared:

- Tyrode's salt solution (pH 7.6) (see appendix H2 for details protocol)
- 1% Triton Solution as per instructions below (see appendix H3 for details protocol)
- 12 mL test tubes with lids that have been soaked overnight in Contrad solution, rinsed and dried
- 100:1 methanol and formic acid (for oxylipins)
- pH 3 water – (water that has had pH adjusted to 3.0 using 1M HCL) (for oxylipins)
- Antioxidant Cocktail (for oxylipins and fatty acids) (see appendix H4 for detailed protocol)

Frozen heart and brain samples were thawed in a large container of ice after removing from the -80C freezer in small batches. Labelled 16 x 125 mm disposable glass test tubes were marked with sample IDs for homogenizing the whole tissues. Weight of the tissues and required volume of Tyrode's salt solution (pH 7.6) calculated according to (350 mg wet tissue = 2000 uL of Tyrode's), were recorded in the lab notebook.

Three disposable glass tubes (16 x 125mm) were prepared and labelled with 100% ethanol and three disposable glass tubes (16 x 125mm) with ultrapure water for cleaning the homogenizer before use, after use, and in between each sample by:

- 3 tubes ethanol x 30 seconds each at **speed 15**
- 3 tubes ultrapure water x 30 seconds each at **speed 15**
- dab with Kimwipe to dry

- change ethanol or water when they become very cloudy

Test tube containing wet heart or brain tissue was placed in a small plastic container (yogurt container) containing an ice slurry (ice plus water). The rotor of the homogenizer was inserted into the test tube and homogenized at **speed 20** for 30 seconds avoiding bubble generation. If bubbles were generated for any samples, it was recorded in the lab notebook for follow-up. Before transferring tissue homogenate into 20 mL glass scintillation vials, the homogenization tube was checked to ensure that no solid tissue was left at the bottom of the tube. The rotor was cleaned with ethanol and ultrapure water before and after homogenization of each sample. Any tools contaminated with biological hazards were wiped off with 10% bleach and then washed normally with Contrad / other detergent. Homogenates were aliquoted for oxylipin and fatty acid extraction in duplicate vials.

For oxylipin extraction, 400 µL of tissue homogenate was aliquoted into a 2 mL plastic microcentrifuge tube. After adding 1 µL of a 1% Triton solution for every 100 µL aliquot for oxylipin analysis, the sample was vortexed for 10 seconds, then incubated and covered on ice for 10 minutes. This vortex and incubation continued for three times after every 10 minutes. Working quickly, the following reagents were added in the same order below to the samples:

a. 500 µL of 100:1 methanol formic acid
b. 600 µL of pH3 water
c. 90 µL of 100% ethanol
d. 10 µL of antioxidant cocktail
e. Vortex for 5 seconds.

These homogenate extracts were then quickly vortexed for 5 seconds and stored in a -80°C freezer for future oxylipin extraction (protocol available on appendix C).

For fatty acid extraction, 250 µL of tissue homogenate was aliquoted into 12 mL glass tubes with screw top (prior to use, tubes were soaked overnight in Contrad solution, rinsed and dried). Immediately, 8.34 µL of the antioxidant cocktail was added to the fatty acid analysis tubes (ratio is 6.67 µL antioxidant for every 200 µL of homogenate) and put back on ice if proceeding directly to fatty acid analysis. Otherwise, after flushing with nitrogen, samples were capped and stored at -80°C for future fatty acid extraction (protocol available on appendix G)

C: Protocol for solid phase extraction of oxylipins in heart and brain tissues

Heart and brain homogenate extracts (from part B) were removed from the -80^{o}C freezer and defrosted on ice. The centrifuge was turned on and set to 4°C. The nitrogen evaporator water bath was turned on to 37°C. For heart and brain homogenate extract, 1mL of pH 3 water was added to a 5 mL centrifuge tube. Once thawed, heart or brain homogenate extracts was vortexed and a volume optimized from a dose response curves (appendices D+E) using practice heart and brain samples was transferred into the tube containing pH 3 water.

A volume of 40 µL and 30 µL internal standard (kept in -20 freezer to each tube) was added to heart and brain homogenate extract containing tubes, respectively, and vortexed. Before putting the internal standard back into the freezer, some nitrogen gas was blown over it before closing the cap. Samples were acidified to pH 3 with 1N HCl (usually ~14 µL of 1N HCl suffices for rat heart and brain) and vortexed before reading pH using pH-indicator strips by dipping the p strip directly into the sample. If pH 3 was less than 3, the pH was raised with 1N NaOH. Samples were centrifuged for 10 min at 6000 rcf at 4°C to remove debris.

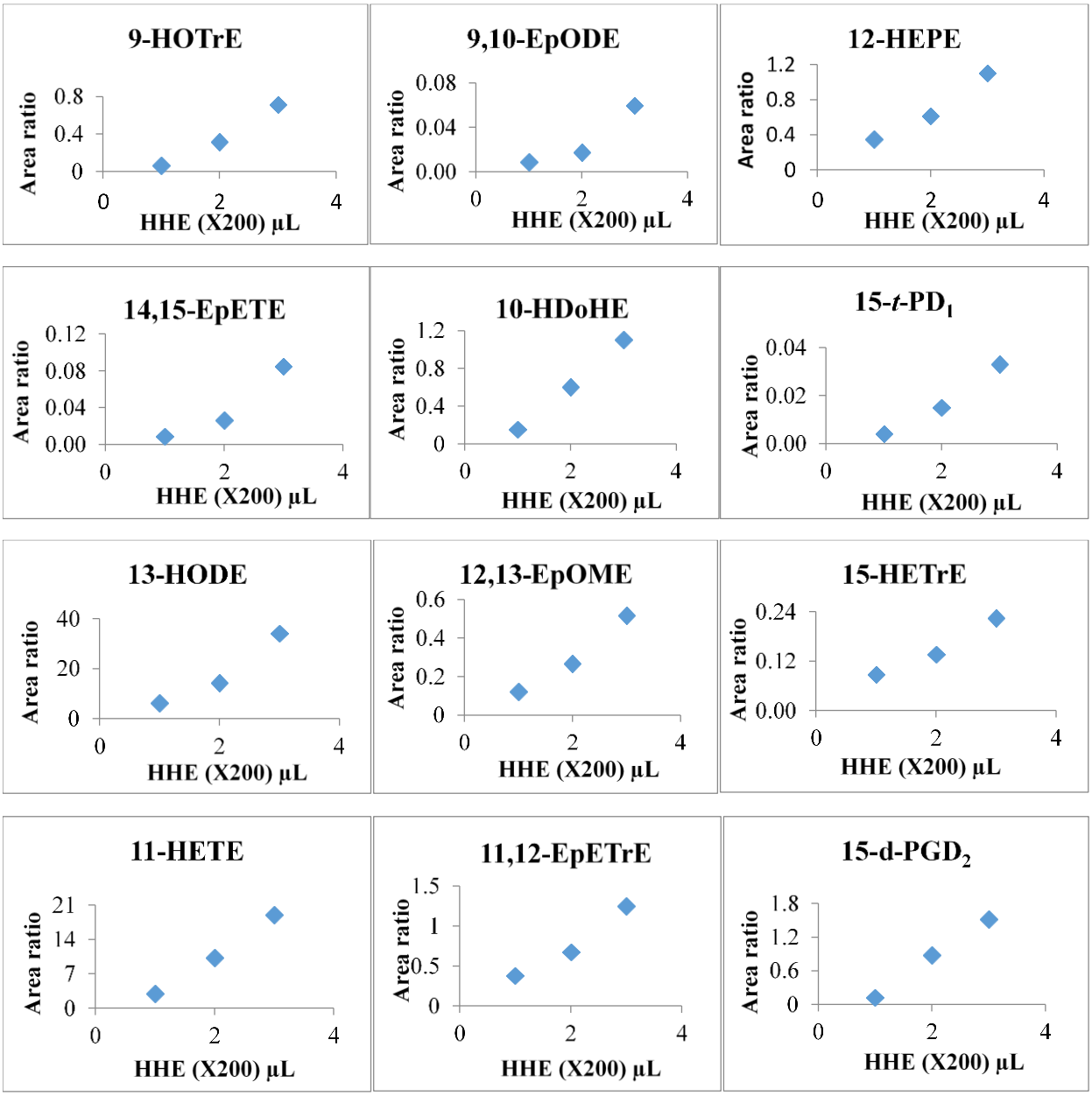
In the fume hood, labelled Strata-X SPE (Phenomenex, 33u, 60 mg/3 mL) columns were set-up for each sample and waste vials were placed under each column. The column was pre-conditioned with 3500 µL methanol. The methanol was allowed to drip through by gravity for 1 minute, then gentle pressure was applied with a BD 10 mL syringe to increase flow (column should go dry). The column was equilibrated with 3500 µL of pH 3 water and pushed through in the same way as the previous step.

The sample was centrifuged and then loaded on the column, avoiding the pellet at the bottom, allowed to drip through by gravity for 1 minute and pushed through as previously described. 1000 µL of 10% methanol in pH 3 water was added to the sample vial, vortexed and

then centrifuged at 4°C, 3000 rpm for 5 minutes. The supernatant was applied again to the column avoiding the pellet and pushed through as before. The column was washed with 2000 µL pH 3 water followed by 1000 µL of hexane to dry the column. At this step, the hexane was pushed through the column until dry. Waste vials were removed and labelled 1500 µL microtubes were placed underneath the columns. Samples were eluted with 1000 µL of 100% methanol.

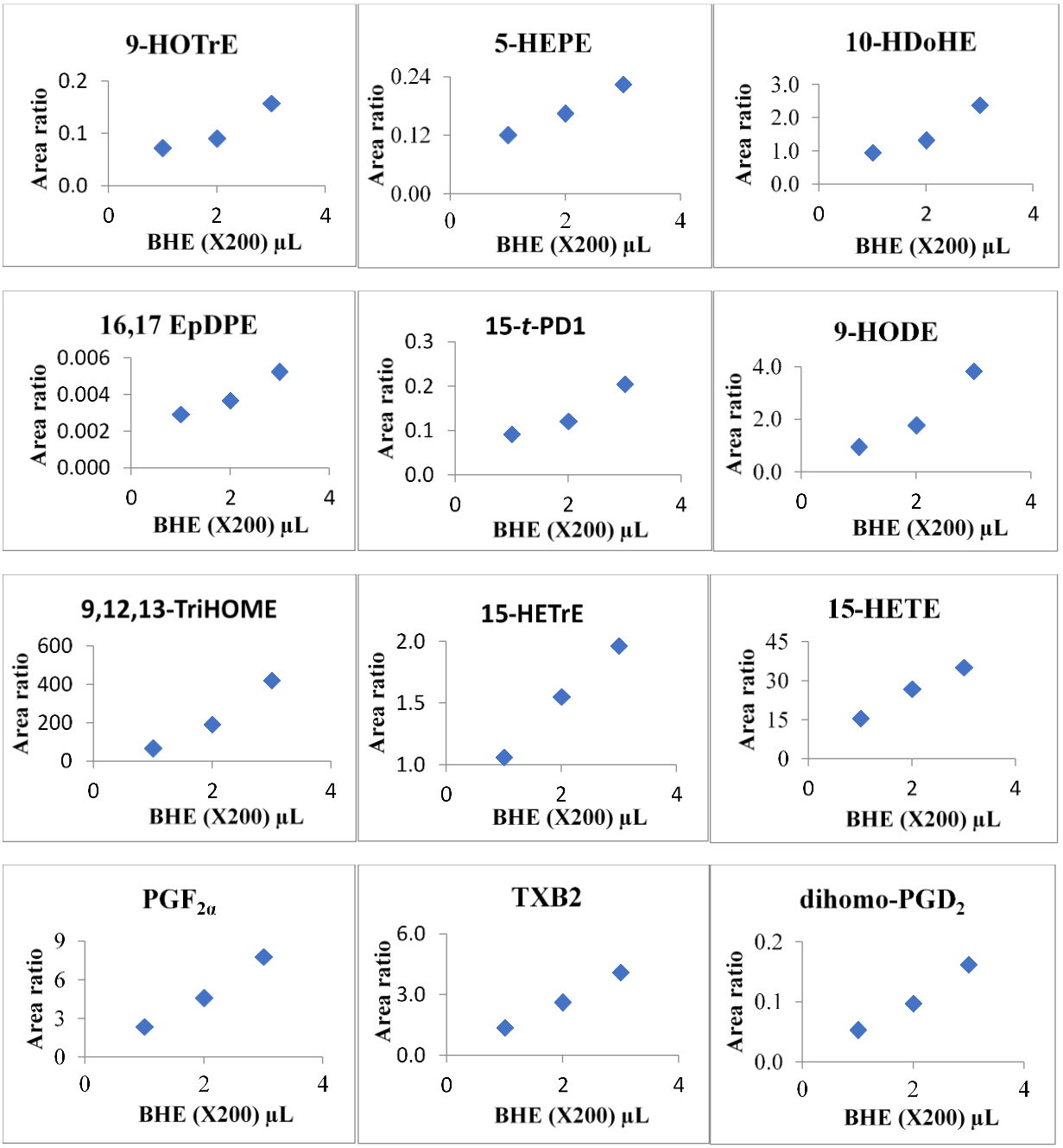
The water bath in the nitrogen evaporator was set at 37°C to dry down samples. When ready to run on HPLC, samples were dried down in a nitrogen evaporator bath at 37°C and reconstituted in 100 µL of Solvent A (water-acetonitrile-formic acid [70:30:0.02 v/v/v]). Samples were then vortexed and centrifuged at 14000 g (rcf) for 10 minutes at 4°C then transferred into labeled GC vials containing a 200 µL polypropylene conical insert. Samples were run on an ABSciex QTRAP 6500 MS with triple quadrapole electrospray ionization (IonDrive Turbo V) and were analyzed using MultiQuant Version 3.0.

D: Optimization of heart homogenate extract (HHE) volume using standard curves



To optimize heart homogenate extract (HHE) volume for oxylipin extraction, a dose response curve had been obtained from the volume of practice HHE and corresponding area ratios (obtained from the ratio of sample are over internal standard area for each oxylipins). A volume of 200µL, 400 µL and 600 µL of HHEs were placed along X-axis against the corresponding area ratios along the Y-axis. A total of 71 oxylipins were quantified from the practice HHE and dose response curves were presented for 12 representative oxylipins form each precursor fatty acid.

E: Optimization of brain homogenate extract (BHE) volume using standard curves



To optimize brain homogenate extract (BHE) volume for oxylipin extraction, a dose response curve was obtained from the volume of practice BHE and their corresponding area ratios (obtained from the ratio of sample area over internal standard area for each oxylipins). A volume of 200 µL, 400 µL and 600 µL of BHEs were placed along X-axis against the corresponding area ratios along the Y-axis. A total of 97 oxylipins were quantified from the practice BHE and dose response curves were presented for 12 representative oxylipins from each precursor fatty acid.

F: Oxylipin mass transitions, retention times, deuterated internal standards and response factors

Component Name	Mass Transition	Retention Time*	Deuterated Internal Standard	Standard Curve Slope
12-HHTrE	279.0 / 217.0	13.53	5-HETE-d_8	0.3646
PGA_2	333.0 / 271.0	9.68	15d-PGJ_2-d_4	11.454
15d-PGA_2	315.0 / 271.0	15.19	15d-PGJ_2-d_4	2.6609
PGB_2	333.0 / 271.0	12.29	15d-PGJ_2-d_4	1.0891
PGD_2	351.0 / 271.0	7.78	PGD_2-d_4	1.6144
15d-PGD_2	333.0 / 271.0	11.81	15d-PGJ_2-d_4	29.722
dhk-PGD_2	351.0 / 207.0	9.15	PGD_2-d_4	2.34
15k-PGD_2	349.0 / 235.0	8.64	PGD_2-d_4	No Primary
Tetranor-PGDM	327.0 / 247.0	2.39	PGD_2-d_4	0.0984
PGE_2	351.0 / 271.0	7.43	PGE_2-d_4	7.8596
11β-PGE_2	351.0 / 271.0	7.58	PGE_2-d_4	4.9206
19oh PGE_2	367.0 / 243.0	3.25	PGE_2-d_4	0.3835
Bicyclo-PGE_2	333.0 / 175.0	11.00/10.17	PGE_2-d_4	0.7943
15k-PGE_2	349.0 / 235.0	7.78	PGE_2-d_4	1.3377
20oh-PGE_2	367.0 / 175.0	3.15	PGE_2-d_4	No Primary
Dhk-PGE_2	351.0 / 207.0	8.44	PGE_2-d_4	0.529
Tetranor-PGEM	327.0 / 291.0	2.30	PGE_2-d_4	0.4032
$PGF_{2\alpha}$	353.0 / 193.0	7.25	$PGF_{2\alpha}$-d_4	3.4181
Dhk-$PGF_{2\alpha}$	353.0 / 291.0	8.64	dhk-$PGF_{2\alpha}$-d_4	4.9299
Dh-$PGF_{2\alpha}$	355.0 / 283.0	7.97	$PGF_{2\alpha}$-d_4	No Primary
19oh-$PGF_{2\alpha}$	369.0 / 192.0	3.25	$PGF_{2\alpha}$-d_4	No Primary
20oh-$PGF_{2\alpha}$	369.0 / 165.0	3.15	$PGF_{2\alpha}$-d_4	No Primary
11β-dhk-$PGF_{2\alpha}$	353.0 / 113.0	8.20	$PGF_{2\alpha}$-d_4	0.8673
11β-$PGF_{2\alpha}$	353.0 / 335.0	6.74	$PGF_{2\alpha}$-d_4	0.1703
15k-$PGF_{2\alpha}$	351.0 / 219.0	7.68	$PGF_{2\alpha}$-d_4	0.6609
6k-$PGF_{1\alpha}$	369.0 / 245.0	5.34	6k-$PGJ_{1\alpha}$-d_4	3.6495
2,3-dinor-6k-$PGF_{1\alpha}$	363.0 / 281.0	6.60	$PGF_{2\alpha}$-d_4	No Primary
2,3-dinor-11β-$PGF_{2\alpha}$	325.0 / 227.0	5.40	$PGF_{2\alpha}$-d_4	1.2716
Tetranor-PGFM	329.0 / 247.0	2.30	$PGF_{2\alpha}$-d_4	No Primary
PGJ_2	333.0 / 189.0	9.87	15d-PGJ_2-d_4	3.6267
15d-PGJ_2	315.0 / 203.0	14.57	15d-PGJ_2-d_4	1.4436
PGK_2	349.0 / 249.0	7.71	PGE_2-d_4	1.8211
TXB_2	369.0 / 169.0	6.61	TXB_2-d_4	6.3658
11d-TXB_2	367.0 / 305.0	7.39	TXB_2-d_4	2.375
2,3-dinor-TXB_2	341.0 / 137.0	5.22	TXB_2-d_4	0.3628
5-HETE	319.0 / 115.0	17.19	5-HETE-d_8	1.169
5-oxoETE	317.0 / 203.0	17.56	5-oxoETE-d_7	0.8498
5,15-DiHETE	335.0 / 201.0	11.46	LTB_4-d_4	0.5267
5,6-DiHETE	335.0 / 115.0	15.29	LTB_4-d_4	0.4905
8-HETE	319.0 / 155.0	16.71	5-HETE-d_8	2.311
8,15-DiHETE	335.0 / 235.0	10.98	LTB_4-d_4	0.1268
9-HETE	319.0 / 123.0	16.84	5-HETE-d_8	0.2492
11-HETE	319.0 / 167.0	16.41	5-HETE-d_8	5.3158
12-HETE	319.0 / 135.0	16.6	15-HETE-d_8	0.189
Tetranor-12-HETE	265.0 / 109.0	13.28	15-HETE-d_8	2.0436
12-oxoETE	317.0 / 153.0	16.60	5-oxoETE-d_7	1.5173
15-HETE	319.0 / 175.0	16.07	15-HETE-d_8	0.9018
15-oxoETE	317.0 / 113.0	16.14	5-oxoETE-d_7	2.2341
HXA_3	335.0 / 195.0	14.6	LTB_4-d_4	No Primary
HXB_3	335.0 / 183.0	14.8	LTB_4-d_4	No Primary

LTB_4	335.0 / 195.0	11.93	LTB_4-d_4	1.2507
6*t*-LTB_4	335.0 / 195.0	11.44	LTB_4-d_4	0.9015
6*t*-12-*epi*-LTB_4	335.0 / 195.0	11.58	LTB_4-d_4	0.6674
12-*epi*-LTB_4	335.0 / 195.0	11.89	LTB_4-d_4	1.4375
12-oxo-LTB_4	333.0 / 179.0	12.60	LTB_4-d_4	1.4903
20cooh-LTB_4	365.0 / 195.0	5.35	LTB_4-d_4	0.1691
20oh-LTB_4	351.0 / 195.0	5.60	LTB_4-d_4	0.6127
LTC_4	624.0 / 272.0	10.75	LTB_4-d_4	0.3364
14,15-LTC_4 (EXC_4)	624.0 / 272.0	7.50	LTB_4-d_4	No Primary
LTD_4	495.0 / 177.0	9.19	LTB_4-d_4	1.0476
14,15-LTD_4 (EXD_4)	495.0 / 177.0	10.98	LTB_4-d_4	No Primary
LTE_4	438.0 / 333.0	10.57	LTB_4-d_4	0.9368
14,15-LTE_4 (EXE_4)	438.0 / 333.0	9.00	LTB_4-d_4	No Primary
15(R)-LXA_4	351.0 / 165.0	8.50	LTB_4-d_4	No Primary
6(R)-LXA_4	351.0 / 217.0	8.50	LTB_4-d_4	0.1535
6(S)-LXA_4	351.0 / 115.0	8.78	LTB_4-d_4	0.292
LXB_4	351.0 / 221.0	7.58	LTB_4-d_4	0.3733
20cooh-AA	333.0 / 271.0	14.38	EPA-d_5	1.1049
5,6-EpETrE	319.0 / 191.0	18.13	11,12-DiHETrE-d_{11}	3.7275
5,6-DiHETrE	337.0 / 145.0	15.56	11,12-DiHETrE-d_{11}	3.6065
8,9-EpETrE	319.0 / 155.0	17.90	8,9-DiHETrE-d_{11}	1.2994
8,9-DiHETrE	337.0 / 127.0	14.85	8,9-DiHETrE-d_{11}	5.5366
11,12-EpETrE	319.0 / 167.0	17.70	11,12-DiHETrE-d_{11}	9.1336
11,12-DiHETrE	337.0 / 167.0	14.27	11,12-DiHETrE-d_{11}	16.4237
14,15-EpETrE	319.0 / 175.0	17.24	14,15-DiHETrE-d_{11}	1.0437
14,15-DiHETrE	337.0 / 207.0	13.54	14,15-DiHETrE-d_{11}	10.939
16-HETE	319.0 / 189.0	15.51	15-HETE-d_8	0.5383
17-HETE	319.0 / 247.0	15.40	15-HETE-d_8	3.0768
18-HETE	319.0 / 261.0	15.28	20-HETE-d_6	3.2074
19-HETE	319.0 / 231.0	14.89	20-HETE-d_6	0.2715
20-HETE	319.0 / 245.0	15.02	20-HETE-d_6	0.3998
2,3-dinor-8-*iso*-$PGF_{2\alpha}$	325.0 / 237.0	5.09	$PGF_{2\alpha}$-d_4	5.3857
5-*iso*-$PGF_{2\alpha}$VI	353.0 / 115.0	7.02	$PGF_{2\alpha}$-d_4	1.5803
8-*iso*-$PGF_{2\alpha}$III	353.0 / 193.0	6.50	$PGF_{2\alpha}$-d_4	1.9211
8-*iso*-15k-$PGF_{2\beta}$	351.0 / 219.0	6.96	$PGF_{2\alpha}$-d_4	3.6218
9-HODE	295.0 / 171.0	15.95	9-HODE-d_4	1.4704
9-oxoODE	293.0 / 185.0	16.28	5-oxoETE-d_7	1.4353
13-HODE	295.0 / 195.0	15.8	13-HODE-d_4	2.9546
13-oxoODE	293.0 / 167.0	15.96	5-oxoETE-d_7	0.2717
9,10,13-TriHOME	329.0 / 171.0	7.32	9-HODE-d_4	3.1287
9,12,13-TriHOME	329.0 / 211.0	7.19	9-HODE-d_4	6.1871
9,10-EpOME	295.0 / 171.0	17.41	9,10-DiHOME-d_4	3.7629
9,10-DiHOME	313.0 / 201.0	13.22	9,10-DiHOME-d_4	16.426
12,13-EpOME	295.0 / 195.0	17.21	12,13-DiHOME-d_4	10.516
12,13-DiHOME	313.0 / 183.0	12.66	12,13-DiHOME-d_4	11.798
15-oxoEDE	321.0 / 223.0	17.92	5-oxoETE-d_7	1.1612
13-HOTrE-γ	293.0 / 193.0	14.89	13-HODE-d_4	1.6998
PGD_1	353.0 / 235.0	7.74	PGD_2-d_4	1.0006
PGE_1	353.0 / 235.0	7.67	PGE_2-d_4	1.4269
6k-PGE_1	367.0 / 331.0	5.65	PGE_2-d_4	0.9207
$PGF_{1\alpha}$	355.0 / 293.0	7.24	$PGF_{2\alpha}$-d_4	3.0675
15k-$PGF_{1\alpha}$	353.0 / 221.0	7.68	$PGF_{2\alpha}$-d_4	No Primary
6,15-dk-dh-$PGF_{1\alpha}$	369.0 / 267.0	6.52	$PGF_{2\alpha}$-d_4	0.4899
PGK_1	351.0 / 251.0	7.76	PGD_2-d_4	4.2079
TXB_1	371.0 / 171.0	6.33	TXB_2-d_4	3.6782
5-HETrE	321.0 / 205.0	18.65	5-HETE-d_8	0.2746

8-HETrE	321.0 / 157.0	17.12	5-HETE-d_8	1.8141
15-HETrE	321.0 / 221.0	16.74	15-HETE-d_8	2.9424
Dihomo-PGD_2	379.0 / 299.0	9.70	PGD_2-d_4	No Primary
Dihomo-15d PGD_2	361.0 / 299.0	14.08	15d-PGJ_2-d_4	No Primary
Dihomo-PGE_2	379.0 / 299.0	9.40	PGE_2-d_4	No Primary
Dihomo-$PGF_{2\alpha}$	381.0 / 337.0	9.20	$PGF_{2\alpha}$-d_4	2.812
Dihomo-PGJ_2	361.0 / 299.0	12.52	15d-PGJ_2-d_4	No Primary
9-HOTrE	293.0 / 171.0	14.46	9-HODE-d_4	2.006
9-oxoOTrE	291.0 / 185.0	14.98	5-oxoETE-d_7	2.2547
13-HOTrE	293.4 / 195.0	14.59	13-HODE-d_4	0.6216
13-oxoOTrE	291.0 / 247.0	14.80	5-oxoETE-d_7	0.0856
12,13-EpODE	293.0 / 183.0	16.19	13-HODE-d_4	2.4048
12,13-DiHODE	311.0 / 183.0	11.12	12,13-DiHOME-d_4	4.4379
15,16-EpODE	293.0 / 235.0	15.49	12,13-DiHOME-d_4	No Primary
15,16-DiHODE	311.0 / 223.0	10.62	12,13-DiHOME-d_4	No Primary
9,10-EpODE	293.0 / 171.0	16.39	9,10-DiHOME-d_4	No Primary
9,10-DiHODE	311.0 / 201.0	11.62	9,10-DiHOME-d_4	No Primary
PGD_3	349.0 / 269.0	6.79	PGD_2-d_4	0.5356
PGE_3	349.0 / 269.0	6.52	PGE_2-d_4	1.3408
$PGF_{3\alpha}$	351.0 / 193.0	6.27	$PGF_{2\alpha}$-d_4	1.2284
TXB_3	367.0 / 169.0	5.73	TXB_2-d_4	5.4993
Δ^{17}-6k-$PGF_{1\alpha}$	367.0 / 163.0	4.68	$PGF_{2\alpha}$-d_4	2.0835
5-HEPE	317.0 / 115.0	15.81	5-HETE-d_8	1.1101
8-HEPE	317.0 / 155.0	15.34	5-HETE-d_8	1.1697
9-HEPE	317.0 / 149.0	15.48	5-HETE-d_8	0.6765
11-HEPE	317.0 / 215.0	15.23	5-HETE-d_8	No Primary
12-HEPE	317.0 / 179.0	15.33	15-HETE-d_8	1.7239
15-HEPE	317.0 / 219.0	15.00	15-HETE-d_8	1.8731
LXA_5	349.0 / 215.0	7.32	LTB_4-d_4	0.3762
RvD_1	375.0 / 141.0	8.36	LTB_4-d_4	0.5531
14,15-EpETE	317.0 / 207.0	16.27	14,15-DiHETrE-d_{11}	2.6835
17,18-EpETE	317.0 / 259.0	15.88	14,15-DiHETrE-d_{11}	1.8647
18-HEPE	317.0 / 215.0	14.50	20-HETE-d_6	1.3858
RvE_1	349.0 / 195.0	5.51	LTB_4-d_4	0.2271
8-*iso*-$PGF_{3\alpha}$	351.0 / 307.0	5.50	$PGF_{2\alpha}$-d_4	No Primary
17k-DHA	341.0 / 297.0	16.31	LTB_4-d_4	1.1412
4-HDoHE	343.0 / 101.0	17.37	5-HETE-d_8	0.4409
7-HDoHE	343.0 / 141.0	16.70	5-HETE-d_8	0.5523
8-HDoHE	343.0 / 109.0	16.77	5-HETE-d_8	0.4476
10-HDoHE	343.0 / 153.0	16.36	5-HETE-d_8	2.1831
11-HDoHE	343.0 / 149.0	16.50	5-HETE-d_8	0.8501
13-HDoHE	343.0 / 221.0	16.18	15-HETE-d_8	0.603
14-HDoHE	343.0 / 205.0	16.30	15-HETE-d_8	0.7806
16-HDoHE	343.0 / 233.0	16.00	15-HETE-d_8	3.3544
17-HDoHE	343.0 / 245.0	16.06	15-HETE-d_8	0.2779
20-HDoHE	343.0 / 241.0	15.72	20-HETE-d_6	1.3316
7R-Maresin-1	359.0 / 177.0	11.33	LTB_4-d_4	0.2837
PD_1	359.0 / 153.0	10.80	LTB_4-d_4	No Primary
15t-PD_1	359.0 / 153.0	11.00	LTB_4-d_4	No Primary
10S,17S-DiHDoHE (PDX)	359.0 / 153.0	11.19	LTB_4-d_4	1.0292
RvD_2	375.0 / 175.0	7.64	LTB_4-d_4	0.3639
RvD_5	359.2 / 199.0	11.34	LTB_4-d_4	No Primary
16,17-EpDPE	343.0 / 193.0	17.36	14,15-DiHETrE-d_{11}	0.2435
19,20-DiHDoPE	361.0 / 229.0	13.31	14,15-DiHETrE-d_{11}	1.1322
19,20-EpDPE	343.0 / 241.0	16.91	14,15-DiHETrE-d_{11}	2.2213

10-Nitrooleate	326.0 / 169.0	19.65	EPA-d_5	No Primary
9-Nitrooleate	326.0 / 168.0	19.65	EPA-d_5	No Primary

*Retention times were based on retention times of primary standards if available. For those with no primary, the retention time was estimated based on comparisons of known and unknown retention time of oxylipins in our samples and the retention times of oxylipins reported by Dumlao DS, Buczynski MW, Norris PC, Harkewicz R, Dennis EA. High-throughput lipidomic analysis of fatty acid derived eicosanoids and N-acylethanolamines. BBA-Mol Cell Biol L 2011;1811(11):724-36. doi: 10.1016/j.bbalip.2011.06.005, and by Wang Y, Armando AM, Quehenberger O, Yan C, Dennis EA. Comprehensive ultra-performance liquid chromatographic separation and mass spectrometric analysis of eicosanoid metabolites in human samples. Journal of Chromatography A 2014;1359:60-9. doi: 10.1016/j.chroma.2014.07.0006.

G: Protocol for heart and brain fatty acids extraction

For heart and brain fatty acid analysis, extraction of phospholipid (PL) and neutral lipid (NL) was conducted from total lipids by a thin-layer chromatography (TLC), followed by methylation, and analyzed by GC. Practice samples were prepared to optimize the correct volume of internal standard to use.

In glass tubes that have been cleaned with Contrad solution, 250 µL of prepared heart or brain homogenate (see appendix B) and 2.5 mL 2:1 chloroform: methanol with 0.01% butylated hydroxytoluene (BHT) (0.03 g BHT, 200 mL chloroform, 100mL methanol) were added. Heart sample was vortexed, and 10 µL of PL and 8 µL of NL internal standard made up in 2:1 chloroform: methanol was added to each sample. For brain sample, 15 µL of PL and 10 µL of NL internal standard made up in 2:1 chloroform: methanol.

Further, 2.25 mL of 2:1 chloroform: methanol was added to the sample, the samples were vortexed, and then 950 µL (0.73% sodium chloride) was added. Samples were then centrifuged at 4°C for 10 minutes at 800 g. The lower phase of the sample was then removed using a Pasteur pipette and transferred into a Contrad cleaned 4.0 mL glass vial. Next, samples were dried down in a nitrogen evaporator water bath at 37°C and once dried,100 µL of 2:1 chloroform: methanol was added to the vial and vortexed each sample.

To make solvent for TLC tank, a glass graduated cylinder (100 mL), in the fume hood was used to fill with 60 mL of heptane, 40 mL of isopropyl ether, and 3 mL of acetic acid. Using the TLC template, 6 or 8 equal and parallel vertical lanes on the silica plate were created using the dull side of a razor blade. At least 1 cm on each side of plate was left.
Using a 25 µL pipette, a total of 50 µL of each sample was drawn and spotted slowly along the horizontal line drop by drop in the center of the lane. After each drop, each spot was dried with

nitrogen. The TLC plate was placed in the tank as vertical as possible until the solvent line reach 1 cm from the top. After removing the plate from solvent tank, it was dried with a gentle stream of nitrogen gas.

Using a hose attached to nitrogen gas, 0.1% ANS (8-Anilino-1-Naphthalene-Sulfonic Acid) solution was used to spray a fine layer onto the TLC plate. 0.1% ANS was prepared using 100 mg ANS in a tin foil covered bottle added with 100 mL of milli-Q water and stirring on stir plate for about 30 minutes.

In a dark room wearing UV safety glasses and hand-held UV light, PL and NL lines were marked using a pencil, indicating where to scrape. Using a razor blade, the indicated portions were scraped onto a creased weigh paper and carefully transferred to the corresponding, contrad cleaned labeled 12 mL screw top test tube.

1.2 mL methanolic sulfuric acid (6.0 mL sulfuric acid, 94.0 mL dry methanol) was added to each sample. Samples were then tightly capped and placed in an 80°C oven for 1.5 hours. After cooling, 1.5 mL toluene was added to the samples and vortexed. Then, 1.0 mL ultrapure water was added to the samples, vortexed, and centrifuged at 800 g for 5 minutes. The top layer of the samples was transferred to a clean 2.0 mL GC vial. Samples were then dried down in a nitrogen evaporator water bath at 37°C and reconstituted with 100 µL and 40 µL of hexane for PL and NL, respectively. Samples were stored at -20°C until analyzed by GC.

Samples were separated on a DB225MS column (30 m X 0.25 mm diameter and 0.25 mm film thickness; Agilent Technologies Canada Inc., Mississauga, Ontario) using a Bruker 450-GC with flame ionization detector. The temperature program was 70°C for 1 min, raised to 180°C at 25°C/min and held for 1 minute, raised to 200°C at 10°C/min and held for 1 min, and raised to 220°C at 2°C/min and held for 6 min. Finally, the temperature was raised to 240°C at

20°C/min and held for 20 minutes. Total run time was 46:40 minutes with a 20:1 split ratio and a column flow of 1.3 mL/min. Hydrogen was used as the carrier gas. Peaks were quantified using the internal standard (C15:0 – Phospholipids and C17:0 – Neutral lipids) and values are expressed as µg/g of heart or brain homogenate.

H: Protocol for reagent preparation

1. Tyrode's salts without NaHCO3

Tyrode's salts powder comes prepackaged from Sigma-Aldrich (Catalogue No. T2145) and is kept in the fridge. As powdered salts are hygroscopic and should be protected from moisture, the entire contents of each package were used immediately after opening. 1L of ultrapure water was measured out in a plastic graduated cylinder and about 800 mL of measured water was transferred into a 2000 mL flask. A large stir bar was added carefully to avoid splash and placed on stir plate to begin gentle stirring. Powdered Tyrode's salt was added into the flask and continued stirring until dissolved. The original Tyrode's salts packaging was rinsed with some of the remaining in 200 mL measured water to remove all traces of powder and added into the flask. Once all the powder was dissolved, the solution was transferred to a 1L volumetric flask and brought to volume using some of the remaining 200 mL measured water in a graduated cylinder. The 2000 mL flask was rinsed up to 3 times to bring the volumetric flask up to volume with water. After inserting a volumetric stopper, the flask was inverted 10 times to mix the solution properly. The Tyrode's salts WITHOUT NaHCO3 was then transferred to a 1L glass bottle covered with tin foil to protect from light and stored in the refrigerator (2-8°C).

2. Tyrode's salt solution (pH 7.6)

In a graduated cylinder, 100 mL of reconstituted Tyrode's salt solution was measured and 100 mg of powdered sodium bicarbonate (Sigma, S5761) was weighed out into a 125 mL Erlenmeyer flask. The flask was covered with tin foil to protect from light and a stir bar added. About 80 mL of measured Tyrode's was transferred from the graduated cylinder to the Erlenmeyer flask. Powder was completely dissolved into solution (in ~ 15 min) by placing the

flask on the stir plate and stirring before adjusting pH. While continuing to stir, the pH of the solution was adjusted to pH 7.6 using 1N HCl or 1N NaOH. The solution was transferred to a 100 mL volumetric flask and brought to volume by using some of the remaining 20 mL measured Tyrode's in graduated cylinder. A volumetric stopper was inserted to invert and to mix the solution and transferred to a 100 mL glass bottle covered with tin foil to protect from light and stored in the refrigerator (2-8°C).

3. 1% Triton

In a 20 mL scintillation vial, 0.02 g of Triton solution was weighed out. 2.0 mL of room temperature Tyrode's (pH 7.6) was added using a 1.0 mL eppendorf pipette. The vial was covered with a cap and vortexed well. Before storing in refrigerator (2-8°C), the vial was covered with tin foil to protect from light.

4. Antioxidant cocktail

50 mL of methanol and 25 mL ethanol were measured out in separate graduated cylinders and mixed together in a 250 mL beaker. The outside of the beaker was covered with tinfoil and placed on a magnetic stirrer. After putting in a stir bar, the beaker was covered with tinfoil to minimize volatilization. 20 mg BHT, 20 mg ethylenediaminetetraacetic acid (EDTA), 200 mg tripolyphosphate TPP and 200 mg indomethacin were weighed out onto separate weigh paper. The ingredients were added to the methanol: ethanol solution and stirred until all dissolved. When all dry ingredients were dissolved, the mixture was transferred to a 100 mL volumetric flask. Using a small amount of deionized distilled H_2O, the sides of the beaker was washed down and poured into the 100 mL flask. The 100 mL volumetric flask was filled up to the mark with deionized distilled H_2O. Using a stopper, the flask was inverted to mix, and the solution was

transferred into a clean, tin foil covered, labeled 125 mL bottle. The appropriate amount of antioxidant cocktail was aliquoted into covered scintillation vials for individual experiments.

5. Solvent A

Water – Acetonitrile – Acetic Acid [70:30:0.02; v/v/v]

*MS Grade

*Prevent evaporation of prepared solutions using paraffin around cap seals

To make 1000 mL:

700 mL water

300 mL acetonitrile

200 uL acetic acid

Vacuum filter through Whatman #4 filter paper

6. Solvent B

Acetonitrile – Isopropyl Alcohol [50:50; v/v]

*MS Grade

To make 1000 mL:

500 mL Acetonitrile

500 mL Isopropyl alcohol

Vacuum filter through Whatman #4 filter paper

I. Research ethics and compliance

UNIVERSITY OF MANITOBA | Research Ethics and Compliance
Office of the Vice-President (Research and International)

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1 March 2013

TO: Dr. H. Aukema, Department of Human Nutritional Sciences

FROM: Dr. R. Hodges, Acting Chair, Fort Garry Campus Animal Care Committee

RE: Your protocol entitled **"Effect of dietary oils on oxylipin composition in normal rat tissues"**

Please be advised that your Animal Use Protocol form was reviewed by the Fort Garry Campus Animal Care Committee (FG ACC) at its February 28 2013 meeting. The committee recommended **APPROVAL** of your protocol **SUBJECT TO A SATISFACTORY RESPONSE TO THE QUERIES NOTED BELOW.**

Protocol Reference Number: **F13-005**
Animals approved for use: **72 rats**
Protocol approval is valid from: **March 1 2013 to February 28 2014**
Category of Invasiveness: **B**

As indicated above, your protocol has been approved, and as such, you are authorized to begin the work described. However, the Committee requires your written response on or before **March 15 2013** to the following queries under which this approval is subject:

a) The committee would like to commend you for submitting a very well written protocol.

b) Block 6A: It is stated that the minimum number of animals per group required is 6. Although the study itself is not expected to cause any health issues or complications, if an animal is lost due to an unrelated health concern, will it cause any problem with the data collected? Does another animal per group need to be added potentially?

c) Block 7C: Can you give a numerical level of weight loss that might be cause for concern and therefore used as a humane endpoint level (15% loss from highest recorded weight is often used for instance.)

d) Schedule 6, Question 3a): There isn't a comment on palatability of the

diets; can you indicate if there is any concern with palatability due to the added fats. Also, any shelf life issues that need to be looked at and, if so, how will they be addressed?

e) Please advise if this is a defined diet or a standard chow diet. If it is not a defined diet, might this have an influence on your experimental data?

f) Please advise if the animals will be single or group housed.

g) Please be advised that J Gauthier and T. Winter need to update their ethics. In order to meet the requirement of all personnel needing to renew their ethics training after five years, they have the option of attending either the May 23 2013 or May 2014 (date tba) Animal Users Professional Development: Advancing Ethical Research. If they are unable to attend either of these sessions, they will have to repeat the online course no later than May 31 2014. Please email autp@cc.umanitoba.ca to register.

If your written response is not received by the date noted above, the protocol will be suspended and no animal experimentation will be allowed to continue until further clarification by you is provided.

Please direct your response to Ms Tracy VanOsch, Co-ordinator, Animal Care, Office of Research Services, 208 Crop Technology Centre, 194 Dafoe Road.

The protocol reference number must be used when ordering animals. It is understood that these animals will be used only as described in your protocol. Failure to follow this protocol will result in the termination of your ability to use animals.

The protocol must be kept current. Minor modifications to the protocol must be submitted in the form of an amendment. Major changes would necessitate preparation and submission of a new protocol. Failure to renew this protocol prior to the expiry date will result in the termination of your ability to continue ordering animals.

On behalf of the Fort Garry Campus Animal Care Committee, I would like to extend our best wishes for the successful completion of your research.

RH/ck

copy: Veterinary Services
Ms J. Nelson, Department of Biological Sciences
Ms D. Borowski, LATC