# Development and application of methods using oxylipins to examine dietary omega-3 requirements and phospholipase $A_2$ activity

by

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# Abstract

Oxylipins are bioactive lipid mediators formed from polyunsaturated fatty acids (PUFA) that are released from membrane phospholipids by phospholipase $A_2$ ($PLA_2$). Since oxylipins mediate PUFA effects, methods using oxylipins were developed to answer key questions in lipid nutrition and metabolism. First, docosahexaenoic acid (DHA), arachidonic acid (ARA) and their derived oxylipins in serum, liver and kidney of male rats, and male and female mice that received graded doses of α-linolenic acid (ALA) were analyzed by mass spectrometry. DHA/ARA oxylipin and fatty acid ratios were then used to demonstrate that the dietary ALA requirement in growing rodents is 0.55g ALA/100g diet, which is higher than that provided by standard laboratory rodent diets. Second, this new method was applied to serum, liver, heart and brain of weanling male and female rats in response to graded doses of dietary ALA and DHA to elucidate that 0.12g DHA/100g diet is bioequivalent to the ALA requirement of 0.55g ALA/100g diet. Third, use of inhibitors to assess ex vivo $PLA_2$ activity towards PUFA and oxylipins in rat hearts revealed calcium-independent (i)$PLA_2$ had activity towards all PUFA and oxylipins analyzed, while secreted (s)$PLA_2$ had greater specificity towards DHA and its oxylipins. The oxylipin data generated may help explain the failure of s$PLA_2$ inhibitor drugs for cardiovascular disease. Fourth, application of this new method in weanling male and female rats showed that dietary DHA increased i$PLA_2$ and s$PLA_2$ activity towards DHA and its oxylipins, whereas dietary ALA increased activity towards ALA, ARA and their oxylipins. i$PLA_2$ and s$PLA_2$ activity towards PUFA was higher in females. In contrast, male hearts had higher cyclooxygenase-derived ARA oxylipin formation, which was attenuated by dietary DHA. In conclusion, the dietary ALA requirement in growing rodents was estimated to be 0.55g ALA/100g diet, and DHA was ~5-times more potent than ALA, with a bioequivalence of 0.12g DHA/100g diet.

Further, the findings herein advance the understanding of how different $PLA_2$ enzymes contribute to diet and sex effects on oxylipins. These findings have implications for potential reformulation of standard rodent diets, and for development of sex-specific dietary omega-3 PUFA guidelines and drugs for cardiovascular health.

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# Dedication

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# Contribution of Authors

Anne Manson was the primary author of this thesis. Since this thesis encompasses four multi-authored manuscripts published or submitted for publication, the contributions of the primary author along with each co-author are included below.

**Chapter 2: A Method to Estimate the Dietary α-Linolenic Acid Requirement Using Non-esterified DHA and Arachidonic Acid Oxylipins and Fatty Acids**

Authors: Anne Manson, Karanbir K. Sidhu, Oleksandra Fedorova, Huy Hoang Khai La, Elizabeth Magaji, Le Kim Long Nguyen, Tanja Winter, Harold M. Aukema

For this manuscript the animal studies were conducted primarily by Karanbir Sidhu and Tanja Winter, with additional help from Oleksandra Fedorova, Huy Hoang Khai La, Elizabeth Magaji and Le Kim Long Nguyen. For oxylipin analysis: Tanja Winter was primarily responsible for the HPLC-MS/MS methodology applied; Anne Manson conducted analysis of mouse serum; Karanbir K. Sidhu conducted analysis of rat liver and serum; Oleksandra Fedorova conducted analysis of rat kidney; Huy Hoang Khai La conducted analysis of mouse liver; Elizabeth Magaji conducted the analysis of mouse kidney; Le Kim Long Nguyen conducted analysis of mouse brain. Oleksandra Fedorova, Huy Hoang Khai La, Elizabeth Magaji, Le Kim Long Nguyen participated in data analysis for the tissues they analyzed. Anne Manson was primarily responsible for data analysis, including statistical coding, and final statistical analysis and interpretation of all data within the study. Anne Manson was primarily responsible for manuscript writing with guidance from Dr. Harold Aukema. Dr. Harold Aukema conceptualized, envisioned and designed the research being conducted.

**Chapter 3: Bioequivalence of docosahexaenoic acid intake to the dietary requirement of alpha-linolenic acid in growing rats using non-esterified oxylipins and fatty acids**

Authors: Anne Manson, Sandrius Mirochnikov, Isabel Delgado Poveda, Hiu Yan Chan, Tanja Winter, Harold M. Aukema

Anne Manson and Dr. Harold Aukema were responsible for conceptualization, envisioning and designing the study. Anne Manson conducted animal studies, with help from Tanja Winter for animal termination. For oxylipin analysis: Tanja Winter was primarily responsible for the HPLC-MS/MS methodology applied; Anne Manson conducted analysis of rat serum and supervised the analysis of all other samples. Isabel Delgado Poveda conducted analysis of liver; Sandrius Mirochnikov conducted analysis of heart; Hiu Yan Chan conducted analysis of brain. Anne Manson was primarily responsible for all data analysis, including statistical coding, and interpretation of all data within the study. Anne Manson was primarily responsible for manuscript writing with guidance from Dr. Harold Aukema.

**Chapter 4: Phospholipase $A_2$ enzymes differently impact PUFA release and oxylipin formation ex vivo in rat hearts**

Authors: Anne Manson, Tanja Winter, Harold M. Aukema

Anne Manson and Dr. Harold Aukema were responsible for conceptualization, envisioning and designing the study. Anne Manson helped Tanja Winter during animal termination to collect tissues. For oxylipin analysis: Tanja Winter was primarily responsible for the HPLC-MS/MS methodology applied; Anne Manson conducted all $PLA_2$ activity analyses

and was primarily responsible for gathering data and decision making in the process of establishing the methodology for $PLA_2$ activity analysis in the study, with guidance from Dr. Harold Aukema. Anne Manson was primarily responsible for all data analysis, including statistical coding, and interpretation of all data within the study. Anne Manson was primarily responsible for manuscript writing with guidance from Dr. Harold Aukema.

**Chapter 5: Sex and dietary ALA and DHA effects on rat heart phospholipase $A_2$ activity mediating fatty acid release and oxylipin formation have cardiovascular implications**

Authors: Anne Manson, Tanja Winter, Harold M. Aukema

Anne Manson and Dr. Harold Aukema were responsible for conceptualization, envisioning and designing the study. Anne Manson conducted animal feeding and helped Tanja Winter during animal termination to collect tissues. For oxylipin analysis: Tanja Winter was primarily responsible for the HPLC-MS/MS methodology applied; Anne Manson conducted all $PLA_2$ activity analyses. Anne Manson was primarily responsible for all data analysis, including statistical coding, and interpretation of all data within the study. Anne Manson was primarily responsible for manuscript writing with guidance from Dr. Harold Aukema.

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# Abbreviations

ADA, adrenic acid; ARA, arachidonic acid; ALA, α-linolenic acid; BEL, bromoenol lactone; BH, Benjamini-Hochberg; COX, cyclooxygenase; CT, cycle threshold; CYP, cytochrome P450; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; $DHA_{OH}$/$ARA_{OH}$, hydroxy-docosahexaenoic acid/hydroxy-eicosatetraenoic acid oxylipins; DiHDoPE, dihydroxy-docosapentaenoic acid; DiHDoHE, dihydroxy-docosahexaenoic acid; DiHETE, dihydroxy-eicosatetraenoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; DiHODE, dihydroxy-octadecadienoic acid; diHOME, dihydroxy-octadecenoic acid; DMSO, dimethyl sulfoxide; EPA, eicosapentaenoic acid; EpDoPE, epoxy-docosapentaenoic acid; EpETrE, epoxy-eicosatrienoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; FA, fatty acid; GC-FID, gas chromatography with flame ionization detection; GLA, γ-linolenic 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; HPLC-MS/MS high performance liquid chromatography in tandem with mass spectrometry; HX, hepoxilin; k, keto; LA, linoleic acid; LOX, lipoxygenase; LT, leukotriene; LX, lipoxin; MAFP, methyl arachidonyl fluorophosphonate; NE, non-esterified; PAF-AH, platelet-activating factor acetylhydrolase; PG, prostaglandin; $PLA_2$, phospholipase $A_2$; PUFA, polyunsaturated fatty acid; PYR, pyrrophenone; triHOME, trihydroxy-octadecenoic acid; RDA, recommended dietary allowance; TX, thromboxane; VAR, varespladib.

# Chapter 1: Thesis Introduction

## *1.1 Oxylipins*

Oxylipins are bioactive lipids with important physiological functions in the regulation of body homeostasis, such as regulation of vascular tone, inflammatory processes, immune responses, cardiac function, blood coagulation, and adipogenesis, among others (Caligiuri et al., 2017; Gabbs et al., 2015). These bioactive lipids are oxygenated metabolites produced from polyunsaturated fatty acids (PUFA). PUFA present in cell membrane phospholipids are cleaved by phospholipase $A_2$ ($PLA_2$) and released as non-esterified (NE) fatty acids (FA), which are then converted to oxylipins either enzymatically by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP), or non-enzymatically (Gabbs et al., 2015).

The most studied oxylipins are the eicosanoids derived from arachidonic acid (ARA) (Dennis & Norris, 2015; Hurley et al., 2011; Leslie, 2004; Moon et al., 2018; Mouchlis & Dennis, 2019); however, oxylipins are also produced from several other PUFAs, such as linoleic acid (LA), gamma-linolenic acid (GLA), dihomo-gamma-linolenic acid (DGLA), alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) (Gabbs et al., 2015). As some oxylipins have protective but others have detrimental roles, it is important to understand how the oxylipin profile can be modulated, as altered oxylipin production is involved in the pathophysiology of several diseases, including cardiovascular diseases (CVD) (Caligiuri et al., 2017; Devassy et al., 2016; Diab et al., 2019; Jenkins et al., 2009; Monirujjaman et al., 2017; Scholte et al., 2019; Solati & Ravandi, 2019). Importantly, several factors are shown to influence the oxylipin profile, including but not limited to diet, sex and pharmaceuticals (Aukema & Ravandi, 2023).

### *1.1.1 Diet effects on oxylipins*

As membrane phospholipids reflect the dietary lipid composition, dietary PUFA are well known for their influence on oxylipin profiles. Consumption of omega-3 fatty acids such as ALA, EPA and DHA will each increase their own fatty acids and oxylipins the most. In addition, adding ALA to a diet high in LA can mitigate potential detrimental effects of a diet high in omega-6 fatty acids (Cayer et al., 2019; Devassy et al., 2017; Ferdouse et al., 2019a; Gabbs et al., 2021; Leng et al., 2017, 2018; S. Li et al., 2023; Mendonça et al., 2018; Pauls et al., 2020; Penner et al., 2021). An interesting finding was that although DHA had the strongest effect, ALA and EPA also increased DHA oxylipins in some tissues, but not always DHA fatty acid (Leng et al., 2018; Mendonça et al., 2018). This provides evidence that dietary effects on oxylipins do not always reflect changes in fatty acids. Additionally, although DHA tended to have the strongest effect, EPA and ALA also often had an impact in reducing ARA and its oxylipins (Ferdouse et al., 2019a; Leng et al., 2018; Mendonça et al., 2018; Pauls et al., 2020; Penner et al., 2021).

The effects described above are thought to mediate the positive outcomes of omega-3 intake, as ALA, EPA, and DHA oxylipins tend to have anti-inflammatory and beneficial functions, whereas ARA oxylipins are generally pro-inflammatory with detrimental effects (Ağagündüz et al., 2024; Caligiuri et al., 2017; Gabbs et al., 2015).

### *1.1.2 ALA intake in the context of dietary requirements*

When evaluating ALA intake, it is important to consider adequacy in relation to dietary requirements. ALA is an essential omega-3 fatty acid, meaning it must be consumed through dietary intake, as it cannot be synthesized *de novo* in mammals (Barceló-Coblijn & Murphy, 2009; Neuringer et al., 1988). ALA deficiency is proven to negatively impact retina function and vision, behaviour, and mortality rates in animal models (Brenna, 2011; Neuringer et al., 1988).

Along with one reported case of human ALA deficiency related to total parental nutrition low in ALA, leading to neurological symptoms and vision blurring (Holman et al., 1982). Even though ALA is widely accepted as an essential fatty acid, there are no established dietary ALA requirements for rodents (Hamilton & Hogan, 1995). For humans, the Adequate Intake guidelines are based on median ALA intake by a healthy population (European Food Safety Authority, 2010; Institute of Medicine, 2005). Therefore, the amount of ALA needed for optimal health remains unknown.

ALA is elongated and desaturated to its long chain counterparts, EPA and DHA. This conversion occurs primarily in the liver and other tissues are supplied via bloodstream (Barceló-Coblijn & Murphy, 2009; Rapoport et al., 2007). Although the conversion rate of ALA to DHA in serum seems to be less than 1% (Brenna et al., 2009; Calder, 2016), a recent study showed that these numbers largely underestimate the whole-body capacity of ALA to DHA conversion, which was 9.5% (Rotarescu et al., 2024). Since there is preferential retention of DHA in comparison with EPA, this suggests DHA's structural and functional roles to be more important than those of EPA (Calder, 2016). This leads to ALA requirement estimates based on the ALA intake at which tissue total or esterified DHA reaches a plateau in response to increasing dietary ALA. Similarly, other nutrient requirements have been established based on the amount of a nutrient intake that no longer increases a biochemical indicator of adequacy (Cashman & Flynn, 1999; Elango et al., 2008; Institute of Medicine, 2005; Willett, 1998).

Previous studies based primarily on esterified DHA suggest the ALA requirement ranges from 0.3 to 1.0% energy in growing rodents (Bourre et al., 1989; Couëdelo et al., 2022; Domenichiello et al., 2017; Gibson et al., 2013; Tu et al., 2010; Whelan et al., 1991). Similarly, the human acceptable macronutrient distribution range (AMDR) is 0.6-1.2% energy from ALA

for both adults and children (Institute of Medicine, 2005). However, these previous studies estimating the dietary ALA requirement were conducted using primarily esterified DHA. Importantly, increasing ALA beyond a concentration considered adequate led to an increase in DHA oxylipins in some tissues, but not always in esterified DHA (Caligiuri et al., 2013; Leng et al., 2018; Mendonça et al., 2018). Additionally, differences on how esterified and non-esterified DHA respond to dietary ALA are reported (Couëdelo et al., 2022; Kim et al., 2011). This suggests that investigation of the ALA requirement through analysis of oxylipins and possibly non-esterified fatty acids could lead to higher ALA adequacy estimates.

### *1.1.3 Important considerations for the ALA requirement*

Using oxylipins to determine the ALA requirement may be important since oxylipins are hormone-like PUFA metabolites that mediate many of the PUFA effects in tissues. Non-esterified fatty acids and oxylipins can activate receptors and elicit physiologic and pathophysiologic responses (Aukema & Ravandi, 2023; Calder, 2016; Duah et al., 2023; Gabbs et al., 2015). Therefore, non-esterified fatty acids and oxylipins may be more directly associated with their functions.

Despite controversies, omega-3 fatty acid intake and/or status are generally accepted as positive in relation to several health outcomes, in particular cardiovascular disease (Abdelhamid et al., 2020; Jiang et al., 2022; Shahidi & Ambigaipalan, 2018; Wang et al., 2006). In contrast, ARA, particularly its concentrations in adipose tissue, have been associated with risk of myocardial infarction and all cause mortality (Baylin & Campos, 2004; Bork et al., 2025; Nielsen et al., 2013). Notably, the ratio of omega-6 to omega-3 oxylipins has been recently suggested as potential markers of cardiometabolic risk in young and middle-aged adults (Jurado-Fasoli et al., 2022, 2023). This is consistent with the evidence on potential benefits of omega-3

derived oxylipins with a contrast to generally detrimental effects of ARA derived ones (Ağagündüz et al., 2024; Caligiuri et al., 2017; Gabbs et al., 2015). Considering that ALA affects both DHA (Bourre et al., 1989; Couëdelo et al., 2022; Domenichiello et al., 2017; Gibson et al., 2013; Tu et al., 2010; Whelan et al., 1991) and ARA (Morise et al., 2004; Tu et al., 2010; Whelan et al., 1991), in a way that both DHA and ARA appear to reach a plateau with increasing dietary ALA (Tu et al., 2010), both DHA and ARA and their oxylipins may be useful in determining an optimal ALA intake.

### *1.1.4 DHA in comparison with ALA*

While increasing dietary ALA appears to either not have an effect or only a small one, consumption of EPA and DHA seem to be beneficial, particularly in the context of cardiovascular disease risk (Abdelhamid et al., 2020; Jiang et al., 2022; Wang et al., 2006). Similar trends can be observed from other studies. ALA has a less pronounced effect in comparison to EPA and DHA on the omega-3 index (EPA+DHA concentration in erythrocytes), a risk factor for cardiovascular disease (Dempsey et al., 2023). In addition, human studies show a high ALA intake does not seem to increase DHA and its oxylipins, but DHA increases DHA and its oxylipins, and possibly decreases ARA oxylipins (Gabbs et al., 2021; Greupner et al., 2018; Schuchardt et al., 2017). Thus, the lack of effects of increasing dietary ALA may be partly explained by DHA reaching a plateau in response to increasing dietary ALA intake (Bourre et al., 1989; Couëdelo et al., 2022; Domenichiello et al., 2017; Gibson et al., 2013; Tu et al., 2010; Whelan et al., 1991). In contrast, DHA and its oxylipins appear to continually increase with increasing dietary DHA intake (Guillot et al., 2009; S. Li et al., 2023; Lust et al., 2023; Saito et al., 1998).

Interestingly, when DHA and ARA are provided as the sole source of dietary PUFA they can reverse or prevent essential fatty acid deficiency in animals (Carlson et al., 2019; Le et al., 2009, 2013; Ling et al., 2012; Strijbosch et al., 2008) and humans under parenteral nutrition (De Meijer et al., 2010). To the best of my knowledge, one study compared the concentrations of ALA and DHA intake that would be required to achieve a similar biological effect. This study with baboons indicated that a maternal diet containing 0.45% energy from ALA was required to maximize fetal brain DHA accretion during pregnancy, while only 0.03% energy from DHA was needed to achieve a similar effect (Greiner et al., 1997). Therefore, the amount of DHA that is required to generate a similar biological effect in comparison with the dietary ALA requirement remains unknown.

### *1.2 Sex effects on oxylipins*

Understanding sex differences is important as there are relevant physiological differences between males and females, which also extend to sex differences in the prevalence of diseases and their pathophysiology (Institute of Medicine., 2001). Sex differences in fatty acid metabolism have long been reported (Christiansen et al., 1969; Decsi & Kennedy, 2011), and also shown to be tissue specific (Kitson et al., 2012) and dependent on the diet (Ranković et al., 2017; Slater-Jefferies et al., 2010). Recent work from our laboratory has shown that not only does the diet influence oxylipin profile, but sex also does, with tissue specific differences in oxylipin profiles between male and female rats, healthy or diseased (Cayer et al., 2019; Devassy et al., 2017; Ferdouse et al., 2019a, 2019b; Leng et al., 2017, 2018; Mendonça et al., 2018).

Importantly, the sex differences found were markedly influenced by diet, with extensive diet by sex interactions shown in muscle, heart, and gonadal, mesenteric and subcutaneous adipose depots (Ferdouse et al., 2019a; Mendonça et al., 2018; Penner et al., 2021). For example,

in the heart, the ALA enriched diet resulted in the highest female/male oxylipin ratios, with females having two to three times more oxylipins than males, while the DHA diet resulted in the lowest ratio, with males and females having very similar concentrations of oxylipins (Ferdouse et al., 2019a).

Although the fatty acid composition influences oxylipin production to some extent, oxylipin profiles do not necessarily reflect the diet and sex changes observed in esterified fatty acids such as phospholipid or neutral lipid fatty acids (Cayer et al., 2019; Ferdouse et al., 2019a; Leng et al., 2017, 2018; Mendonça et al., 2018). Therefore, more studies are necessary to further elucidate the mechanisms beyond phospholipid fatty acid composition that impacts oxylipin production. Taking a closer look at the previous diet by sex interaction findings, oxylipins derived from different downstream enzymatic pathways appeared to follow a similar pattern (Ferdouse et al., 2019a). Therefore, it seems unlikely that these diet by sex effects would be due to different activities of COX, LOX, and CYP. Thus, potential enzymes that could be influencing the amount of NE FA released are $PLA_2$, which are thought to be the rate limiting enzymes for the production of oxylipins (Dennis et al., 2011; Jenkins et al., 2009; Leslie, 2004).

### *1.2.1 Phospholipase $A_2$*

Phospholipase $A_2$ ($PLA_2$) is a superfamily of enzymes with six types of enzymes discovered up to date, which are further classified into 16 groups, and subgroups, described in Table 1. The six types of $PLA_2$ include: secreted ($sPLA_2$), cytosolic ($cPLA_2$), calcium-independent ($iPLA_2$), lipoprotein-associated ($Lp\text{-}PLA_2$) also known as platelet activating factor acetyl hydrolase (PAF-AH), lysosomal ($LPLA_2$), and adipose-specific ($AdPLA_2$) (Mouchlis & Dennis, 2019). Although the first $PLA_2$ was identified late in the $19^{th}$ century from snake venom (Dennis et al., 2011), and the first $iPLA_2$ was identified from canine myocardium in 1985

**Table 1-1.** Phospholipase $A_2$ superfamily

| Type | Group | Subgroup |
|---|---|---|
| $sPLA_2$ | I | A, B |
| | II | A, B, C, D, E, F |
| | III | |
| | V | |
| | IX | |
| | X | |
| | XI | A, B |
| | XII | A, B |
| | XIII | |
| | XIV | |
| $cPLA_2$ | IV | A(α), B(β), C(γ), D(δ), E(ε), F(ζ) |
| $iPLA_2$ | VI | A(β), B(γ), C(δ), D(ε), E(ζ), F(η) |
| PAF-AH | VII | A($LpPLA_2$), B(PAF-AH II) |
| | VIII | A($\alpha_1$), B($\alpha_2$), β |
| $LPLA_2$ | XV | |
| AdPLA | XVI | |

Adapted from (Dennis et al., 2011).

(Wolf & Gross, 1985), some of these enzymes, such as Ad$PLA_2$ and certain types of c$PLA_2$ and i$PLA_2$, have been only identified fairly recently (Dennis et al., 2011; Duncan et al., 2008; Ohto et al., 2005; Ramanadham et al., 2015).

Most of these $PLA_2$ enzymes have different structures and mechanisms regulating activity (Leslie, 2015; Mouchlis & Dennis, 2019). While c$PLA_2$ acts intracellularly on perinuclear membranes, i$PLA_2$ acts in the mitochondria or other organelles, and s$PLA_2$ can act both intra or extracellularly on plasma membrane (Kinsey et al., 2005; Mancuso et al., 2004; Mouchlis & Dennis, 2019). $PLA_2$s are present in several tissues and cell types (Moon et al., 2016; Ramanadham et al., 2015), but expression of the enzyme types and subtypes vary among different cells and tissues (Bonventre, 1999; Dennis, 2000; Hara et al., 2019; Kinsey et al., 2005; Leslie, 2015). In platelets, most of the $PLA_2$ activity comes from c$PLA_2$ (Duvernay et al., 2015), whereas in brain, heart, and skeletal muscle, most of the activity seems to be from i$PLA_2$ (Jenkins et al., 2002). Therefore, this could explain differences in diet by sex effects on oxylipins in different tissues, as distinct $PLA_2$ types may be differently affected by diet and sex, and their expression varies depending on the tissue being analyzed.

$PLA_2$ has an important function in lipid metabolism, particularly in the hydrolysis of membrane phospholipids, releasing a NE FA and a lysophospholipid (Mouchlis & Dennis, 2019). NE FA can be released from lysophospholipids by lysophospholipase activity, which is also a function of at least some $PLA_2$ enzymes (Leslie, 2015; Ramanadham et al., 2015; Wolf & Gross, 1996; Yan et al., 2005). The NE FA released are then available for COX, LOX, and CYP to be converted into oxylipins (Vasquez et al., 2018). In addition, there is recent suggestion that $PLA_2$ enzymes can release esterified oxylipins directly from phospholipids (Annevelink et al., 2022). The main $PLA_2$s thought to contribute to oxylipin production are the group IVA c$PLA_2\alpha$,

group VIA iPLA$_2$β, group VIB iPLA$_2$γ, and groups IIA, V, and X of sPLA$_2$s (Dennis et al., 2011).

### *1.2.2 Heart and PLA$_2$*

Heart tissue, also called myocardium, includes several cell types, including cardiac myocytes, smooth muscle cells, endothelial cells, macrophages and fibroblasts (Moon et al., 2016). The majority of iPLA$_2$γ activity in the heart comes from cardiomyocytes (Moon et al., 2016). Whereas, in endothelial cardiac cells most PLA$_2$ activity is owing to iPLA$_2$ enzymes, with more activity from iPLA$_2$β than from iPLA$_2$γ (Sharma et al., 2011). Although most PLA$_2$ activity in the heart has been reported as coming from iPLA$_2$, there could be some activity from cPLA$_2$α, and possibly from cPLA$_2$ζ in humans, which may be species specific (Haq et al., 2003; Jenkins et al., 2009; Moon et al., 2018). It is important to note that cPLA$_2$ activity in the heart is thought to be low (Cedars et al., 2009; Jenkins et al., 2009; S. J. Liu & McHowat, 1998). Interestingly, the heart has been reported as a major source of sPLA$_2$ (Berry et al., 2015; Hernandez-Anzaldo et al., 2015). However, sPLA$_2$ activity could not be detected in a cardiomyocyte cell culture or in the media (van Dijk et al., 2009). Therefore, it is still unclear whether sPLA$_2$ activity towards fatty acid release and oxylipin formation is present in the whole heart or if sPLA$_2$ is mostly secreted by the heart, not acting locally.

### *1.2.3 PLA$_2$ activity towards fatty acid release and oxylipin formation*

Although cPLA$_2$α is the only enzyme demonstrated to have specificity for the release of ARA, other isoforms also contribute to the release of ARA as NE FA (Leslie, 2015; Mouchlis et al., 2018). Recently, group IVF cPLA$_2$ζ was identified as the phospholipase in human myocardium mitochondria to account for most of the ARA release in non-failing hearts (Moon et al., 2018), which emphasise that there may be other types of PLA$_2$ displaying high activity

towards NE FA release that are still unknown or largely unstudied to date. *In vitro* studies with human recombinant enzymes indicate that iPLA$_2$β and sPLA$_2$V appear to prefer LA (Mouchlis et al., 2018). However, additional recent studies focused on omega-3 fatty acids showed sPLA$_2$V to release more DHA and iPLA$_2$β to release more EPA in comparison with both cPLA$_2$α and iPLA$_2$β or sPLA$_2$V (Hayashi et al., 2021). Therefore, research on PLA$_2$ preference for PUFA release is limited, particularly on the release of omega-3 fatty acids, and even less is known about how PLA$_2$ inhibition impacts oxylipin formation.

PLA$_2$ studies on oxylipin production also shows oxylipins to not always reflect PUFA release from PLA$_2$ (Miki et al., 2013; Moon et al., 2016; Murase et al., 2016), demonstrating the need to examine the effects of PLA$_2$ isoforms specifically on oxylipins. For example, most studies support a role of sPLA$_2$ in ARA release (Hayashi et al., 2021; Miki et al., 2013; Murase et al., 2016). However, ARA oxylipins are not always impacted (Miki et al., 2013; Murase et al., 2016). Several studies consistently reported that sPLA$_2$ isoforms seem to have a preference for DHA release in human sPLA$_2$ V (in-vitro) (Hayashi et al., 2021), mouse lymphoid tissue sPLA$_2$ IID (Miki et al., 2013), mouse recombinant sPLA$_2$ IIF (Yamamoto et al., 2015), mouse colon and sperm sPLA$_2$ X (Murase et al., 2016) with an impact on DHA oxylipin formation (Miki et al., 2013; Murase et al., 2016). And some studies support a role of sPLA$_2$ enzymes on EPA release and their oxylipin formation (Hayashi et al., 2021; Miki et al., 2013; Murase et al., 2016) However, none of these studies investigated sPLA$_2$ activity in the heart.

A cardiac myocyte specific iPLA$_2$γ knockout study recently showed that ARA, LA, and DHA oxylipins were impacted; however, other oxylipins were not analyzed (Moon et al., 2016). Additionally, this is the only study on PLA$_2$ specific fatty acid release and oxylipin formation in the heart. Human iPLA$_2$ has a preference for EPA release *in vitro* (Hayashi et al., 2021), while

others suggest that $iPLA_2$ is a major mediator of DHA release in rodent brain (Cheon et al., 2012; Strokin et al., 2007) and liver (Deng et al., 2016). $iPLA_2$ is the main $PLA_2$ type in the heart; however, there is very little information on the effects of $iPLA_2$ enzymes on PUFA release and oxylipin formation in the heart. This highlights the need for further studies to understand how fatty acid release and oxylipin formation are affected by $PLA_2$. For example, there are no studies on ALA release or oxylipin formation to date.

### *1.2.4 Diet and sex and $PLA_2$*

Different dietary lipid composition seems to influence different $PLA_2$ enzymes (Z. Li et al., 2015; Y. Liu et al., 2014; Rao et al., 2007; Rudkowska et al., 2013; Smesny et al., 2014; K.-P. Su et al., 2018; Tyagi et al., 2014; Vincentini et al., 2011). EPA and DHA supplementation significantly changed gene expression of different $PLA_2$ in peripheral blood mononuclear cells, with an increase in $cPLA_2$ and in group IIF $sPLA_2$, and a decrease in group IIA $sPLA_2$ (Rudkowska et al., 2013). Similarly, EPA increased $cPLA_2\alpha$ gene expression in peripheral blood mononuclear cells of both the control group and in patients with depression, but DHA had no effect (K.-P. Su et al., 2018). Another study evaluating fish oil supplementation and $iPLA_2$ activity in plasma found that there was a significant decrease in $iPLA_2$ activity with fish oil consumption (Smesny et al., 2014). Although the dietary effects on different $PLA_2$ seem to be consistent in different tissues, the overall effect of diet on different $PLA_2$ activity may result in tissue specific differences, as expression of different types of $PLA_2$ is tissue specific.

With respect to sex differences, several studies have shown sex differences in $PLA_2$ concentrations, activity, and gene expression (Boekholdt et al., 2005; Keeting et al., 1998; Mallat et al., 2007; Pop et al., 2013; Stone et al., 2019; Ward et al., 2018; Xu et al., 2017). While one study showed a sex difference in human serum for type II $sPLA_2$ (Boekholdt et al., 2005),

several reports did not find sex differences for $sPLA_2$ (Chalbot et al., 2009; Mallat et al., 2007; Pop et al., 2013; C. Zhang et al., 2015); however, all these studies have included mostly women at an older age. Moreover, sex differences in $sPLA_2$ group IIA levels were found in atherosclerotic carotid arteries (Ward et al., 2018), in left ventricles (Newman et al., 2017), and a study on ventricular myocardium showed that $sPLA_2$ group IIA gene expression changed with age, sex, and heart failure (Boheler et al., 2003). A study evaluating $cPLA_2\alpha$ activity on human male and female osteoblast-like cells found that both $PGE_2$ and $cPLA_2\alpha$ were higher in the male cells when compared to female (Keeting et al., 1998). This study is from over 25 years ago; however, no other reports on $cPLA_2$ and no studies on $iPLA_2$ sex differences were found. Although both diet and sex have been shown to influence $PLA_2$ activity and levels separately, the relationship between diet, sex, and $PLA_2$ remains largely unexplored. This is particularly true in regard to the effects of dietary lipid composition and sex on $PLA_2$ preference for fatty acid release and oxylipin formation.

### *1.2.5 $PLA_2$ activity considerations*

Evaluating activity of different types of $PLA_2$ can provide insight into the mechanisms regulating the sex differences in oxylipin production. In addition, analyzing enzyme preference towards different phospholipid fatty acid compositions achieved through dietary interventions, may provide the opportunity to determine general $PLA_2$ selectivity towards PUFA release. For example, ALA containing phospholipids are greatly enhanced by the dietary consumption of ALA, and this is also true for other PUFA (Ferdouse et al., 2019a; Leng et al., 2018; Mendonça et al., 2018). Thus, it is important to identify if there would be a change in activity of a particular $PLA_2$ isoform when the phospholipid membrane is enriched with a certain PUFA. It has been a challenge to study the preference of different types of $PLA_2$ enzymes, mainly because changes in

the substrate form can change the enzyme activity (Leslie & Gelb, 2004; Reynolds et al., 1991). This further demonstrates the importance of determining how enzyme activity changes according to different substrate composition to better understand *in vivo* activity of $PLA_2$ enzymes.

$PLA_2$ activity and isoform preference for PUFA are typically assessed using synthetic or labelled phospholipids and recombinant $PLA_2$ enzymes. Alternatively, phospholipids or $PLA_2$ are extracted from tissues and used for assessing $PLA_2$ isoform activity *in vitro* (Leslie & Gelb, 2004; Mouchlis et al., 2019; Reynolds et al., 1991). Although significant information has been obtained from these studies, these *in vitro* methods do not always completely reflect the situation *in vivo* (Astudillo et al., 2023). For example: single or synthetic phospholipids do not reflect the effects of the complex phospholipid compositions present in tissue on enzyme activity and single enzyme assay conditions do not account for the complex interactions between and for the relative amounts of multiple $PLA_2$ isoforms *in vivo*. These methods are also limited in their capability to account for diet and sex effects, requiring an alternative to explore this question.

Lipidomic analysis allow us to look at several lipid components and metabolites in a single run (Dumlao et al., 2011). Lipidomic based methodologies have been used, validated and shown to be effective to analyse $PLA_2$ activity (Mouchlis et al., 2018), which is commonly done by looking at either lysophospholipids or NE FA released over an incubation time (Mouchlis et al., 2019). Oxylipin analysis by mass spectrometry have been recently utilized when considering $PLA_2$ activity using knockout mouse models (Moon et al., 2012, 2016, 2018). All further demonstrating the possibility for development of a method to evaluate ex vivo activity of $PLA_2$. This is also possible due to recent advances in oxylipin analysis by mass spectrometry, allowing to scan for over 100 oxylipins at once. Until recently, oxylipin analysis was limited to a single molecule by use of enzyme-linked immunosorbent assays (EIA) (Dumlao et al., 2011).

## *1.3 Hypotheses and Objectives*

The overarching hypothesis of this thesis was that oxylipins can be used to provide novel information that will be crucial in the understanding of dietary omega-3 fatty acid requirements, and $PLA_2$ activity when considering diet and sex. The overarching objective of this thesis was to develop and apply two methods using oxylipin analysis to aid in the understanding of: omega-3 fatty acid requirements for optimal health; and $PLA_2$ activity in relation to diet and sex in rat hearts. In addition, each of the four studies encompassed in this thesis have their own specific hypotheses and objectives which are discussed below.

For the first study presented in chapter 2 on the dietary ALA requirement, the hypothesis was that oxylipins can be used as a biomarker of the dietary ALA requirement leading to higher dietary ALA requirement estimates than previously encountered by analysis of esterified DHA. The objective of this study was to 1) determine the dietary ALA requirement using oxylipins in serum, liver and kidney of male rats as a proof of concept; 2) determine the dietary ALA requirement using oxylipins in serum, liver, kidney and brain of male and female mice. This first study established a new method for assessment of the dietary ALA requirement using non-esterified DHA/ARA fatty acids and oxylipins.

For the second study presented in chapter 3 on the DHA bioequivalence to the dietary ALA requirement, the hypothesis was that by using the method from chapter 2 to determine the ALA requirement, we could determine the dose of DHA that would lead to a similar biological effect to the dietary ALA requirement. The objectives of this study were to 1) determine the dietary ALA requirement using non-esterified DHA/ARA fatty acids and oxylipins in serum, liver, heart, and brain of male and female rats; 2) determine the dose of DHA that would achieve the same biological effect on non-esterified DHA/ARA fatty acids and oxylipins as the dietary

ALA requirement established in the first study objective. This second study demonstrates the applicability of the ALA requirement method established in the first study in chapter 2.

The third and fourth studies, presented in chapters 4 and 5, respectively, had a common overarching objective of determining diet and sex effects on $PLA_2$ activity. However, to achieve this objective, we first had to develop a method that would allow for assessment of diet and sex effects on $PLA_2$ activities ex vivo in rat hearts. Therefore, the hypothesis for the third study was that different $PLA_2$ types would have distinct activities towards PUFA release and oxylipin formation in rat hearts. The study objective was to develop a method to determine $sPLA_2$, $iPLA_2$ and $cPLA_2$ activities on PUFA release and oxylipin formation ex vivo in rat hearts. This was achieved by incubation of rat heart homogenates with and without $PLA_2$ inhibitors.

For the fourth study presented in chapter 5, the hypothesis was that differences in sex effects on oxylipins between dietary ALA and DHA in rat hearts could be explained by distinct effects of diet and sex on $PLA_2$ activities. Therefore, the objectives of this study were to 1) determine the effects of sex and dietary ALA and DHA on $sPLA_2$ and $iPLA_2$ activities towards PUFA release and oxylipin formation in rat hearts; and 2) determine if the patterns of diet and sex effects on $PLA_2$ activities could explain diet by sex interactions previously observed in oxylipin analysis. This study demonstrates the applicability of the method developed in the third study in chapter 4 to assess $PLA_2$ activities ex vivo.

# Chapter 2: A Method to Estimate the Dietary α-Linolenic Acid Requirement Using Non-esterified DHA and Arachidonic Acid Oxylipins and Fatty Acids

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## *2.1 Abstract*

**Background:** The dietary requirement for ALA remains unclear, as evidenced by the absence of an RDA for this essential fatty acid (FA). In previous studies we observed that the amount of dietary ALA required to maximize non-esterified (NE) DHA oxylipins appears to be higher than the amount required to maximize tissue esterified DHA concentrations, which have classically been used to estimate the ALA requirement. Further, we observed that dietary ALA reduces esterified arachidonic acid (ARA) and its NE oxylipins.

**Objective**: Since NE oxylipins and FA mediate biologic activities of FA, we examined whether these DHA and ARA pools could be used to determine the dietary ALA requirement.

**Methods***:* Nine groups of 4-week-old male Sprague-Dawley rats (n=5) and 10 groups of male and female CD1 mice (n=6) were provided 0.1 to 2.5g ALA and 2g of linoleic acid per 100g of AIN93G-based diets. NE DHA and ARA and their oxylipins in serum, liver, kidney and brain homogenates underwent solid phase extraction and were quantified by HPLC-MS/MS. Breakpoint analysis of transitions from increase to plateau were conducted using piecewise regression.

**Results**: In response to increasing dietary ALA, NE DHA oxylipins and DHA in serum, liver and kidney (but not brain) initially increased rapidly and then plateaued whereas ARA oxylipins and ARA tended to decrease and plateau. Thus, breakpoints were calculated for the ratios of DHA/ARA and hydroxy-DHA/hydroxy-ARA ($DHA_{OH}/ARA_{OH}$), which consisted of oxylipins synthesized via pathways common to both FA. In serum, liver and kidney the highest estimated breakpoint indicated an ALA requirement of ~0.7g/100g diet (1.7%energy), approximately twice that of previous estimations.

**Conclusion:** This study supports the use of NE $DHA_{OH}/ARA_{OH}$ or DHA/ARA as biochemical indicators of the ALA requirement. Applying this method in rats and mice indicates that the requirement is higher than previously estimated using esterified DHA alone.

Keywords: α-linolenic acid; dose response; dietary requirement; fatty acids; oxylipins; docosahexaenoic acid; arachidonic acid; mice; rats; sex.

## *2.2 Introduction*

The omega-3 fatty acid α-linolenic acid (ALA) is an essential fatty acid (FA) for rodents and humans due to their inability to synthesize ALA de novo, thus requiring dietary consumption. The health benefits of ALA are believed to be due to its elongation and desaturation into longer chain PUFA, such as EPA and/or DHA. Absence or low concentrations of ALA have been shown to negatively impact retina function and vision, behaviour, and mortality rates in animal models [reviewed in (Brenna, 2011; Neuringer et al., 1988)]. The only reported case of ALA deficiency in humans was a case of total parental nutrition with very low ALA which resulted in neurological symptoms and vision blurring (Holman et al., 1982). Despite ALA being considered an essential FA, there is no official recommendation on dietary intake for rats and mice (Hamilton & Hogan, 1995). For humans there are only Adequate Intake reference values, which are based on the usual dietary intake of a population that is assumed to be maintaining an adequate ALA status (European Food Safety Authority, 2010; Institute of Medicine, 2005). Therefore, the minimum amount of dietary ALA required for optimal health remains unclear.

ALA requirements in rodents have classically been estimated by determining the point at which tissue total DHA reaches a plateau in response to increasing dietary ALA, an approach that is similar to how other nutrient requirements have been set (Willett, 1998). These studies have suggested that the ALA requirement ranges from 0.3 to 1.0% energy (~0.1 to 0.4g ALA/100g of diet in AIN-93G based diets) (Bourre et al., 1989; Couëdelo et al., 2022; Domenichiello et al., 2017; Gibson et al., 2013; Tu et al., 2010; Whelan et al., 1991). On a % energy basis this is similar to humans, where the dietary recommendation is 0.4-0.5% energy from ALA for both adults and children (Institute of Medicine, 2005).

Oxylipins, bioactive metabolites formed via multiple enzymatic and non-enzymatic pathways from PUFA, activate receptors that mediate many of the PUFA effects in tissues (Aukema & Ravandi, 2023; Gabbs et al., 2015). Compared to the use of maximal tissue or phospholipid DHA to estimate the ALA requirement, several of our prior studies on oxylipins suggested that tissue DHA oxylipins may reach a maximum concentration with higher dietary ALA intake, indicating a potentially higher requirement. In a rat study with high fat diet-induced obesity and varying amounts and ratios of dietary ALA and linoleic acid (LA), the concentration of ALA that resulted in the highest concentrations of renal DHA oxylipins was much higher than the amount required to maximize DHA (Caligiuri et al., 2013). In subsequent studies in healthy rats a similar pattern was observed (Leng et al., 2017, 2018). Since oxylipins mediate physiological effects of FAs and more directly reflect FA functions and their requirements (Gabbs et al., 2015), a potentially higher ALA requirement based on oxylipin data is of interest.

Also of potential relevance to the dietary ALA requirement is the fact that dietary ALA also affects other oxylipins (Ferdouse et al., 2019a; Leng et al., 2018; Mendonça et al., 2018). The balance of omega-6/omega-3 oxylipins determines their physiological effects (Shearer & Walker, 2018), and inclusion of oxylipins derived from other FA affected by dietary ALA could be used to provide a more comprehensive assessment of the ALA requirement. Of particular interest are the arachidonic acid (ARA) oxylipins due to their predominantly opposing effects on physiology when compared to DHA oxylipins (Gabbs et al., 2015), as well as being the main omega-6 oxylipins altered by dietary ALA (Cayer et al., 2019; Ferdouse et al., 2019a; Greupner et al., 2018; Leng et al., 2017, 2018; Mendonça et al., 2018).

One of the reasons that dietary ALA effects on DHA compared to its oxylipins may differ is because oxylipins are primarily analyzed in their free, non-esterified (NE) form, while

previous studies that used DHA as the indicator of adequacy have analyzed esterified DHA. If it is the NE compared to esterified pool that is responsible for this difference, NE FA may respond similarly to NE oxylipins. This is plausible, as differences in the response to the concentration of dietary ALA on esterified and NE DHA have been reported in at least two studies (Couëdelo et al., 2022; Kim et al., 2011). However, these studies were not examining the dietary ALA requirement, nor have there been any studies that have included ARA as an indicator of this requirement. Therefore, the objective of this work was to test whether the NE pools of both DHA and ARA oxylipins, or the NE pools of both DHA and ARA could be used to provide a more comprehensive and functionally relevant assessment of the dietary ALA requirement.

### *2.3 Methods*

#### *2.3.1 Animal studies*

First, 4-week-old Sprague Dawley male rats were single caged and randomized into 9 diet groups (n=5, 45 rats total) and provided ad libitum access to diets ranging from 0.07 to 1.86g of ALA/100g diet for 5 weeks. The diets were the same as the AIN93G diet (Reeves et al., 1993) except that soybean oil was replaced by an equal total concentration (7g oil/100g of diet) of combinations of flaxseed, safflower, coconut, and olive oils to maintain LA and MUFA concentrations constant at ~2g and ~1g/100g diet, respectively, and to increase ALA at the expense of saturated FA. The concentration of LA at 2g/100g of diet meets the LA requirement of 1.2g/100g of diet in rodents (Bourre et al., 1989), and represents 4.8% of energy in these diets, which is similar to the ~5% energy reflected in the Adequate Intake recommendation for this FA in humans (Institute of Medicine, 2005). The formulated diet including the oils used and the analyzed FA compositions are shown in **Table 1**. All diet ingredients were purchased from Dyets (Bethlehem, PA).

**Table 2-1.** Oils used and analyzed fatty acid composition of diets provided to Sprague-Dawley rats

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Dietary oils used (g/100 g diet)* | | | | | | | | |
| Flax oil | 0.16 | 0.25 | 0.34 | 0.53 | 1.06 | 1.61 | 2.15 | 2.68 | 3.58 |
| Coconut oil | 3.46 | 3.41 | 3.34 | 3.21 | 2.86 | 2.49 | 2.12 | 1.78 | 1.17 |
| Safflower oil | 2.48 | 2.46 | 2.45 | 2.42 | 2.33 | 2.25 | 2.17 | 2.08 | 1.94 |
| Olive oil | 0.90 | 0.88 | 0.87 | 0.84 | 0.75 | 0.65 | 0.56 | 0.46 | 0.31 |
| | *Analyzed fatty acids (g/100 g diet)* | | | | | | | | |
| **ALA (g/100g diet)** | **0.09** | **0.11** | **0.14** | **0.48** | **0.54** | **0.75** | **1.10** | **1.30** | **1.86** |
| ALA (energy %) | 0.22 | 0.26 | 0.34 | 1.15 | 1.29 | 1.80 | 2.63 | 3.11 | 4.45 |
| LA | 2.23 | 1.86 | 1.64 | 2.38 | 2.00 | 1.88 | 1.90 | 1.80 | 1.95 |
| SFA | 2.34 | 2.32 | 2.06 | 3.78 | 1.82 | 1.80 | 1.17 | 0.90 | 1.23 |
| MUFA | 1.24 | 1.03 | 0.90 | 1.31 | 0.99 | 1.00 | 1.14 | 0.96 | 1.18 |

Total oil was 7g/100 g of diet. Other ingredients in the AIN93G-based diets were as follows: cornstarch (39.8 g/100 g), casein (20 g/100 g), dytrose (13.2 g/100 g), sucrose (10 g/100 g), fibre (5 g/100 g), AIN93G-MX (3.5 g/100 g), AIN93G-VX (1 g/100 g), L-cystine (0.3 g/100 g), choline bitartrate, 41.1% choline (0.25 g/100 g), TBHQ (0.0014 g/100 g). α-linolenic acid (ALA), linoleic acid (LA). Diet fatty acids were analyzed in duplicate.

Second, 4-week-old CD1 male and female mice, were double caged and randomized into 10 similar diet groups and provided diets ranging from 0.08 to 2.56g of ALA/100g diet (n=6, 120 mice total) for 6 weeks using similar diets **(Table 2)**.

After the dietary intervention period, non-fasting animals were anesthetized with isoflurane and terminated by decapitation. Trunk blood was collected to obtain serum, which was stored at -80°C until analysis. After exsanguination, kidney and liver were harvested from rats, and brain, kidney and liver were harvested from mice. Tissues were immediately frozen in liquid nitrogen and stored at -80°C until analysis. One kidney sample for one male mouse in the 0.82 ALA diet, and one serum sample for one female mouse in the 0.56 ALA diet were lost during sample preparation for analyses. All procedures were done in accordance with the Canadian Council for Animal Care and ARRIVE guidelines (Percie du Sert et al., 2020) and approved by the University of Manitoba Animal Care Committee.

### *2.3.2 Fatty acid analysis of oils and diets*

Total FA composition of all the oils and the final diets were confirmed by analyzing diet samples in duplicate using gas chromatography with flame ionization detection (GC-FID), as previously described (Gabbs et al., 2021). Briefly, 25mg of diet sample was analyzed following a liquid-liquid extraction based on the Folch method (Folch et al., 1957) for total lipids using chloroform/methanol (2:1/vol:vol) with 0.01% BHT, and 0.73% sodium chloride. Phosphatidylcholine C15:0 (Avanti Polar Lipids, AL, USA) and triacylglycerol C17:0 (NuCheck, MN, USA) were added as internal standards. Lipid extracts were dried down and FA were transmethylated using methanolic $H_2SO_4$ (Aukema & Holub, 1989). The resulting methylated FAs were extracted using hexane and ultrapure water, dried down and re-suspended in hexane.

**Table 2-2.** Oils used and analyzed fatty acid composition of diets provided to CD1 mice

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Dietary oils (g/100 g diet)* | | | | | | | | | |
| Flax oil | 0.16 | 0.27 | 0.36 | 0.56 | 1.15 | 1.73 | 2.32 | 2.91 | 3.69 | 4.87 |
| Coconut oil | 3.15 | 3.08 | 3.05 | 2.91 | 2.54 | 2.17 | 1.80 | 1.43 | 0.94 | 0.20 |
| Safflower oil | 2.89 | 2.87 | 2.84 | 2.81 | 2.68 | 2.57 | 2.45 | 2.33 | 2.18 | 1.93 |
| Olive oil | 0.80 | 0.78 | 0.76 | 0.72 | 0.63 | 0.53 | 0.43 | 0.33 | 0.19 | 0.00 |
| | *Analyzed Fatty acids (g/100 g diet)* | | | | | | | | | |
| **ALA (g/100g diet)** | **0.08** | **0.13** | **0.14** | **0.27** | **0.56** | **0.82** | **1.20** | **1.52** | **1.88** | **2.56** |
| ALA (energy %) | 0.19 | 0.31 | 0.34 | 0.65 | 1.34 | 1.96 | 2.87 | 3.64 | 4.50 | 6.13 |
| LA | 2.01 | 1.98 | 2.01 | 1.83 | 1.94 | 2.10 | 2.10 | 2.01 | 2.05 | 2.04 |
| SFA | 2.51 | 2.51 | 2.49 | 2.63 | 2.31 | 1.93 | 1.62 | 1.53 | 1.25 | 0.77 |
| MUFA | 1.03 | 1.02 | 1.03 | 0.96 | 0.99 | 1.04 | 1.08 | 1.05 | 1.07 | 1.09 |

Total oil was 7 g/100 g of diet. Other ingredients in the AIN93G-based diets were as follows: cornstarch (39.8 g/100 g), casein (20 g/100 g), dytrose (13.2 g/100 g), sucrose (10 g/100 g), fibre (5 g/100 g), AIN93G-MX (3.5 g/100 g), AIN93G-VX (1 g/100 g), L-cystine (0.3 g/100 g), choline bitartrate, 41.1% choline (0.25 g/100 g), TBHQ (0.0014 g/100 g). α-linolenic acid (ALA), linoleic acid (LA). Diet fatty acids were analyzed in duplicate.

Samples were separated on a 30m DB-225MS column (Agilent Technologies, CA, USA) using a Bruker 450 GC-FID (Gabbs et al., 2021).

### *2.3.3 NE oxylipin and FA analysis of tissues and serum*

Analyses of NE oxylipins and FAs were performed as described (Aukema et al., 2016; Leng et al., 2017; Monirujjaman et al., 2017) based on the method from (Dumlao et al., 2011a). Prior to homogenization whole frozen kidneys were lyophilized, and representative portions of whole lyophilized kidney and of frozen livers, and whole frozen brains were homogenized in Tyrode's buffer (pH 7.6) (Sigma-Aldrich, MA, USA) (dry tissue: 1/28, wet tissue: 1/5.7, wt/vol). Triton X-100 to achieve a final concentration of 0.01% and an antioxidant cocktail containing 0.2mg/ml BHT, 0.2mg/ml EDTA, 100µM indomethacin, 100µM trans-AUCB in methanol/water (50/50, vol/vol) (Rund et al., 2018) were added to the homogenates and serum samples. Lipids in these samples were then solubilized by adding methanol and HCl to achieve a 20% methanol in pH3 water solution. Deuterated internal standards were added to each sample (Cayman Chemical, MI, USA) [a full list of standards can be found in (Manson et al., 2023)]. Solid phase extraction was performed using 33 µm polymeric reverse-phase Strata-X solid phase extraction columns (Phenomenex, CA, USA) (28). NE oxylipins and FAs were eluted from these columns with methanol, dried down under a nitrogen stream, resuspended in the mobile phase (water/acetonitrile/acetic acid, 70/30/0.02, vol/vol/vol), and analyzed using HPLC-MS/MS (QTRAP 6500; Sciex, ON) with a reverse phase C18 column. Quantitation was based on the stable isotope dilution method (Hall & Murphy, 1998). The limit of quantification (LOQ) was set at peak heights of 5 times baseline and an oxylipin was considered quantifiable if this criterion was met for at least 4 animals in a diet group. **Supplemental Table 1** shows the oxylipins

quantified in each tissue. Samples were prepared in random order, and both sample preparation and peak selection were blinded.

### *2.3.4 Statistical analysis*

Statistical analysis to determine diet and sex effects were conducted by one-way (diet) or two-way (diet, sex) ANOVA as appropriate. Normality was tested prior to ANOVA using a Shapiro-Wilk test. If data was not normal, log-transformation was applied, and if data contained zeros, (data + 1) was log transformed. Interactions were evaluated using a type 3 ANOVA; if the interaction was not significant, a type 2 ANOVA was conducted using diet and sex. Protected Fisher's LSD test was used as post hoc test to determine diet and sex differences when diet or diet*sex interactions were present. Data are shown as mean ± SE. Significance was set at $p<0.05$.

Ratios were expressed as the $\log_2$ of the ratio (or fold-change) so that the reciprocals of the ratio are symmetric and have equal weighting. Ratio data were analyzed using piecewise regression (also known as segmented regression) to first determine whether two linear regressions fit the data better than one linear regression (Muggeo, 2008; Ryan & Porth, 2007). If two linear regressions were a better fit using hypothesis testing (Muggeo, 2016), determination of the breakpoint where there is a change in slope within the dataset was conducted. Piecewise regression with breakpoint estimates incorporating bootstrap was conducted using RStudio (2023.06.0+421, 2023 Posit Software, PBC) using the package segmented by Vito M. R. Muggeo version 1.6-4 from April 13, 2023 (Muggeo, 2023). Breakpoints were considered different if their 95% CIs did not overlap.

## *2.4 Results*

### *2.4.1 Animal and tissue weights*

Male mice had higher body, kidney, and liver weights than female mice, but diet had no effect on any of these parameters in rats or mice **(Supplemental Tables 2-3)**.

### *2.4.2 Dietary ALA effects on NE oxylipins and FAs*

Dietary ALA consistently altered the concentrations of NE oxylipins derived from ALA **(Supplemental Fig 1A)**, EPA **(Supplemental Fig 2A)**, DHA (**Figure 1A**) and ARA **(Figure 2A)**, but not LA oxylipins **(Supplemental Fig 3A)** in kidneys, livers and serum of both rats and mice; the only exception to this was rat liver DHA, which was not significantly altered by diet (ANOVA P value and complete statistic results shown in **Supplemental Tables 4-5**). Both ALA and EPA oxylipins progressively increased in response to increasing dietary ALA. This pattern was distinct from DHA oxylipins, which initially increased and then leveled off as dietary ALA content increased. ARA oxylipin effects were opposite to that for DHA oxylipins, as ARA oxylipins initially decreased and then leveled off as dietary ALA content increased. In the brain, by contrast, increasing dietary ALA only increased EPA oxylipins (**Figure 3**).

Diet effects on the NE FA pools were similar to effects on oxylipins. In kidney, liver and serum, diet had effects on ALA (**Supplemental Fig 1B**), EPA (**Supplemental Fig 2B**), DHA (**Fig 1B**) and ARA (**Fig 2B**), although the effects were less consistent than for the oxylipins as no diet effects were observed for DHA and ARA in rat kidneys and for ARA in mouse kidneys. However, the patterns of FA concentrations in response to dietary ALA were similar to the oxylipin patterns, including the brain where only EPA increased with increasing dietary ALA.

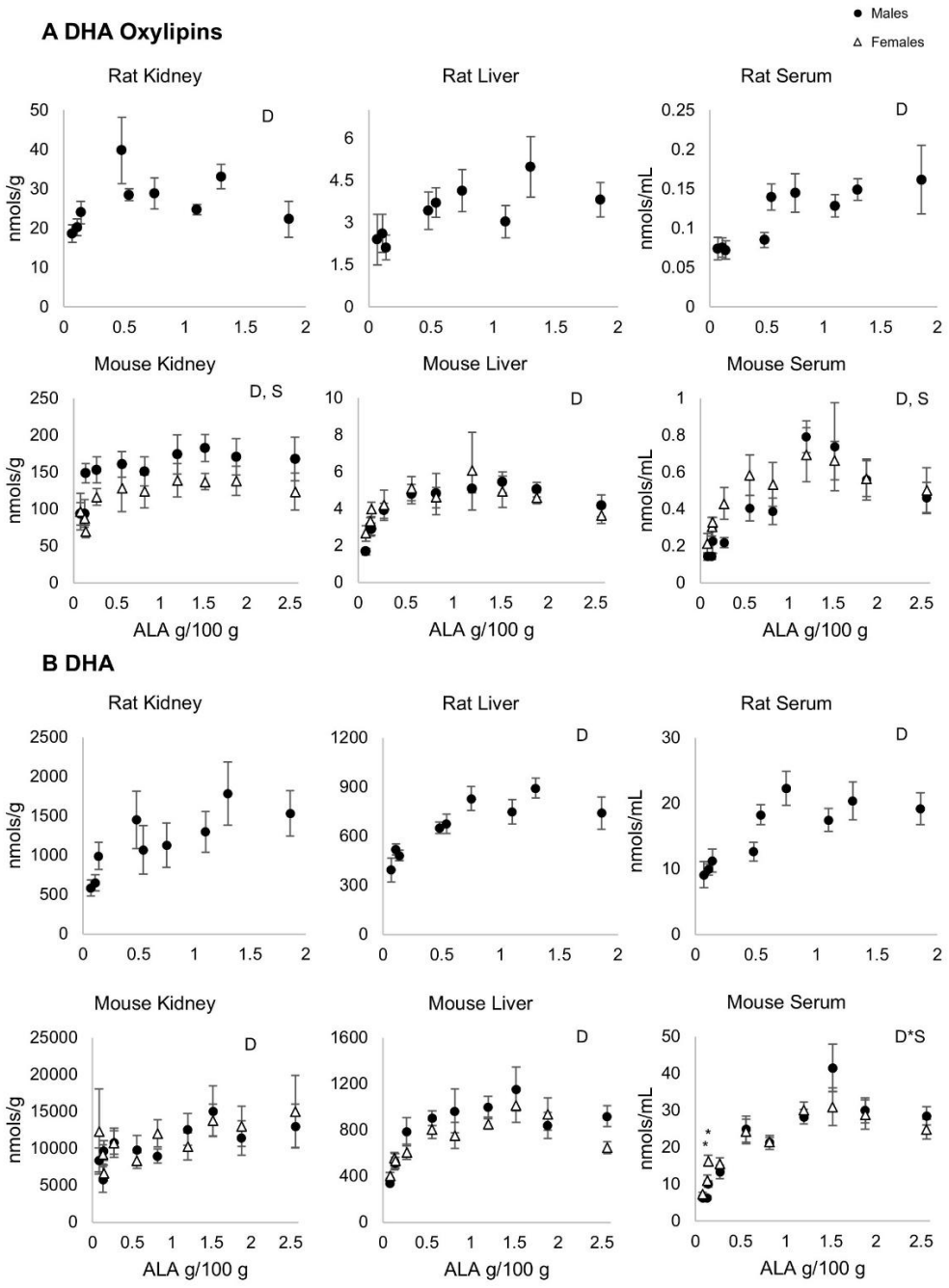


**Figure 2-1.** Total NE DHA oxylipins (A) and NE DHA (B) in response to dietary α-linolenic acid (ALA) in kidney, liver and serum.
Females: white triangles with black outline; Males: black-filled circles. Data shown as mean and SE (N=119-120, n=5-6 mice; N=45, n=5 rats). ANOVA was conducted to evaluate diet, sex and diet*sex effects. D, S and D*S denote diet and sex main effects and their interactions, respectively. Where interactions are present, sex differences are indicated by an * above each diet group on the graph, and diet effects within each sex are reported in Supplemental Tables 4 and 5.

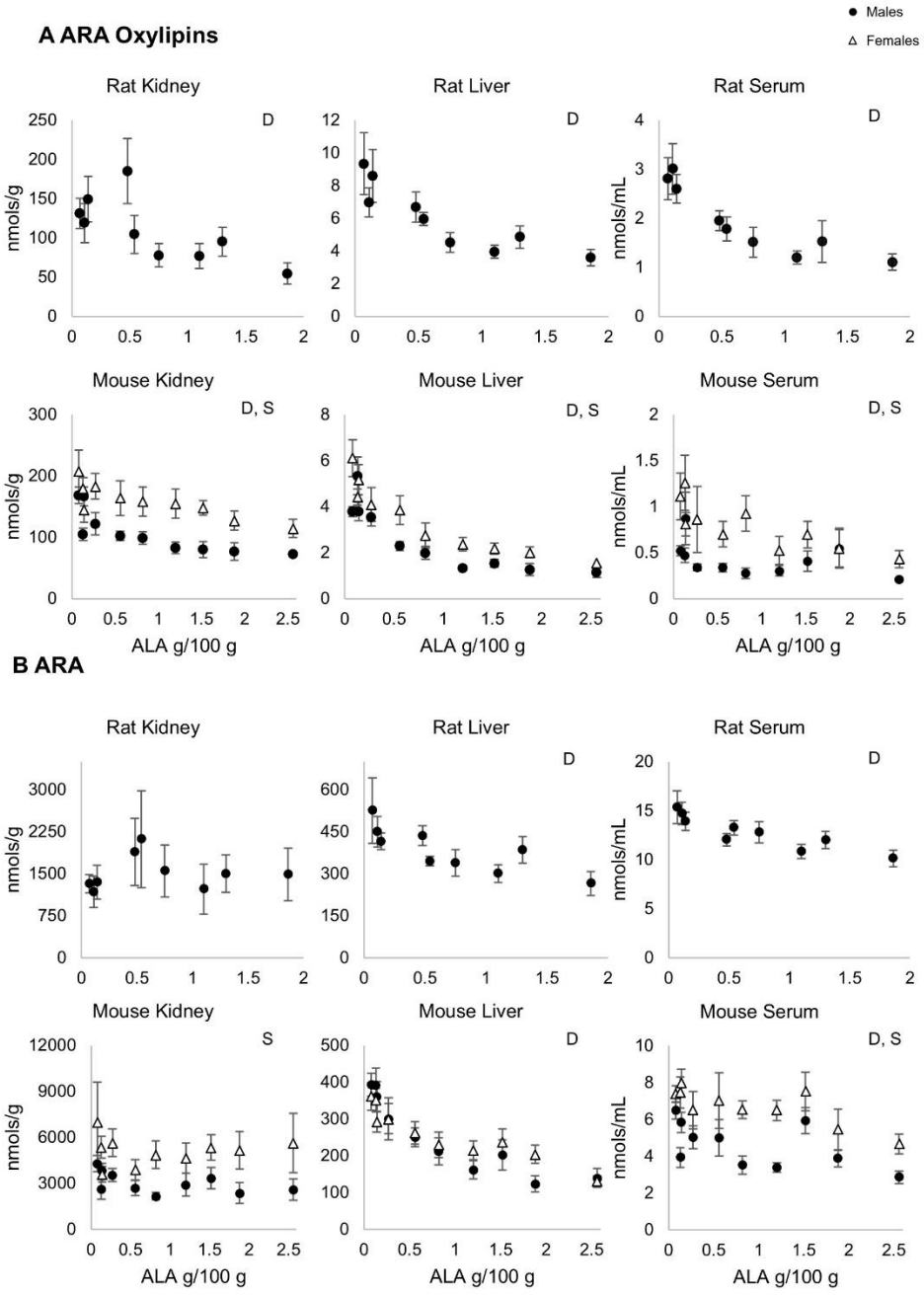


**Figure 2-2.**Total NE arachidonic acid (ARA) oxylipins (A) and NE ARA (B) in response to dietary α-linolenic acid (ALA) in kidney, liver and serum.
Females: white triangles with black outline; Males: black-filled circles. Data shown as mean and SE (N=119-120, n=5-6 mice; N=45, n=5 rats). ANOVA was conducted to evaluate diet, sex and diet*sex effects. D, S and D*S denote diet and sex main effects and their interactions, respectively. Where interactions are present, sex differences are indicated by an * above each diet group on the graph, and diet effects within each sex are reported in Supplemental Tables 4 and 5.

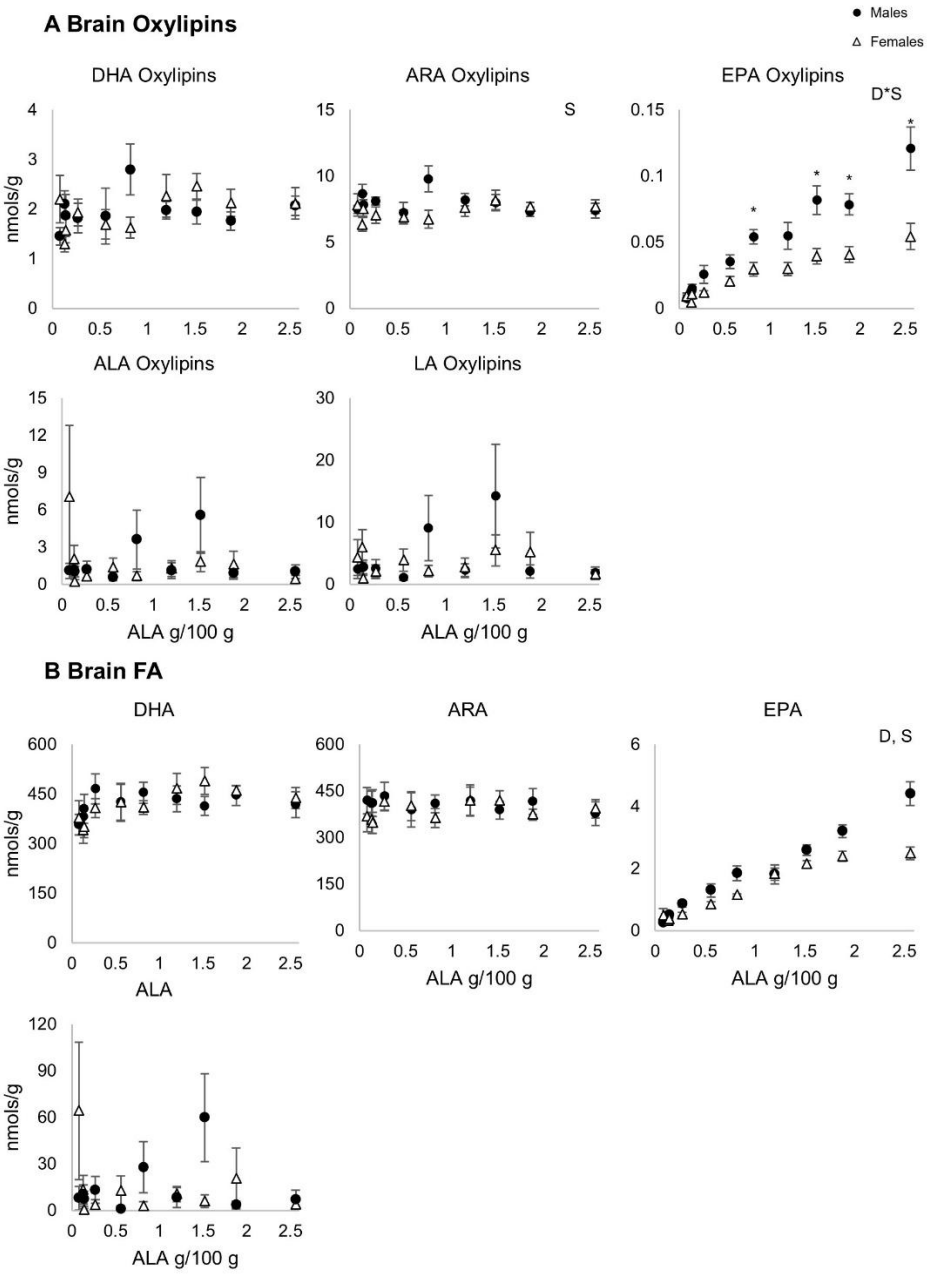


**Figure 2-3.** NE oxylipin and FA concentrations in response to dietary α-linolenic acid (ALA) in the brain.
Females: white triangles with black outline; Males: black-filled circles. Data shown as mean and SE (N=120, n=6 mice). ANOVA was conducted to evaluate diet, sex and diet*sex effects. D, S and D*S denote diet and sex main effects and their interactions, respectively. Where interactions are present, sex differences are indicated by an * above each diet group on the graph, and diet effects within each sex are reported in Supplemental Tables 4 and 5. Arachidonic acid (ARA); α-linolenic acid (ALA); linoleic acid (LA).

### *2.4.3 Ratios of NE pools of DHA and ARA and their oxylipins as biomarkers of adequacy*

In all three tissues and serum, the concentration of ALA and EPA and their oxylipins (except brain ALA oxylipins were not affected by dietary ALA) progressively continue to increase in response to dietary ALA, so these cannot be used to establish a dietary requirement for ALA. Similarly, LA oxylipins are not useful in this regard since they are not altered by dietary ALA. On the other hand, in the kidney, liver and serum (but not brain) the concentration of DHA and ARA and their oxylipins reach a point where they approach a maximum and minimum, respectively, with increasing concentrations of dietary ALA, potentially allowing them to be used as indicators of the ALA requirement.

Since a balance of DHA and ARA and their oxylipins both contribute to physiologic function and health, both should be used to assess the dietary ALA requirement. Therefore, the requirement would be the minimum dietary ALA needed to achieve a plateau in the ratio of DHA to ARA and/or their oxylipins. For DHA and ARA, this is simply DHA/ARA. For DHA and ARA oxylipins, multiple pathways produce oxylipins, including some that are exclusive to one FA (e.g. ARA is converted by cyclooxygenase to prostanoid type oxylipins, but not DHA), or are found in different numbers between tissues (i.e. cytochrome P450 epoxygenase oxylipins). Therefore, an unbiased ratio should be comprised of oxylipins that are formed by pathways shared by both FA and are commonly present across multiple tissues. The major pathways that meet this criterion include the lipoxygenase and cytochrome P450 ω-hydroxylase pathways that produce hydroxy-FA (Supplemental Table 1). These are the hydroxy-docosahexaenoic acids (HDoHEs) from DHA and hydroxy-eicosatetraenoic acids (HETEs) from ARA. Hence, the hydroxy-DHA/hydroxy-ARA ($DHA_{OH}/ARA_{OH}$) ratio was used for the ratio of DHA to ARA oxylipins.

As seen in **Figure 4A**, $DHA_{OH}/ARA_{OH}$ in both rat and mouse kidney, liver and serum increased rapidly in response to lower dietary ALA concentrations and then reached a breakpoint after which little or no further increase occurred with further increases in dietary ALA. The tissue that has the highest breakpoint determines the overall dietary requirement, since intake below the value would be insufficient for that tissue. In rats the highest $DHA_{OH}/ARA_{OH}$ breakpoint was at 0.75g ALA/100g diet in both the kidney and serum, but this did not differ from liver as all three of the 95% CIs overlapped with each other (**Table 3**). In female mice the $DHA_{OH}/ARA_{OH}$ breakpoint in the liver (0.68g) was higher than in serum, with the kidney breakpoint being in between. In male mice, the oxylipin ratio in the liver (0.63g) and serum (0.66g) yielded similar estimates, with the kidney breakpoint being significantly lower (**Table 4**).

Using the DHA/ARA ratios, in rats the highest breakpoints were in the liver (0.75g ALA/100g diet) and serum (0.70g), with no breakpoint estimable in the kidney (Table 3). In female mice the 95% CI overlapped for all tissues, but the highest breakpoint was in the kidney (0.68g). In male mice, the breakpoint in serum (0.60g) was higher than in either liver or kidney (Table 4).

When comparing $DHA_{OH}/ARA_{OH}$ and DHA/ARA, the only significantly different breakpoints were in rat kidney (no breakpoint estimable) and mouse liver (where the $DHA_{OH}/ARA_{OH}$ breakpoint was higher). When comparing male rats and mice, $DHA_{OH}/ARA_{OH}$ breakpoints were not different between species, and when comparing DHA/ARA ratios the breakpoint was lower only in mouse liver. Hence overall, whether using the $DHA_{OH}/ARA_{OH}$ or DHA/ARA ratios in kidney, liver or serum, the highest breakpoint estimates were consistently ~0.7g/100g diet in both rats and mice.

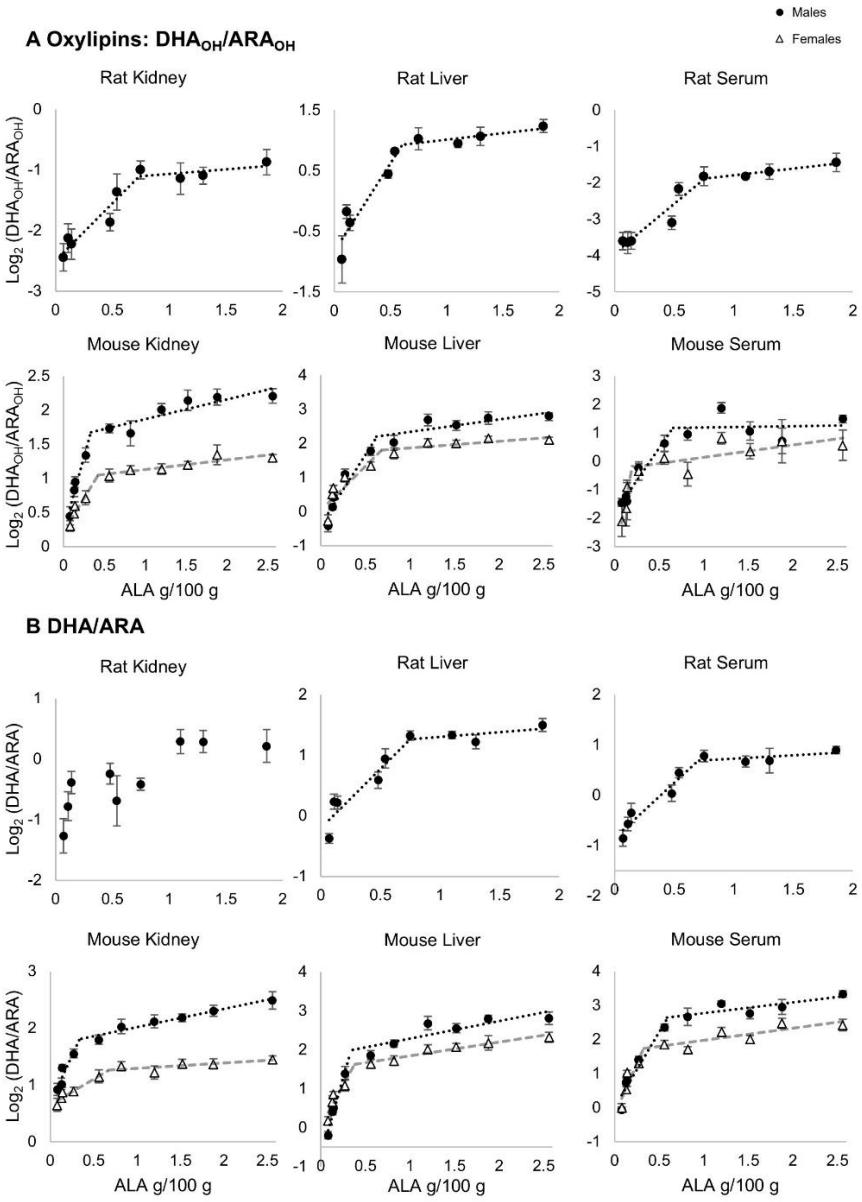


**Figure 2-4.** DHA$_{OH}$/ARA$_{OH}$ (A) and DHA/ARA (B) in response to dietary α-linolenic acid (ALA) in kidney, liver and serum.
Females: white triangles with black outline; Males: black-filled circles. Log2 (fold change) data shown as mean and SE (N=119-120, n=5-6 mice; N=45, n=5 rats). Two lines indicate that two different linear regressions fit the dataset better than one, with the common point between the lines being the breakpoint. Data was analyzed by piecewise regression to determine a breakpoint. Full description of breakpoints and confidence intervals are found in Tables 3-4. DHA/ARA represents the ratio of NE DHA/ARA and DHA$_{OH}$/ARA$_{OH}$ represents the ratio of hydroxy DHA/hydroxy ARA oxylipins. Oxylipins in the DHA$_{OH}$/ARA$_{OH}$ ratio are: 4, 7, 8, 10, 11, 13, 14, 16, and 17-hydroxy-docosahexaenoic acid (HDoHE) and 5, 8, 9, 11, 12, and 15- hydroxy-eicosatetraenoic acid (HETE), where present.

**Table 2-3.** Tissue breakpoints for $DHA_{OH}/ARA_{OH}$ and DHA/AA from rats provided increasing concentrations of dietary ALA.

| | BP (g ALA/100g diet) | 95% CI | P value |
|---|---|---|---|
| | *Kidney* | | |
| $DHA_{OH}/ARA_{OH}$ | 0.75 ± 0.20 | 0.34, 1.16 | 0.0482 |
| DHA/ARA | No breakpoint | | 0.1762 |
| | *Liver* | | |
| $DHA_{OH}/ARA_{OH}$ | 0.60 ± 0.09 | 0.43, 0.78 | 0.0004 |
| DHA/ARA | 0.75 ± 0.10 | 0.55, 0.95 | 0.0002 |
| | *Serum* | | |
| $DHA_{OH}/ARA_{OH}$ | 0.75 ± 0.16 | 0.44, 1.06 | 0.0133 |
| DHA/ARA | 0.70 ± 0.10 | 0.50, 0.91 | 0.0005 |

Data are reported as breakpoint (BP) ± SE, 95% confidence interval (CI) and P value derived from piecewise regression analysis to generate a BP. DHA/ARA represents the ratio of NE DHA/ARA and $DHA_{OH}/ARA_{OH}$ represents the ratio of hydroxy DHA/hydroxy ARA oxylipins. α-linolenic acid (ALA), arachidonic acid (ARA).

**Table 2-4.** Tissue breakpoints for $DHA_{OH}/ARA_{OH}$ and DHA/AA from mice provided increasing concentrations of dietary ALA.

| | **Female** | | | **Male** | | |
|---|---|---|---|---|---|---|
| | BP (g ALA/100g diet) | 95% CI | P value | BP (g ALA/100g diet) | 95% CI | P value |
| | | | *Kidney* | | | |
| $DHA_{OH}/ARA_{OH}$ | 0.42 ± 0.10 | 0.23, 0.61 | <0.0001 | 0.34 ± 0.05 | 0.25, 0.42 | <0.0001 |
| DHA/ARA | 0.68 ± 0.16 | 0.36, 0.99 | 0.0059 | 0.36 ± 0.06 | 0.24, 0.47 | <0.0001 |
| | | | *Liver* | | | |
| $DHA_{OH}/ARA_{OH}$ | 0.68 ± 0.09 | 0.50, 0.87 | <0.0001 | 0.63 ± 0.06 | 0.50, 0.75 | <0.0001 |
| DHA/ARA | 0.38 ± 0.06 | 0.26, 0.50 | <0.0001 | 0.34 ± 0.03 | 0.28, 0.39 | <0.0001 |
| | | | *Serum* | | | |
| $DHA_{OH}/ARA_{OH}$ | 0.20 ± 0.05 | 0.09, 0.30 | 0.0269 | 0.66 ± 0.12 | 0.42, 0.90 | <0.0001 |
| DHA/ARA | 0.32 ± 0.04 | 0.25, 0.39 | <0.0001 | 0.60 ± 0.05 | 0.49, 0.70 | <0.0001 |

Data are reported as breakpoint (BP) ± SE, 95% confidence interval (CI) and P value derived from piecewise regression analysis to generate a BP. DHA/ARA represents the ratio of NE DHA/ARA and $DHA_{OH}/ARA_{OH}$ represents the ratio of hydroxy DHA/hydroxy ARA oxylipins. α-linolenic acid (ALA), arachidonic acid (ARA).

### *2.4.4 Sex differences in mouse tissues*

For mouse oxylipins, sex differences in response to dietary ALA were observed at least once for all tissues for oxylipins derived from DHA, ARA and EPA, but not for ALA and LA. There were main effects of sex for DHA (Figure 1) oxylipins in kidney and serum, with higher concentrations of these oxylipins in male kidney and female serum, and on ARA oxylipins in all tissues, with these oxylipins being higher in females in kidney, liver and serum (Figure 2A), and lower in females in brain (Figure 3). There was a diet by sex interaction in EPA oxylipins in mouse kidney, with higher oxylipins in males at lower concentrations of dietary ALA.

For FA, sex differences were observed in all tissues at least once for ALA, EPA and ARA, but for DHA there was only a sex difference in two diet groups in mouse serum. Kidney and serum ARA was higher in females (Figure 2B), while liver, serum and brain EPA was lower in females, and kidney EPA was higher in males (Figure 3, Supplementary Fig 2). LA cannot be quantified with the HPLC/MS-MS method used, so is not reported herein.

The only breakpoints which displayed a sex effect were in serum. For both $DHA_{OH}/ARA_{OH}$ and DHA/ARA, the breakpoints were lower in females (Table 4).

## *2.5 Discussion*

This study demonstrates that NE $DHA_{OH}/ARA_{OH}$ or DHA/ARA can be used to estimate the dietary ALA requirement in rats and mice. In contrast to prior methods based on the concentration of esterified DHA, this approach uses the NE pools of oxylipins or FA. These free forms of oxylipins and FA are the biologically active forms that bind to their cognate receptors and elicit physiologic and pathophysiologic responses, so are more directly associated with function. In addition, these ratios also incorporate the effects on ARA or its oxylipins, neither of which have previously been used in the assessment of the dietary ALA requirement. ARA and its

oxylipins generally have opposing effects to DHA or its oxylipins, and therefore incorporating both in this assessment is more encompassing of the physiologic and health effects elicited by dietary ALA.

The estimated requirement for dietary ALA in rats and mice when using this approach was found herein to be ~0.7g ALA/100g of diet (~1.7% energy). This estimate is approximately twice as high as previous estimates that were determined by examining the point at which esterified DHA plateau in response to increasing dietary ALA in several tissues. An early study of various tissues and brain cells resulted in an estimate of ~0.2g ALA/100g of diet (~0.4% energy) regardless of which tissue was examined (Bourre et al., 1989), and the 0.5g ALA/100g diet provided by soybean oil in the AIN93G diet is based on these findings (Reeves et al., 1993). Likewise, another early study measuring DHA in mouse liver and peritoneal macrophages suggested a plateau at or below the lowest dietary ALA provided (0.29g ALA/100g of diet) (Whelan et al., 1991). More recent reports examining the concentration at which esterified (phospholipid) DHA reaches a plateau in plasma or erythrocytes reveal that this occurs between 0.3-1% energy ALA (Couëdelo et al., 2022; Gibson et al., 2013; Tu et al., 2010). Similarly, estimates of the esterified DHA and DHA synthesis rates were found to plateau at or below 0.3g ALA/100g diet in serum (Domenichiello et al., 2017). Together, these studies supported an ALA requirement between 0.3-1% energy ALA, which is equivalent to 0.1-0.4g ALA/100g in AIN-93G based diets. It should be noted that the estimated dietary ALA requirement herein was set in the context of the AIN93G diet with a fixed concentration of dietary LA and MUFA, and that different concentrations of LA and MUFA may lead to different results.

The method herein to determine the ALA requirement uses the point where an indicator reaches an optimal value or plateau beyond which it is no longer affected by an increase in ALA.

This point indicates additional benefits are unlikely or limited with further increase in nutrient intake. This is a common approach for determining nutrient requirements (Cashman & Flynn, 1999; Willett, 1998) that has been applied to several nutrients, including amino acid requirements (Elango et al., 2008; Institute of Medicine, 2005). ALA and EPA and their oxylipins continue to increase in response to increasing dietary ALA, consistent with previous studies reporting on the esterified concentrations of these FA in response to dietary ALA (Couëdelo et al., 2022; Morise et al., 2004; Portolesi et al., 2007; Tu et al., 2010). Since a plateau in these fatty acids or their oxylipins did not occur within the dietary ALA concentrations tested, there is no point which could be used to indicate an adequate concentration. Even if their concentrations did eventually start to level off with >2g ALA/100g diet, these amounts would clearly be in excess of the dietary requirement (14). Similarly, in a 12-week human study in which 14g of dietary ALA was consumed daily, plasma concentrations of ALA and EPA and their oxylipins continued to increase during the entire 12-week period, while DHA and ARA oxylipins were unaffected since the baseline amounts of ALA were already adequate (Greupner et al., 2018). Thus, until an objective indicator of health linked to a specific concentration of ALA or EPA or their oxylipins is identified, they cannot be used to set the requirement.

In contrast to ALA and EPA and their oxylipins, DHA and its oxylipins appear to reach a plateau in response to increasing ALA, likely because of competition for the conversion to DHA at two $^{\Delta 6}$desaturase steps, namely the conversion of ALA to stearidonic acid (18:4n3) and the conversion of 24:5n3 to 24:6n3 (D'andrea et al., 2002; Gibson et al., 2013; Portolesi et al., 2007; Voss et al., 1991). The second step appears to be rate limiting (Portolesi et al., 2007), with the resulting plateau in DHA being attributed to a higher enzymatic affinity for 18-carbon FA, when compared to 24-carbon FA (Geiger et al., 1993). ARA and its oxylipins also are used in our

method because although ARA is not in the same part of the PUFA metabolic pathway, it is the major metabolite of the omega-6 pathway, like DHA is for the omega-3 pathway. Further, ARA and its oxylipins decrease with increasing dietary ALA, reflecting their generally opposing effects to those of DHA. Thus, this method incorporates the effects of ALA on the major metabolites of both the omega-3 and omega-6 pathways. The opposite trends are likely due to substrate competition for the pathways that metabolize ALA and LA to longer-chain PUFA (Brenner & Peluffo, 1966; Geiger et al., 1993; Gibson et al., 2013). The decrease of NE ARA in response to ALA in serum and tissues, but not in brain herein, is consistent with its effect on esterified ARA in some (Greupner et al., 2018; Morise et al., 2004; Tu et al., 2010), but not other studies (Domenichiello et al., 2014; Portolesi et al., 2007), while for NE ARA oxylipins we have found that they decrease in response to dietary ALA in rat kidney, liver and serum, as well as other tissues such as the heart, spleen and muscle (Ferdouse et al., 2019a; Leng et al., 2018; Pauls et al., 2020; Penner et al., 2021), but not in the brain (Ferdouse et al., 2019b). Such changes in ARA oxylipins in response to dietary ALA have not been observed in human studies (Gabbs et al., 2021; Greupner et al., 2018).

It is unclear from the current findings whether the oxylipin ratio is a more sensitive indicator than the FA ratio or if they are equivalent. The breakpoint when using the oxylipin compared to the FA ratio was similar in most cases, except in rat kidney and mouse liver. In these exceptions, the oxylipin ratio provided the only or the highest breakpoint, but that alone does not demonstrate that it is superior. Nevertheless, the breakpoint determined by either NE pool provides a higher breakpoint than when using the esterified FA pool that has been historically used (Bourre et al., 1989; Couëdelo et al., 2022; Domenichiello et al., 2017; Gibson et al., 2013; Tu et al., 2010). The higher breakpoint found when using the NE pools is consistent with

previous studies in which we observed that the amount of dietary ALA needed to maximize DHA oxylipins was greater than the amount needed to maximize esterified DHA in kidneys of rats with and without obesity (Caligiuri et al., 2013; Leng et al., 2017, 2018). More dietary ALA also has been shown to be required to maximize NE DHA compared to phospholipid DHA in rat plasma in two other studies (Couëdelo et al., 2022; Kim et al., 2011). However, comparison of NE to esterified pools of DHA and ARA and their oxylipins in response to dietary ALA remains to be examined in studies designed specifically for this purpose.

The tissue that has the highest ALA requirement determines the overall dietary requirement, since intake below the value would be insufficient for that tissue. Overall, the highest breakpoints derived in kidney, liver and serum were similar in both rats and mice. On the other hand, the brain was not a good indicator of the requirement, as there was no effect of dietary ALA on the concentration of DHA or ARA or their oxylipins. This is consistent with several reports that show that esterified and NE DHA concentrations in the adult rat brain are maintained unless dietary ALA is reduced to at least less than 0.03 g ALA/100g diet (~0.07 %en ALA), whereas the amount of ALA that is needed to maintain DHA concentration is much higher in other tissues such as liver, heart and testes (Bourre et al., 1992; Igarashi et al., 2007; Kim et al., 2011; A. Kulkarni et al., 2022; Taha et al., 2016). This is also consistent with our oxylipin studies that showed that brain oxylipins were resistant to change in response to dietary PUFA, with DHA oxylipin concentration being no different whether the diet contained either 0.3 or 3.4g ALA/100g diet (Ferdouse et al., 2019b). The brain, therefore, appears to have priority for DHA over other tissues. Whether there are differences in the ALA requirement in kidney, liver, serum or other yet to be studied tissues remains to be elucidated.

Whether serum could be a proxy for the body ALA requirement also remains to be determined, as the breakpoints in serum were different than in kidney or liver in a few instances. This is important because serum is much more accessible and could be more readily employed in large human studies. This is also important because the estimated dietary ALA requirement in the mouse study displayed a sex effect when serum was used, but not when liver or kidney were used. Sex differences in breakpoints are plausible, as there were sex differences in the concentrations of FA and oxylipins in the current mouse study. Interestingly, sex differences in the concentrations of oxylipins in these same tissues in rats have been previously observed (Leng et al., 2017, 2018), but the effects were not always consistent with the findings in mice herein. For example, previous study on rats showed that ARA oxylipin concentration in serum, kidney and liver were higher in males, whereas in the current study on mice, they were higher in females. Hence, further studies are needed to elucidate species differences and whether the estimated dietary ALA requirement does differ by sex. Determining whether serum can be used as a proxy for tissues is important, as it has recently been shown that the estimated conversion of ALA to DHA in serum is markedly lower than in liver or whole body (Rotarescu et al., 2024).

In conclusion, this study supports the use of NE oxylipins or FA as biomarkers of ALA intake, specifically the use of NE $DHA_{OH}/ARA_{OH}$ or DHA/ARA as biochemical indicators of the ALA requirement. Further studies examining whether the $DHA_{OH}/ARA_{OH}$ or the DHA/ARA ratio is more useful, which tissue has the highest requirement, or whether serum can be used as a proxy for tissues, remain to be explored further. Regardless, our results suggest that the ALA requirement may be higher than previously found by analysis based on esterified DHA alone. This has implications for whether the AIN93-G diet should be reformulated from its current 0.5g ALA/100g of diet. Further, in addition to determining the ALA requirement, we posit that this is

a method that also could be used to address several other key questions in lipid metabolism, such as estimating the ALA equivalency to DHA, the effect of LA concentrations on the ALA requirement, determining the optimal dietary omega-3 to omega-6 FA ratio in the absence of EPA or DHA intake, or determining the potentially altered ALA requirement in specific life stages or disease states.

### *2.6 Chapter transition*

Following the future directions from this study, in the next chapter, the method developed using NE DHA/ARA fatty acids and oxylipins to determine the dietary ALA requirement was applied to determine the bioequivalence between dietary DHA to the dietary ALA requirement.

There were some considerations taken from the findings in chapter 2. In the following study re-assessment of the dietary ALA requirement was conducted with rats to address the gap of female rats not being evaluated in this study. In addition, brain was evaluated, since rat brain was not included herein. The ALA diets were formulated to have more concentrations around the expected breakpoint of 0.7g ALA/100g diet, as this could improve piecewise regression breakpoint estimates. Lastly, the concentrations of dietary DHA were based on previous studies including graded concentrations of DHA (Le et al., 2013; S. Li et al., 2023; Ostermann et al., 2019; Schuchardt et al., 2017), particularly when they had a comparison group that only included dietary ALA. However, none of these previous studies were focused on determining the bioequivalence of dietary DHA to dietary ALA.

# Chapter 3: Bioequivalence of docosahexaenoic acid intake to the dietary requirement of alpha-linolenic acid in growing rats using non-esterified oxylipins and fatty acids

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## *3.1 Abstract*

Alpha-linolenic acid (ALA) is an essential omega-3 fatty acid that can be converted to docosahexaenoic acid (DHA), which by itself can also meet the dietary omega-3 fatty acid requirement. However, the amount of dietary DHA that is bioequivalent to ALA remains unknown. The study objective was to estimate the DHA dose that is equivalent to the ALA requirement, using non-esterified oxylipins and fatty acids. 168 male and female Sprague-Dawley rats received graded doses of dietary ALA (0.10 to 2.0g/100g diet) or DHA (0.07 to 1.3g/100g diet). All diets contained 2g of linoleic acid/100g diet and were based on the AIN93G. Non-esterified fatty acids and oxylipins were analyzed in serum, liver, heart, and brain by HPLC-MS/MS. Using piecewise regression, breakpoints were calculated for ALA diets using DHA/arachidonic acid (ARA) and hydroxy-DHA/hydroxy-ARA oxylipins ($DHA_{OH}/ARA_{OH}$). Breakpoints in serum and liver were highest and most suitable to determine the dietary ALA requirement, with no differences between sexes or whether the DHA/ARA or $DHA_{OH}/ARA_{OH}$ ratio was used. These breakpoints indicated an estimated average dietary ALA requirement of 0.55g ALA/100g diet (1.32% energy) 95% CI [0.42, 0.69], which is higher than what is provided by the AIN93G diet. DHA was ~4-6 (mean ± SE: 4.74 ± 0.22) times more potent than ALA, with 0.12g DHA/100g diet (0.3% energy) being equivalent to the 0.55g ALA/100g diet that meets the ALA requirement. Hence, dietary DHA is ~5 times more potent than ALA in meeting the dietary ALA requirement in the growing rat.

Keywords: alpha-linolenic acid, docosahexaenoic acid, oxylipins, fatty acids, dose response, dietary requirement.

## *3.2 Introduction*

Alpha-linolenic acid (ALA, 18:3n3) is an essential omega-3 fatty acid that must be consumed through dietary intake, since it cannot be synthesized *de novo* in mammals (Barceló-Coblijn & Murphy, 2009; Neuringer et al., 1988). In the body, ALA is elongated and desaturated to eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3). Incorporation and loss of EPA appears to be faster than of DHA, which suggests a preferential retention of DHA due to its structure and function (Calder, 2016). Some tissues such as the brain have limited capability of conversion, and the heart is unable to convert ALA to DHA due to a lack of elongase-2 (Barceló-Coblijn & Murphy, 2009; Demar et al., 2005; Igarashi et al., 2008; Rapoport et al., 2007). Most of the ALA to DHA conversion occurs in the liver, supplying other tissues via blood (Barceló-Coblijn & Murphy, 2009; Rapoport et al., 2007). Conversion of dietary ALA to DHA based on serum and plasma studies have long been believed to be low (<1%), which along with a stronger effect of DHA in comparison to ALA on DHA concentration, leads to recommendations for consumption of pre-formed DHA (Brenna et al., 2009; Calder, 2016). However, a recent study has shown that whole-body ALA to DHA conversion was much higher than expected at 9.5% (Rotarescu et al., 2024). This adds to the rationale to support consumption of ALA alone as sufficient to supply DHA to the brain and maintain adequate nutritional status for most people (Barceló-Coblijn & Murphy, 2009; Burdge, 2022; Domenichiello et al., 2015).

Currently there is no set dietary requirement of ALA for rodents (Hamilton & Hogan, 1995) or humans, although Adequate Intake recommendations exist for humans based on median amounts of ALA consumption by a healthy population (European Food Safety Authority, 2010; Institute of Medicine, 2005). Recently, we proposed the use of non-esterified DHA/arachidonic acid (ARA, 20:4n6) and their hydroxy oxylipins as biomarkers of the ALA requirement (Manson

et al., 2024). Oxylipins are hormone-like polyunsaturated fatty acid (PUFA) metabolites, and along with non-esterified fatty acids, activate receptors and mediate many of the PUFA effects in tissues (Aukema & Ravandi, 2023; Gabbs et al., 2015). In response to dietary ALA, DHA and its oxylipins increase and reach a plateau, whereas the opposite effect is often seen with ARA and its oxylipins (Bourre et al., 1989; Couëdelo et al., 2022; Domenichiello et al., 2017; Gibson et al., 2013; Manson et al., 2024; Tu et al., 2010; Whelan et al., 1991). Since DHA and ARA and their oxylipins reflect a balance of omega-6 to omega-3 fatty acids (Shearer & Walker, 2018) and metabolites with opposing effects (Gabbs et al., 2015), both were included in determining the ALA requirement. In this previous analysis using male and female mice and male rats, and examining liver, kidney and serum, we estimated the ALA requirement to be higher than previous estimates (Bourre et al., 1989; Couëdelo et al., 2022; Domenichiello et al., 2017; Gibson et al., 2013; Tu et al., 2010; Whelan et al., 1991), which were based on esterified DHA concentrations and did not include ARA in the calculation. We therefore revisited this estimate in the current study, expanding our analysis to also include heart and brain tissues in male and female rats.

In contrast with the initial increase followed by a plateau in DHA and its oxylipins in response to increasing dietary ALA, when dietary DHA is increased, its oxylipins continue to increase (Guillot et al., 2009; S. Li et al., 2023; Lust et al., 2023; Saito et al., 1998). To the best of our knowledge, only one study has directly compared the bioequivalence of ALA to DHA in terms of the amount of dietary intake needed to achieve a biological effect. This study in fetal baboons indicated that a maternal diet containing 0.45% energy from ALA was required, whereas 0.03% energy from DHA was sufficient to meet the requirements for fetal brain DHA accretion (Greiner et al., 1997). Additionally, DHA and ARA as the sole PUFA source are sufficient to

reverse or prevent essential fatty acid deficiency in animals (Carlson et al., 2019; Le et al., 2009, 2013; Ling et al., 2012; Strijbosch et al., 2008) and humans under parenteral nutrition (De Meijer et al., 2010). However, the concentration of DHA to achieve a similar biological effect as the ALA requirement is unknown.

Therefore, the objective of this study was to use non-esterified DHA/ARA and their oxylipins, as in our published method (Manson et al., 2024), to estimate the ALA requirement in growing male and female rats. This was then directly compared to increasing doses of DHA to determine the dose of DHA that meets the dietary ALA requirement.

### *3.3 Methods*

#### *3.3.1 Animal study*

After 1 week of acclimation, 84 male and 84 female (total 168) 4-week-old Sprague-Dawley double-caged rats were randomized into 14 diet groups (n=6). Cage position was randomly assigned, and all cages received standard bedding, a tunnel and paper towel as enrichment. Animals received water and diet *ad libitum*. The 14 diets followed the AIN93G guidelines (Reeves et al., 1993) except that soybean oil was replaced by an equal total concentration (7g oil/100g of diet) of combinations of flaxseed, safflower, coconut, and olive oils (**Tables 1-2**).

Out of the 14 diets, 9 diet groups contained graded concentrations of ALA ranging from 0.10 to 1.98 g/100g diet but contained no DHA or EPA. The remaining 5 diets had graded concentrations of DHA ranging from 0.07 to 1.35g/100g of diet. These 5 diet groups had concentrations of ALA as low as possible (~0.005g/100g of diet or 0.01%energy) and the source of omega-3 fatty acids was primarily DHA, which was provided in purified form containing 98% DHA in ethyl ester form (Maxomega DHA 95 EE, BASF, Germany). The DHA oil composition

**Table 3-1.** Oil amounts and fatty acid composition of diets used for DHA diets

| **Formulated DHA level** | **0.050** | **0.125** | **0.250** | **0.500** | **1.00** |
|---|---|---|---|---|---|
| *Dietary oils (g/100g diet)* | | | | | |
| DHA oil | 0.058 | 0.144 | 0.290 | 0.580 | 1.15 |
| Coconut oil | 3.38 | 3.29 | 3.1 | 2.81 | 2.19 |
| Safflower oil | 2.80 | 2.80 | 2.8 | 2.81 | 2.82 |
| Olive oil | 0.762 | 0.770 | 0.78 | 0.800 | 0.840 |
| *Analyzed Fatty acids (g/100g diet)* | | | | | |
| SFA | 3.14 | 3.38 | 2.85 | 2.52 | 2.16 |
| MUFA | 1.01 | 1.04 | 1.16 | 1.04 | 1.08 |
| PUFA | 2.03 | 2.19 | 2.58 | 2.71 | 3.54 |
| LA | 1.94 | 2.01 | 2.18 | 2.05 | 2.18 |
| ALA | 0.005 | 0.005 | 0.005 | 0.005 | 0.009 |
| EPA | 0.001 | 0.001 | 0.002 | 0.003 | 0.005 |
| DHA (g/100g diet) | 0.074 | 0.165 | 0.392 | 0.646 | 1.35 |
| DHA (energy %) | 0.177 | 0.395 | 0.938 | 1.55 | 3.22 |

AIN93G-based diets were formulated as follows: cornstarch (39.8g/100g), casein (20g/100g), dytrose (13.2g/100g), sucrose (10g/100g), fibre (5g/100g), AIN93G-MX (3.5g/100g), AIN93G-VX (1g/100g), L-cystine (0.3g/100g), choline bitartrate, 41.1% choline (0.25g/100g), TBHQ (0.0014g/100g), oil (7g/100g). Diet fatty acids were analyzed in duplicate.

**Table 3-2.** Oil amounts and fatty acid composition of diets used for ALA diets

| **Formulated ALA level** | **0.100** | **0.200** | **0.300** | **0.500** | **0.700** | **0.900** | **1.20** | **1.50** | **1.90** |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *Dietary oils (g/100g diet)* | | | | | |
| Flax oil | 0.180 | 0.370 | 0.560 | 0.950 | 1.33 | 1.71 | 2.29 | 2.86 | 3.43 |
| Coconut oil | 3.33 | 3.20 | 3.08 | 2.84 | 2.59 | 2.35 | 1.98 | 1.62 | 1.26 |
| Safflower oil | 2.77 | 2.75 | 2.71 | 2.64 | 2.58 | 2.52 | 2.42 | 2.32 | 2.23 |
| Olive oil | 0.720 | 0.680 | 0.650 | 0.570 | 0.500 | 0.420 | 0.310 | 0.200 | 0.080 |
| | | | | *Analyzed Fatty acids (g/100g diet)* | | | | | |
| SFA | 2.96 | 2.94 | 2.75 | 2.64 | 2.63 | 2.48 | 2.35 | 2.1 | 1.69 |
| MUFA | 1.06 | 1.06 | 1.06 | 1.10 | 1.12 | 1.08 | 1.15 | 1.25 | 1.12 |
| PUFA | 2.19 | 2.28 | 2.37 | 2.67 | 2.94 | 3.07 | 3.56 | 4.17 | 4.19 |
| LA | 2.09 | 2.06 | 2.06 | 2.13 | 2.17 | 2.12 | 2.24 | 2.39 | 2.21 |
| EPA | 0.0003 | 0.0004 | 0.0003 | 0.0003 | 0.0004 | 0.0004 | 0.0003 | 0.0003 | 0.0005 |
| ALA (g/100g diet) | 0.097 | 0.212 | 0.305 | 0.537 | 0.764 | 0.946 | 1.32 | 1.78 | 1.98 |
| ALA (energy %) | 0.239 | 0.503 | 0.742 | 1.29 | 1.82 | 2.27 | 3.16 | 4.26 | 4.74 |

AIN93G-based diets were formulated as follows: cornstarch (39.8g/100g), casein (20g/100g), dytrose (13.2g/100g), sucrose (10g/100g), fibre (5g/100g), AIN93G-MX (3.5g/100g), AIN93G-VX (1g/100g), L-cystine (0.3g/100g), choline bitartrate, 41.1% choline (0.25g/100g), TBHQ (0.0014g/100g), oil (7g/100g). Diet fatty acids were analyzed in duplicate.

was analyzed as described below and is shown in **Supplemental Table 1**. ALA or DHA were increased at the expense of saturated fatty acids. All diets contained constant amounts of linoleic acid (LA) and monounsaturated fatty acids at ~2g and ~1g/100g diet, respectively. The concentration of LA meets the LA requirement of 1.2 g/100 g of diet (Bourre et al., 1989), and represents 4.8% of energy. This is similar to the ~5% energy reflected in the Adequate Intake recommendation for humans (Institute of Medicine, 2005). All diets were in pelleted form and were purchased from Dyets (Bethlehem, PA). Results of fatty acid compositions (as described below) are shown in Tables 1-2.

After the 6-week dietary intervention period, non-fasting animals were anesthetized with isoflurane and terminated by decapitation. Trunk blood was collected to obtain serum. After exsanguination, liver, heart and brain were harvested and immediately frozen in liquid nitrogen. Serum and tissues were stored at -80°C until analysis. All samples were analyzed except two serum samples for male rats in the 0.165 DHA diet group and one brain sample of a female rat in the 0.305 ALA diet group were lost during analyses. All procedures were approved by the University of Manitoba Animal Care Committee and conducted in accordance with the Canadian Council for Animal Care and ARRIVE guidelines (Percie du Sert et al., 2020).

### *3.3.2 Diet fatty acid analysis*

Fatty acid compositions of the DHA oil and of the final diets were analyzed by gas chromatography with flame ionization detection (GC-FID), as described (Gabbs et al., 2021). Briefly, 5mg of oil or 25mg of diet was extracted via liquid-liquid extraction based on the Folch method (Folch et al., 1957) for total lipids with chloroform/methanol (2:1/vol:vol), 0.01% BHT, and 0.73% sodium chloride. Phosphatidylcholine C15:0 (Avanti Polar Lipids, AL, USA) was used as an internal standard and pipetted and weighed for confirmation in each sample using an

analytical scale. Lipid extracts were dried down and fatty acids were transmethylated using methanolic $H_2SO_4$ (Aukema & Holub, 1989). The resulting methylated fatty acids were extracted using hexane and ultrapure water, dried down and re-suspended in hexane. Samples were separated on a 30m DB-225MS column (Agilent Technologies, CA, USA) using a Bruker 450 GC-FID.

### *3.3.3 Non-esterified oxylipin and fatty acids analysis*

Analyses of non-esterified oxylipins and fatty acids were based on the method from (Dumlao et al., 2011a) and conducted as previously described (Aukema et al., 2016; Leng et al., 2017; Monirujjaman et al., 2017). Representative portions of frozen livers, whole frozen hearts, and whole frozen brains were homogenized in Tyrode's buffer (pH 7.6) (Sigma-Aldrich, MA, USA) (1/5.7, wt/vol). Lipids in these samples were solubilized by adding methanol formic acid to liver, heart and brain, or methanol only to serum, followed by pH3 water (adjusted with HCl) to achieve a 20% methanol in pH3 water solution. Antioxidant cocktail [0.2mg/ml BHT, 0.2mg/ml EDTA, 100µM indomethacin, 100µM trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (t-AUCB) in methanol/water (50/50, vol/vol) (Rund et al., 2018)] and deuterated internal standards (Cayman Chemical, MI, USA) were added to each sample. A complete list of standards and their concentrations was described in (Manson et al., 2023).

All samples were adjusted to pH3 using 1N HCl or NaOH prior to solid phase extraction with 33 µm polymeric reverse-phase Strata-X solid phase extraction columns (Phenomenex, CA, USA). Non-esterified oxylipins and fatty acids were eluted with methanol, dried down under a nitrogen stream, and resuspended in the mobile phase (water/acetonitrile/acetic acid, 70/30/0.02, vol/vol/vol). Analysis was conducted using HPLC-MS/MS (HPLC: Nexera-XR LC-20AD XR, Shimadzu, Japan; mass spectrometer: QTRAP 6500; AB Sciex, ON) with a reverse phase

column: Luna 5u C18(2) 100A; 250 x 2.00 mm (Phenomenex, CA, USA). The software used for the instrument was Analyst 1.6.2 (AB Sciex, ON) and for data analysis MultiQuant 3.0.5373.0 (AB Sciex, ON). Quantitation was based on the stable isotope dilution method (Hall & Murphy, 1998). Peak heights of 5 times baseline were set as the lower limit of quantification (LLOQ) and an oxylipin was considered quantifiable if at least 5 samples in any diet group met this criterion. Sample preparation and peak selection were blinded and conducted in random order. **Supplemental Table 2** shows all quantifiable oxylipins in each tissue.

### *3.3.4 Statistical analysis*

First, all zeroes were replaced by half of the lowest limit of detection (LLOD) for each component. Piecewise regression analysis was then conducted to determine a breakpoint in rats that received ALA diets. This analysis was conducted on $\log_2$ of the ratio of non-esterified $DHA_{OH}/ARA_{OH}$ oxylipins (representing the ratio of total hydroxy-docosahexaenoic acid/total hydroxy-eicosatetraenoic acid oxylipins) or non-esterified DHA/ARA fatty acids, generating a fold-difference value, so that the reciprocals of the ratio are symmetric and have equal weighting, as previously described (Manson et al., 2024). Piecewise regression with breakpoint estimates incorporating bootstrap was conducted using RStudio (2023.06.0+421, 2023 Posit Software, PBC) using the package segmented by Vito M. R. Muggeo version 1.6-4 from April 13, 2023 (Muggeo, 2023). Breakpoints were considered different if their 95% confidence intervals (CI) did not overlap. This analysis was conducted in segregated male and female data.

Once a breakpoint was determined, the equation values were extracted from the regression analysis, which were for the linear increase in the component of interest in response to dietary ALA (i.e. the first part of the graph), and the value of "y" for x equal to the breakpoint of ALA was calculated. The DHA data was plotted, and a trendline curve and equation was

generated using all data (i.e. not just the means). The same "y" value found for the breakpoint of ALA was inputted into the DHA equation to determine the amount of DHA that would be equivalent to the breakpoint of ALA (i.e. the ALA requirement). The upper and lower confidence interval values for ALA breakpoint were used to generate a confidence interval for the DHA bioequivalent dose.

Statistical analysis to determine dose and sex effects were conducted using multiple linear regression, followed by Fisher's Protected LSD tests. Data were separated into ALA and DHA diets for analysis. Normality was tested a priori using the Shapiro-Wilk test. If data were not normal, log-transformation was applied. Interaction of fatty acid dose and sex were evaluated and if the interaction was not significant it was removed from the model and a regression was conducted using the main effects of fatty acid dose and sex only. Fatty acid dose was included in the model as a continuous variable. To test for differences in effect of ALA vs DHA diet, multiple linear regression was applied with all ALA and DHA diets. In this model the main effects of diet (ALA vs. DHA) were analyzed when controlling for both dose and sex effects. Brain samples were run in three separate batches; thus, the effect of run was included in the model for these analyses when significant. Data are shown as mean ± SE. Significance was set at $p<0.05$.

### *3.4 Results*

#### *3.4.1 Animal body and tissue weight*

Rat body weight, growth and tissue weight (liver, heart, brain) were not different according to ALA or DHA dose. Male rats had higher body and tissue weights than female rats (Supplemental Tables 3-4).

### *3.4.2 Determination of the ALA requirement*

The ALA requirement was determined using $DHA_{OH}/ARA_{OH}$ oxylipins and DHA/ARA to generate breakpoints in separate analysis for each ratio, for males and females, and for each tissue (serum, liver, heart) (**Figure 1, Table 3**). Although breakpoints generated using oxylipin ratios were numerically higher than the ones generated using fatty acid ratios in 5 out of 6 analyses, there were no differences between the use of $DHA_{OH}/ARA_{OH}$ or DHA/ARA when determining breakpoints (Figure 1, Table 3, **Supplemental Table 5**). Similarly, females had generally higher breakpoints than males in serum and liver; however, a marginally significant sex difference was only identified in liver and only when using the $DHA_{OH}/ARA_{OH}$ but not the DHA/ARA ratio. The heart generally had lower breakpoints (0.33-0.42g of ALA/100g of diet) in comparison with serum and liver (0.38-0.95g of ALA/100g of diet); this was significant compared to both serum and liver in females when using $DHA_{OH}/ARA_{OH}$, and when compared to serum in females when using DHA/ARA. Brain was clearly different than all other tissues, due to a lack of a breakpoint at the dietary ALA concentrations provided, indicating that its breakpoint was much lower than for serum, liver and heart, and even at very low dietary ALA concentrations it has sufficient ALA to maximize $DHA_{OH}/ARA_{OH}$ and DHA/ARA ratios.

When determining the requirement for dietary ALA, it is ideal to use the breakpoint in the tissue with the highest breakpoints since this determines the concentration that is sufficient for the tissue(s) with the highest requirements. The breakpoints for liver and serum were therefore used to determine the requirement, since they were not different from each other, but were higher than in the heart and brain; hence the requirement was estimated from the 8 breakpoints for liver and serum in males and females and for both ratios. The lowest breakpoint indicated a minimum requirement of 0.38 ALA/100g of diet (0.91% energy), while the highest breakpoint was at 0.95g

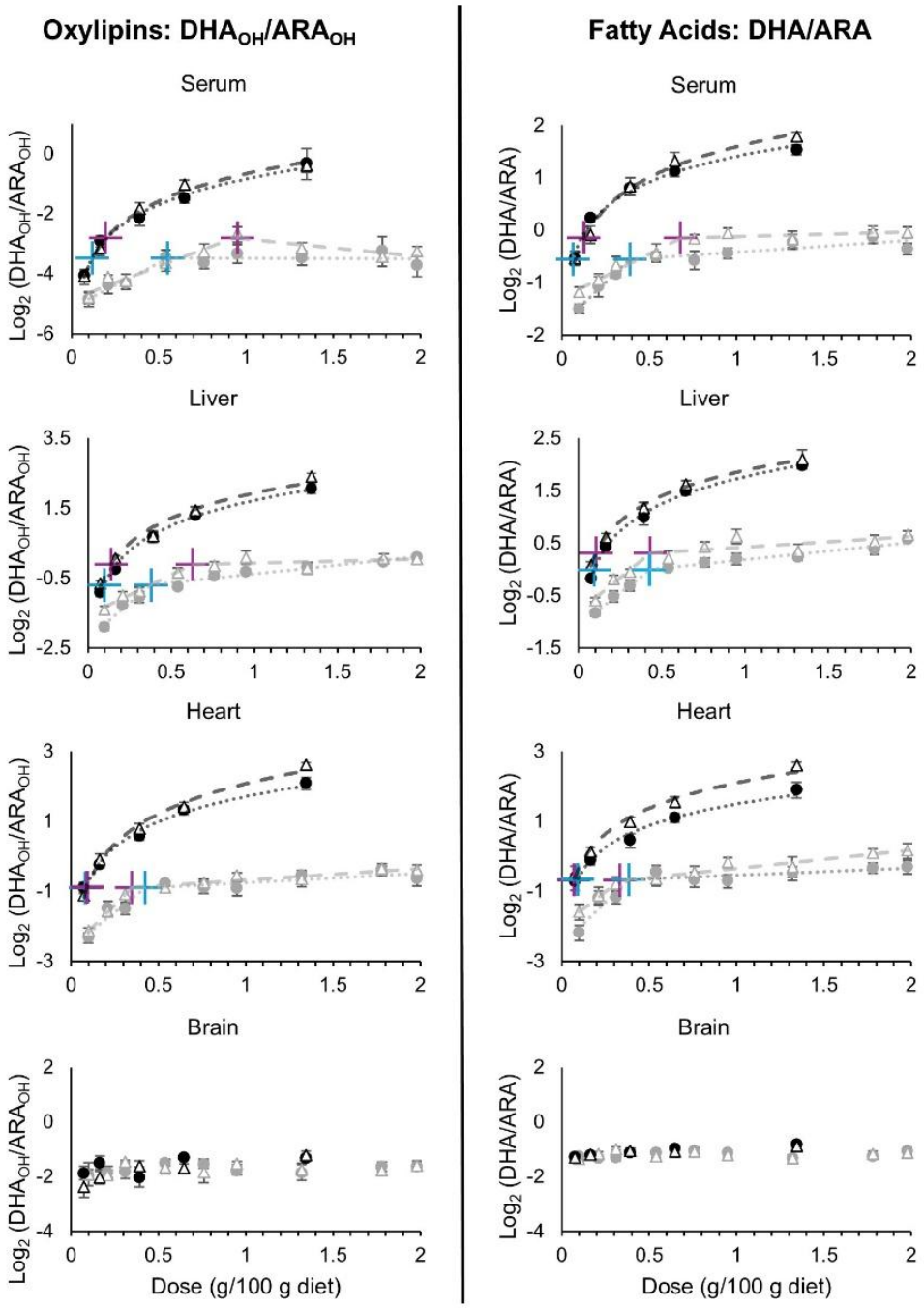


**Figure 3-1.** Non-esterified DHA$_{OH}$/ARA$_{OH}$ (left) and DHA/ARA (right) in response to dietary ALA and DHA in serum, liver, heart, and brain (mean±SE; n=6).
The breakpoint of ALA was analyzed by piecewise regression, and the breakpoint was applied to the DHA equation to determine the DHA bioequivalence to the ALA requirement.
Bioequivalence between ALA and DHA is shown with blue crosses for males and pink crosses for females. ALA: grey; DHA: black; Males: filled circles; Females: outlined triangles.

**Table 3-3.** ALA Breakpoints (BP) with 95% confidence intervals (CI) and DHA equivalency.

| | Female | | | | | | Male | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BP (ALA g/100g) | 95% CI | P value | DHA Eq. (DHA g/100g) | 95% CI | Equipotency (fold difference) | BP (ALA g/100g) | 95% CI | P value | DHA Eq. (DHA g/100g) | 95% CI | Equipotency (fold difference) |
| | | | | | *Serum* | | | | | | | |
| $DHA_{OH}/ARA_{OH}$ | 0.95 ± 0.11 | 0.72, 1.17 | 0.0002 | 0.197 | 0.14, 0.28 | 4.82 | 0.55 ± 0.12 | 0.31, 0.80 | 0.0211 | 0.121 | 0.07, 0.22 | 4.57 |
| DHA/ARA | 0.67 ± 0.11 | 0.45, 0.91 | 0.0006 | 0.128 | 0.08, 0.20 | 5.32 | 0.39 ± 0.07 | 0.25, 0.52 | 0.0014 | 0.064 | 0.04, 0.12 | 6.09 |
| | | | | | *Liver* | | | | | | | |
| $DHA_{OH}/ARA_{OH}$ | 0.63 ± 0.08 | 0.46, 0.80 | 0.0001 | 0.139 | 0.10, 0.20 | 4.54 | 0.38 ± 0.04 | 0.30, 0.46 | <0.0001 | 0.099 | 0.07, 0.14 | 3.87 |
| DHA/ARA | 0.43 ± 0.08 | 0.27, 0.59 | <0.0001 | 0.105 | 0.06, 0.19 | 4.09 | 0.43 ± 0.07 | 0.29, 0.56 | <0.0001 | 0.093 | 0.06, 0.15 | 4.57 |
| | | | | | *Heart* | | | | | | | |
| $DHA_{OH}/ARA_{OH}$ | 0.35 ± 0.04 | 0.26, 0.43 | 0.0003 | 0.091 | 0.07, 0.13 | 3.8 | 0.42 ± 0.12 | 0.26, 0.59 | 0.004 | 0.081 | 0.04, 0.15 | 6.11 |
| DHA/ARA | 0.33 ± 0.08 | 0.18, 0.49 | 0.0241 | 0.07 | 0.04, 0.12 | 4.76 | 0.38 ± 0.06 | 0.26, 0.51 | 0.0021 | 0.089 | 0.05, 0.18 | 4.31 |

Data is reported as breakpoint±SE followed by the confidence interval and the p value. P values represent statistical analysis from R package segmented (function selgmented). Following determination of a breakpoint, and respective response variable at an ALA concentration equal to the breakpoint, the equivalent DHA concentration was calculated by inputing the "y" value for the ALA breakpoint into the DHA equation. Equations used can be found in Supplemental Table 5.

ALA/100g diet (2.27%energy). The average of for all 8 breakpoints indicated a requirement of 0.55 ± 0.07g of ALA/100g of diet (1.32% energy) 95% CI [0.42, 0.69].

### *3.4.3 Equivalence of DHA to the requirement of ALA*

Based on the dietary ALA requirement, the DHA equivalence was determined in serum, liver, and heart for each ratio and sex. The DHA equivalence was the concentration of dietary DHA that results in the $DHA_{OH}/ARA_{OH}$ or DHA/ARA value that is equivalent to the corresponding value for the ALA breakpoint. Regardless of which breakpoint was used ($DHA_{OH}/ARA_{OH}$ or DHA/ARA, in serum, liver, or heart, in males or females), the dose of DHA that was bioequivalent to the ALA requirement was consistently 4 to 6-fold (mean ± SE: 4.74 ± 0.22) lower than the ALA requirement dosage (Figure 1, Table 3, Supplemental Table 5). Even when the heart was not included in this calculation the mean difference was 4.74 ± 0.25, since both the ALA breakpoint and the equivalent DHA dose were correspondingly lower. The DHA bioequivalence in the brain cannot be determined as ALA breakpoints could not be generated for this tissue. The dosages of DHA to achieve the same effect as the ALA requirement in serum and liver of females were higher than in males but were not significantly different [0.11 – 0.20g DHA/100g of diet (0.25 – 0.47% energy) vs. 0.06 – 0.12g of DHA/100g of diet (0.15 –0.29% energy), respectively (Figure 1, Table 3)]. The average of the 8 DHA bioequivalence dosage estimates among liver and serum, for males and females, using both $DHA_{OH}/ARA_{OH}$ and DHA/ARA is 0.12 ± 0.04g of DHA/100g of diet (0.28% energy) 95% CI [0.09, 0.15], which is 4.7-fold less than the average amount of ALA (0.55g/100g diet) that meets the dietary requirement for ALA.

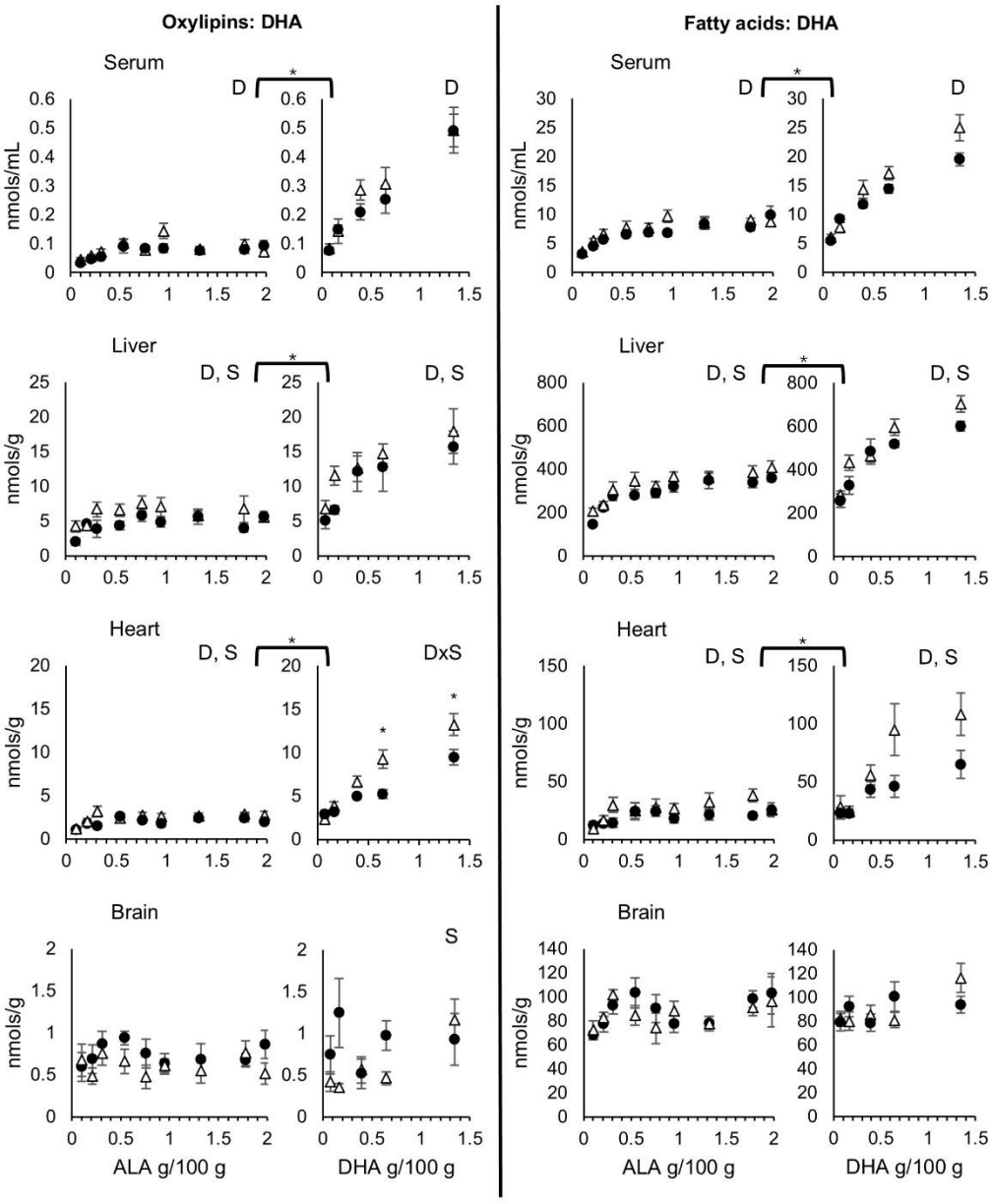


**Figure 3-2.** Non-esterified total DHA oxylipins (left) and DHA (right) in response to dietary ALA and DHA in serum, liver, heart, and brain (mean±SE; n=6).
Multiple linear regression was applied separately to dietary ALA and DHA groups to determine dose and sex effects. Results are shown on the upper right corner (D: Dose; S:Sex; DxS interaction – for the interaction * denotes significant difference). A separate multiple linear regression analysis was used to determine difference in effect between dietary ALA and DHA while controlling for dose and sex, with significance indicated by * above brackets between the dietary ALA and DHA graphs. Males are shown in circles and females in outlined triangles. Complete statistical results are reported in Supplemental Tables 6-7.

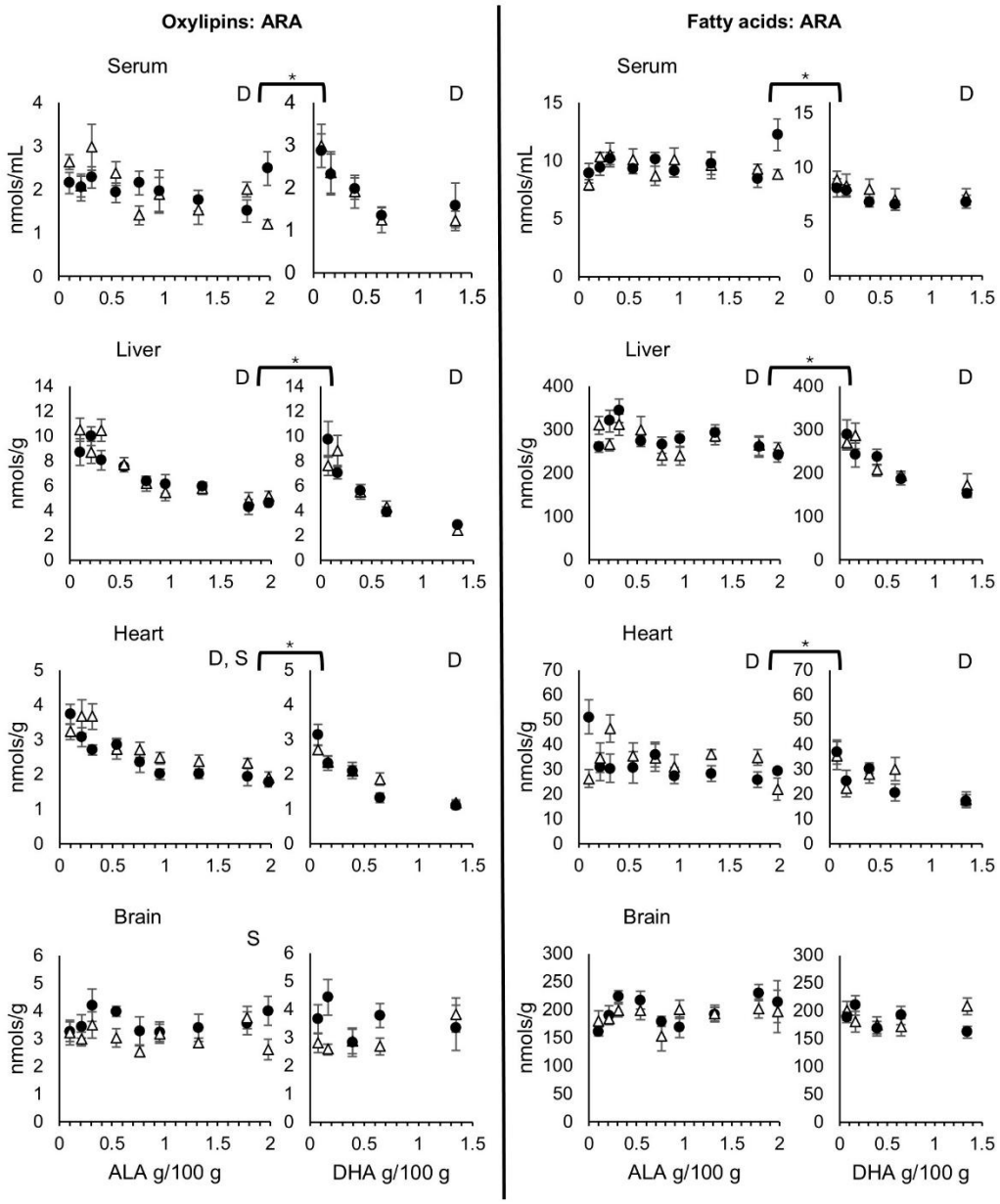


**Figure 3-3.** Non-esterified total ARA oxylipins (left) and ARA (right) in response to dietary ALA and DHA in serum, liver, heart, and brain (mean±SE; n=6).
Multiple linear regression was applied separately to dietary ALA and DHA groups to determine dose and sex effects. Results are shown on the upper right corner (D: Dose; S:Sex). A separate multiple linear regression analysis was used to determine difference in effect between dietary ALA and DHA while controlling for dose and sex, with significance indicated by * above brackets between the dietary ALA and DHA graphs. ALA: grey; DHA: black; Males: filled circles; Females: outlined triangles. Complete statistical results are reported in Supplemental Tables 6-7.

### *3.4.4 Overall effects of ALA and DHA on fatty acids and oxylipins*

In serum, liver, and heart, but not in the brain, dietary ALA and DHA increased DHA and its oxylipins (**Figure 2**), and decreased ARA and its oxylipins (except ARA in serum with dietary ALA) (**Figure 3**), with a stronger effect of DHA in comparison with ALA. With respect to other PUFA and oxylipins, dietary ALA and DHA linearly increased EPA and its oxylipins in all tissues (**Supplemental Fig 1**), with a stronger effect of ALA in comparison with DHA on EPA in the heart, but a stronger effect of DHA on EPA oxylipins in the liver, and on EPA in the brain. In serum, liver, and heart, but not in the brain, ALA and its oxylipins increased linearly in response to dietary ALA, but did not change in response to dietary DHA (**Supplemental Fig 2**). Dietary ALA compared to DHA had a much stronger effect on ALA oxylipins.

LA oxylipins were decreased with increasing dietary ALA in serum, and by both ALA and DHA diets in liver, with no difference in the effects of ALA vs. DHA on these oxylipins (**Supplemental Fig 3**). Adrenic acid (ADA) and gamma-linolenic acid (GLA) were detected in serum and liver, and ADA decreased in response to both dietary ALA and DHA, with a stronger effect of DHA on reducing ADA concentrations in serum. GLA concentrations were decreased with increasing doses of ALA or DHA in female livers only (Supplemental Fig 3), with GLA concentrations being lower for dietary DHA groups in both liver and serum.

### *3.4.5 Sex effects on fatty acids and oxylipins*

Considering all analysis, including total oxylipins (DHA, EPA, ALA, ARA, LA) as well as fatty acids (DHA, EPA, ALA, ADA), for all four tissues, which constituted 40 analyses, 18 (45%) of them had a sex difference for at least one of the ALA and/or DHA diets. When considering each ALA and DHA diet separately for all tissues (80 sex comparisons), 28 (35%) of the comparisons displayed a sex difference (Figures 2-3; Supplemental Figures 1-3;

Supplemental Tables 6-7). Of these, 24/28 (86%) sex differences were higher in females and 20/28 (72%) were noted in oxylipins. Sex differences varied by tissue: heart had the most sex differences (13/28 – 46%), followed by liver (10/28 – 35%), while serum (3/28 – 11%) and brain (2/28 – 7%) had the least. All of the sex differences in the heart and serum were higher in females, 80% were higher in females in liver, and both differences in the brain were higher in males. In the heart, sex effects were dose dependent (dose*sex interaction) in three instances (DHA oxylipins with the DHA diet, EPA oxylipins with both diets), in which females had higher concentrations of these oxylipins only at higher concentrations of ALA or DHA, and our data suggest these differences increase with dosage (Figure 2; Supplemental fig 1).

### *3.5 Discussion*

This study reveals that 0.12g DHA/100g diet is bioequivalent to the 0.55g ALA/100 g diet that meets the estimated average dietary ALA requirement in the growing rat, resulting in a ~5-fold higher potency of DHA compared to ALA. Dietary DHA also generates higher $DHA_{OH}/ARA_{OH}$ and DHA/ARA ratios than the plateaus achieved with ALA alone, so any DHA above the dietary requirement generates an additional increase in these ratios. These conclusions are based on our method which utilizes $DHA_{OH}/ARA_{OH}$ or DHA/ARA ratios to determine the ALA requirement, which was herein further validated and expanded from prior estimates in serum, liver and kidneys of male and female mice and male rats (Manson et al., 2024) to estimates from male and female rat serum, liver, heart and brain herein. Together these findings indicate that serum and liver provide the best estimate of the dietary ALA requirement, which was found to be 0.55g ALA/100g diet in the growing rat.

Several previous studies have shown that DHA compared to ALA increases DHA and its oxylipins to a greater extent (Ferdouse et al., 2019a; Gabbs et al., 2021; Greiner et al., 1997;

Leng et al., 2018; S. Li et al., 2023; Mendonça et al., 2018; Pauls et al., 2020; Penner et al., 2021; H. -M. Su et al., 1999), and although not as clearly, reduces ARA and its oxylipins more strongly (Ferdouse et al., 2019a; Gabbs et al., 2021; Leng et al., 2018; Mendonça et al., 2018; Pauls et al., 2020; Penner et al., 2021). In addition, the more potent effect of DHA (or fish) relative to a diet with only ALA is suggested by research with a single DHA dose (Schuchardt et al., 2017), graded doses of DHA (Le et al., 2013; S. Li et al., 2023) and graded portions of fish (Ostermann et al., 2019). However, only Grenier et al (1997) (Greiner et al., 1997) directly compared ALA and DHA with the goal of estimating bioequivalence. In this study, a maternal diet with 0.45% energy from ALA was sufficient to support normal fetal brain DHA accretion in baboons whereas only 0.03% energy from DHA was needed (Greiner et al., 1997). This higher DHA potency estimate may be due to the higher ALA requirement for the fetal baboon brain compared to the current findings in the growing rat brain, in which the brain breakpoint was likely less than the lowest dose of ALA (0.10g/100g diet, 0.24% energy). It also may be due to species differences, or a greater dependency of the brain on pre-formed DHA at an earlier stage of development that displays a higher DHA accretion rate during this period of peak brain growth (Clandinin et al., 1980; Greiner et al., 1997; Lapillonne et al., 2013; S. L. Smith & Rouse, 2017).

The amount of dietary DHA that meets the requirement for ALA is low, close to the lowest DHA dose provided of 0.07g DHA/100g diet. Hence, in the context of adequate or marginal ALA intake, most DHA intake will lead to additional effects on DHA and ARA and their oxylipin ratios. This is similar to the lower effect of ALA compared to EPA and DHA on the omega-3 index (EPA+DHA concentration in erythrocytes), a risk factor for cardiovascular disease (Dempsey et al., 2023). While intake of EPA and DHA is sometimes associated with a

reduction in cardiovascular disease and mortality, ALA intake is not or has a smaller effect (Abdelhamid et al., 2020; Jiang et al., 2022; Wang et al., 2006). Since ALA deficiency is uncommon, the already maximal DHA/ARA oxylipin and fatty acid ratios may explain the lack of effect of ALA on cardiovascular outcomes. On the other hand, the ability of DHA to increase these ratios beyond the maximum achieved with dietary ALA may explain how dietary DHA (and possibly EPA) could improve cardiovascular disease. In this regard, suboptimal ALA intake in some individuals or studies may help explain the variability in EPA/DHA study outcomes, since supplementation with EPA/DHA in ALA deficiency would increase low DHA/ARA oxylipin and fatty acid ratios to a concentration associated with adequacy. This is similar to the suggestion that differences in baseline EPA/DHA concentrations may help explain the variability in the cardiovascular benefits of EPA/DHA (Bae et al., 2023).

The dietary ALA requirement estimate in the current study was 0.55 ± 0.07g of ALA/100g of diet (1.32% energy) 95% CI [0.42, 0.69]. When combining our results of serum and liver herein, with our previous estimates (Manson et al., 2024) in serum, liver, and kidney in male and female mice and male rats, the average of the 25 breakpoint estimates generated is 0.54 ± 0.04g of ALA/100g of diet [0.47, 0.62], demonstrating consistency among studies, species, sexes and ratios used. The liver and serum were used in the current study to estimate the requirement because their breakpoints were higher than the heart in several cases, indicating that the heart may have priority for DHA uptake and therefore not reflect overall body requirement. Similarly, the brain appears to have even greater priority for DHA, as the breakpoint appears to be lower than the ALA provided in the lowest ALA diet. This is consistent with studies showing that brain DHA concentrations are maintained unless dietary ALA is reduced to at least less than 0.03 g ALA/100g diet (~0.07 % energy ALA), in contrast with other tissues (Bourre et al., 1992;

Igarashi et al., 2007; Kim et al., 2011; A. Kulkarni et al., 2022; Taha et al., 2016). The brain is similarly resistant to change in oxylipins with up to 3g DHA/100g of diet (Ferdouse et al., 2019b). The fact that serum has similar breakpoints to liver in the current rat study and to liver and kidney in mice and male rats previously (Manson et al., 2024), suggests that blood could be used to estimate ALA status when using this method. This remains to confirmed, as there may be other yet unexplored tissues that may exhibit a higher requirement.

Higher conversion of ALA into DHA in females has been observed in liver (Rezaei et al., 2024), even when liver synthesis rates of ALA to DHA in ALA fed mice were not shown to be different between sexes (Rotarescu et al., 2024). However, although the liver $DHA_{OH}/ARA_{OH}$ breakpoint was marginally higher in females, and the dose of DHA that met the ALA requirement was numerically higher in females, a sex difference in the ALA requirement or DHA bioequivalence was not shown herein. Further, in contrast to the numerically higher breakpoints in serum $DHA_{OH}/ARA_{OH}$ and DHA/ARA in the current rat study, these breakpoints were significantly lower in female serum in our previous mouse study (Manson et al., 2024). This may reflect a species difference, as DHA concentrations have been reported to be higher (Cohen & Ward, 2005; Rezaei et al., 2024), or not different (ALA diet) (Rotarescu et al., 2024) in male mouse serum, compared to no sex differences in rat serum herein and previously (Leng et al., 2018), or higher DHA in female rat serum (Kitson et al., 2012; Leng et al., 2017). Nevertheless, potential sex differences in the ALA requirement or DHA bioequivalence remain to be investigated further.

Overall, the estimated ALA requirement herein and previously using the same method (Manson et al., 2024), suggest a higher ALA requirement than previous studies using esterified DHA only. These estimated ranged between 0.3 to 1.0% energy (~0.1 to 0.4g ALA/100g of diet

in AIN-93G based diets) (Bourre et al., 1989; Couëdelo et al., 2022; Domenichiello et al., 2017; Gibson et al., 2013; Tu et al., 2010; Whelan et al., 1991), which is similar to the human acceptable macronutrient distribution range (AMDR) of 0.6-1.2% energy from ALA for both adults and children (Institute of Medicine, 2005). Non-esterified oxylipins and fatty acids may be better biomarkers because they are the biologically active forms that bind to their cognate receptors and elicit physiologic and pathophysiologic responses. Previous data suggests that esterified DHA reaches a plateau at lower concentrations than non-esterified DHA (Couëdelo et al., 2022; Kim et al., 2011) or its oxylipins (Caligiuri et al., 2013; Leng et al., 2017, 2018), but this remains to be investigated. Including ARA also adds strength to the assessment of the ALA requirement, since ARA oxylipins decrease and plateau in response to ALA intake (Bourre et al., 1989; Couëdelo et al., 2022; Domenichiello et al., 2017; Gibson et al., 2013; Manson et al., 2024; Tu et al., 2010; Whelan et al., 1991). ARA oxylipins often have opposing effects to DHA oxylipins (Gabbs et al., 2015) and influence health, as recently emphasized by ARA adipose tissue content being associated with higher all-cause mortality in a large prospective cohort study (Bork et al., 2025). Therefore, incorporating ARA and its oxylipins is more encompassing of the health effects elicited by ALA and may contribute to the higher requirement estimates herein.

Collectively, the current study suggests a potential need for reformulation of dietary omega-3 guidelines. This includes the AIN93G diet for the growing laboratory rodent (Reeves et al., 1993), as it contains ~0.50g ALA/100g diet. An estimated average requirement of 0.55g of ALA/100g of diet would be expected to meet the needs of approximately half of the rodent population (Institute of Medicine, 2005), potentially leading to variability in research results, particularly between strains of animals. In addition, an estimate of 1.3% of calories would reflect a mean dietary intake of 2.3-2.9g of ALA/day in a child or teenage human diet of 1600-2000kcal.

This is slightly higher than the recommended intake of 0.6-1.2% energy and the median ALA intake in US and Canada for children (0.9g/day) and adolescents (1.0-1.6g/day) (Institute of Medicine, 2005). Notably, pediatric populations are expected to have higher dietary ALA requirements (Barceló-Coblijn & Murphy, 2009; Bourre et al., 1989, 1993; Sinclair et al., 2022). Since recent studies have reported potentially inadequate omega-3 status in teenagers (Almasri et al., 2023; Vyncke et al., 2012) and even essential fatty acid deficiency in subset populations of children and teenagers (Cardino et al., 2024), further research may be warranted in pediatric populations at risk of dietary ALA deficiency, such as those with malabsorption and/or risk of malnutrition.

Retroconversion of DHA to EPA is reportedly very low (Metherel et al., 2017), so the similar and sometimes greater effect of DHA in comparison to ALA on EPA (except heart) and its oxylipins was unexpected. This may be explained by the concentrations of EPA present in the DHA oil, which contributed to the higher EPA content (0.0006 to 0.005g vs. 0.0003g of EPA/100g diet in DHA vs ALA diets, respectively) in the DHA diet. The low concentrations of ALA oxylipins when dietary ALA is low and DHA is the primary source of omega-3 fatty acids is consistent with a previous study in laying hens (S. Li et al., 2023), and should also be noted. Since ALA oxylipins may have beneficial effects on their own (Cambiaggi et al., 2023; Eccles & Baldwin, 2022; Gabbs et al., 2015), this could be a potential detrimental effect of a diet containing primarily DHA, although this is rather uncommon.

In conclusion, dietary DHA is ~5-fold more potent than ALA in meeting the dietary ALA requirement as assessed by DHA/ARA oxylipin and fatty acid ratios. When the dietary ALA requirement is met, the effect of any further DHA induced increase in the DHA/ARA oxylipin and fatty acid ratios on health remains to be elucidated. The higher dietary ALA requirement of

0.55g ALA/100 g diet that is estimated from using the ratios of both DHA and ARA and their oxylipins suggests the AIN93G diet may need to include moderately higher ALA concentrations. These studies also illustrate how $DHA_{OH}/ARA_{OH}$ or DHA/ARA as biochemical indicators of dietary ALA adequacy can be applied to answer other key questions in lipid metabolism, such as comparing the bioequivalence of EPA to ALA and DHA, determining how LA influences the ALA requirement, DHA bioequivalence and the optimal dietary omega-3 to omega-6 ratio, and determining the ALA requirement and DHA equivalence in other life stages and disease states.

### *3.6 Chapter transition*

In the study presented in chapter 3 we show that in rat hearts increasing doses of dietary DHA have a more pronounced effect on DHA oxylipins in females than males. Similarly, in previous studies the sex differences found were dependent on the diet (Ferdouse et al., 2019a; Mendonça et al., 2018). Since these sex differences observed in oxylipins did not reflect fatty acid data, and the effects observed were consistent among oxylipins from COX, LOX, and CYP pathways, this suggests these sex differences could be mediated by changes in $PLA_2$ activity according to diet.

However, there was a lack of studies on $PLA_2$ activity towards fatty acid release and even less was known about how this further impacts oxylipin formation. In addition, using traditional $PLA_2$ activity methods we would be limited in how we could explore this hypothesis. This is since these studies use synthetic phospholipids and recombinant enzymes, making it difficult to reflect diet and sex differences observed in vivo. Furthermore, $PLA_2$ activity assays in which we would be able to use our samples to reflect diet and sex effects conditions as in vivo would be limited in how $PLA_2$ activity is assessed, as this is typically done by analysis of a single product. This would limit our ability to evaluate and gain insights into enzyme activity towards PUFA

release and oxylipin formation, which would be important in the context of the hypothesis to be investigated. Therefore, we developed a method presented in chapter 4 to be able to evaluate $PLA_2$ activity towards PUFA release and oxylipin formation ex vivo in rat hearts using inhibitors.

# Chapter 4: Phospholipase $A_2$ enzymes differently impact PUFA release and oxylipin formation ex vivo in rat hearts

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*4.1 Abstract*

Phospholipase $A_2$ ($PLA_2$) enzymes cleave cell membrane phospholipids and release polyunsaturated fatty acids (PUFA), which can be converted into oxylipins. However, little is known about $PLA_2$ preference for PUFA, and even less is known about how this further impacts oxylipin formation. Therefore, we investigated the role of different $PLA_2$ groups in PUFA release and oxylipin formation in rat hearts. Sprague-Dawley rat heart homogenates were incubated without or with varespladib (VAR), methyl arachidonyl fluorophosphonate (MAFP) or EDTA. Non-esterified PUFA and oxylipins were determined by HPLC-MS/MS, and isoform expressions by RT-qPCR. Inhibition of $sPLA_2$ IIA and/or V by VAR reduced the release of ARA and DHA, but only DHA oxylipins were inhibited. MAFP reduced the release of ARA, DHA, ALA, and EPA, and the formation of ARA, LA, DGLA, DHA, ALA, and EPA oxylipins. Interestingly, cyclooxygenase and 12-lipoxygenase oxylipins were not inhibited. mRNA expression levels of $sPLA_2$ and $iPLA_2$ isoforms were highest whereas levels of $cPLA_2$ were low, consistent with activity. In conclusion, $sPLA_2$ enzymes lead to the formation of DHA oxylipins, while $iPLA_2$ is likely responsible for the formation of most other oxylipins in healthy rat hearts. Oxylipin formation cannot be implied from PUFA release, thus, both should be evaluated in $PLA_2$ activity studies.

*Keywords*: phospholipase $A_2$; polyunsaturated fatty acids; oxylipins; omega-3 fatty acids; fatty acid metabolism; eicosanoids.

## *4.2 Introduction*

Phospholipase $A_2$ ($PLA_2$) is a superfamily of enzymes involved in the hydrolysis of membrane phospholipids (Dennis et al., 2011; Mouchlis & Dennis, 2019). The polyunsaturated fatty acids (PUFA) released by $PLA_2$ can then be converted by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) enzymes into oxylipins. These bioactive lipid mediators are involved in important physiological and pathophysiological cardiovascular functions, such as regulation of vascular tone, inflammation, cardiac function, and blood coagulation (Caligiuri et al., 2017; Dennis & Norris, 2015; Dietrich et al., 2010; Fujioka et al., 2008; Gabbs et al., 2015; Kerkelä et al., 2011; Micova et al., 2016; Moon et al., 2018; Yano et al., 2011). Notably, oxylipins derived from different fatty acid precursors and/or pathways have distinct functions that can be protective or detrimental (Astudillo et al., 2019; Dennis & Norris, 2015; Gabbs et al., 2015). For example, oxylipins derived from arachidonic acid (ARA) via COX can stimulate or inhibit platelet aggregation, mediate tachycardia, and increase vascular resistance, while LOX and CYP oxylipins derived from docosahexaenoic acid (DHA) generally have anti-inflammatory effects and can inhibit COX activity and platelet aggregation (Gabbs et al., 2015). Thus, specific $PLA_2$ isoform inhibitors that target oxylipin formation are being widely explored as potential drugs to target diseases such as cardiovascular disease (Jantan et al., 2014; Shukla et al., 2015).

Importantly, research on $PLA_2$ preference for PUFA release is limited, particularly on the release of omega-3 fatty acids, and even less is known about how $PLA_2$ inhibition impacts oxylipin formation. Three main $PLA_2$ families are primarily involved in PUFA metabolism and oxylipin synthesis: secreted (s)$PLA_2$, cytosolic (c)$PLA_2$, and calcium-independent (i)$PLA_2$ [also known as patatin-like phospholipases (PNPLA)] (Dennis & Norris, 2015; Mouchlis & Dennis,

2019; Ramanadham et al., 2015). *In vitro* studies to date indicate that cPLA$_2$α preferentially mediates release of ARA, while iPLA$_2$β and sPLA$_2$V appear to prefer LA. In addition, sPLA$_2$V has been shown to release more DHA and iPLA$_2$β to release more EPA in comparison with both cPLA$_2$α and iPLA$_2$β or sPLA$_2$V (Hayashi et al., 2021; Mouchlis et al., 2018). Although most PLA$_2$ activity in mouse heart has been reported as coming from iPLA$_2$β and iPLA$_2$γ (Jenkins et al., 2009; Moon et al., 2016; Sharma et al., 2011), some activity has been reported from cPLA$_2$α in mouse myocardium, and possibly from cPLA$_2$ζ in human heart (Cedars et al., 2009; Jenkins et al., 2009; Moon et al., 2018). The heart also is a major source of sPLA$_2$ (Berry et al., 2015; Hernandez-Anzaldo et al., 2015). With respect to PUFA metabolism and oxylipin formation, the role of iPLA$_2$γ in the mouse heart has been explored (Moon et al., 2016), but the roles of other PLA$_2$s, including sPLA$_2$, in the heart remain to be elucidated. Tissue compositions of oxylipins do not always reflect phospholipid or neutral lipid fatty acid content (Cayer et al., 2019; Ferdouse et al., 2019a; Leng et al., 2017, 2018; Mendonça et al., 2018), and in PLA$_2$ studies oxylipin production does not always reflect PUFA release from PLA$_2$ (Miki et al., 2013; Moon et al., 2016; Murase et al., 2016), so it is necessary to examine the effects of PLA$_2$ isoforms specifically on oxylipins.

The current methods available to assess PLA$_2$ activity and isoform preference for PUFA commonly use synthetic or labelled phospholipids and recombinant PLA$_2$ enzymes. Alternatively, phospholipids or PLA$_2$ are extracted from tissues and used for assessing PLA$_2$ isoform activity *in vitro* (Leslie & Gelb, 2004; Mouchlis et al., 2019; Reynolds et al., 1991). Much information has been obtained from these studies, but these *in vitro* methods do not always completely reflect the situation in vivo. For example: single or synthetic phospholipids do not reflect the effects of the complex phospholipid compositions present in tissue on enzyme activity

and single enzyme assay conditions do not account for the complex interactions between and for the relative amounts of multiple $PLA_2$ isoforms in vivo. Further, assessing only PUFA release does not necessarily predict oxylipin production, and limited analyses of PUFA provides an incomplete picture of overall activity (Astudillo et al., 2019; Hayashi et al., 2021; Miki et al., 2013; Moon et al., 2016; Mouchlis et al., 2019; Murase et al., 2016; Offer et al., 2005). In vivo effects of $PLA_2$ isoforms on oxylipin production have been evaluated in a limited number of knockout models (Miki et al., 2013; Moon et al., 2016; Murase et al., 2016), but these also are limited in their ability to account for interactions between $PLA_2$ isoforms.

In the current study we utilized an ex vivo approach used previously in COX isoform studies (C. J. Smith et al., 1998; Warford-Woolgar et al., 2006), which assesses $PLA_2$ isoform activities in heart tissue homogenates containing all phospholipid substrates and intrinsic $PLA_2$ isoforms present in the tissue. To determine how different $PLA_2$ isoforms impact PUFA release and oxylipin formation in rat hearts, we quantified the release of non-esterified PUFA and production of non-esterified oxylipins simultaneously in the presence and absence of selective $PLA_2$ inhibitors. We found that $sPLA_2$ IIA and/or V released ARA and DHA, but only DHA oxylipins were affected, and that $iPLA_2$ enzymes are likely primarily involved in the release of ARA, ALA, EPA and the formation of ARA, LA, DGLA, ALA, and EPA oxylipins.

## *4.3 Materials and Methods*

### *4.3.1 Tissue procurement*

Rat hearts were obtained from 8-week-old male Sprague-Dawley that were the healthy untreated control rats provided a nutritionally adequate diet in a separate experiment. Rats were anesthetized with isoflurane and terminated by decapitation. After exsanguination, hearts were

removed, immediately frozen in liquid nitrogen and stored at -80°C until use. All procedures were done in accordance with the Canadian Council for Animal Care and ARRIVE guidelines (Percie du Sert et al., 2020) and approved by the University of Manitoba Animal Care Committee.

### *4.3.2 Phospholipase $A_2$ activity*

Frozen hearts were thawed on ice, weighed, rinsed in ice-cold Tyrode's buffer (pH 7.6) and homogenized in 7.4 volumes of Tyrode's (1.8mM $CaCl_2$, 1.05mM $MgCl_2$, 2.68mM KCl, 137mM NaCl, 0.417mM $NaH_2PO_4$, 11.9mM $NaHCO_3$, and 5.55mM D-Glucose; Sigma-Aldrich, MA, USA). The homogenate was then stored at -80°C until analyses. On the day of assay, heart homogenates were thawed on ice and 200 uL aliquots were pre-incubated with inhibitor or vehicle [dimethyl sulfoxide (DMSO)] on ice for 15 min prior to incubation at 37°C. For each heart homogenate, aliquots were taken at time 0 (baseline) and after 20 min of incubation, with and without inhibitors. All incubations were done in a shaking water bath at 37°C. The incubation time was based on a time course incubation that showed that the curve for PUFA release and oxylipin formation was linear at 20 min (**Supplemental Figure 1**). Reactions were stopped by the addition of 500 µL of methanol formic acid (100:1/v:v), followed by addition of 800 µL pH 3 water, 90 µL ethanol, 10 µL antioxidant cocktail [0.2 g/L butylated hydroxytoluene, 0.2 g/L EDTA, 2 g/L triphenylphosphine, and 2 g/L indomethacin in methanol:ethanol:water (2:1:1, v:v:v)], and 100 µL of deuterated internal standards diluted in 50% ethanol (**Supplemental Table 1**) (Cayman Chemical, MI, USA). Samples were adjusted to pH 3, centrifuged at 14000 g, and the supernatant was loaded onto 33 µm polymeric reverse-phase Strata-X solid phase extraction columns (Phenomenex, CA, USA) that were pre-conditioned with 3.5 mL of methanol, followed by 3.5 mL of pH 3 water. Sample tubes were rinsed with 1

mL of 10% methanol in pH 3 water, centrifuged again and this supernatant was also loaded onto the column. The column was then rinsed with 2 mL pH 3 water, followed by 1 mL hexane. Non-esterified oxylipins and PUFA were eluted with 1 mL methanol, filtered through a Pasteur glass pipette with glass wool and sodium sulfate, and rinsed with 300 μL of methanol. Samples were then dried down under a nitrogen stream and re-suspended in the mobile phase (water/acetonitrile/acetic acid, 70/30/0.02, v/v/v) for analysis. Analysis of non-esterified oxylipins and PUFA was performed by HPLC-MS/MS (QTRAP 6500; Sciex, ON), using a reverse phase C18 column, as described (Aukema et al., 2016; Dumlao et al., 2011a; Leng et al., 2017; Monirujjaman et al., 2017). Activity was calculated as the result of the incubated sample (with or without inhibitor) minus the sample at baseline (not-incubated – time 0min), divided by the incubation time (20min). This was to account for non-esterified PUFA and oxylipin amounts already present in the homogenate as well as those released during the processes of homogenizing, freezing and thawing.

### *4.3.3 Inhibitors*

The inhibitors used in this study were R-bromoenol lactone (R-BEL) for iPLA$_2$γ, S-bromoenol lactone (S-BEL) for iPLA$_2$β, varespladib (LY315920) for sPLA$_2$ IIA, V and X, LY311727 for sPLA$_2$ IIA, V and X, methyl arachidonyl fluorophosphonate (MAFP) for cPLA$_2$ and iPLA$_2$ (all purchased from Cayman Chemical, MI, USA), and pyrrophenone (PYR) (Calbiochem, MA, USA) for cPLA$_2$α. EDTA (Promega Corporation, WI, USA) was used to chelate calcium and inhibit activity of Ca-dependent PLA$_2$ isoforms (cPLA$_2$ and sPLA$_2$). All inhibitors were diluted in DMSO. Before addition to the sample, the inhibitor in DMSO was diluted in Tyrode's to achieve a final concentration of 0.1% DMSO. DMSO alone at a concentration of 0.1% was added to the control samples without inhibitor.

### *4.3.4 $PLA_2$ isoform expression*

Primers for 25 $PLA_2$ isoforms were designed based on NCBI primer-blast tool (Ye et al., 2012). A primer was only selected if the position strand matched the rat genome database (J. R. Smith et al., 2019), and if there was no predicted formation of self-dimers, primer-dimers, hairpins, or self-annealing of 3' and 5' ends (Breslauer et al., 1986; Kibbe, 2007). GAPDH was used as housekeeping gene and its sequence was obtained from a previous paper (Vesentini et al., 2012). A detailed list of $PLA_2$ primers is provided in **Supplemental Table 2**. For RNA extraction, a Monarch Total RNA miniprep kit (New England Biolabs, ON, Canada) was used. RNA quality and concentration were determined by NanoDrop Spectrophotometer (ThermoFisher Scientific, ON, Canada). RNA was reverse transcribed using iScript Advanced cDNA Synthesis Kit and iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, ON, Canada) was used for reverse transcription quantitative PCR (RT-qPCR) using 25 ng of cDNA and the cycling protocol for Applied Biosystems StepOne. Melt curves were analyzed to assess primer suitability, and amplicon length confirmed by 2.5% agarose gel electrophoresis of the PCR product. $PLA_2$ mRNA that were not at detectable levels in the heart were tested with rat brain and testis as positive controls to confirm that the primers were functional. The relative expression of mRNA was calculated based on the cycle threshold (CT) using the formula $2^{-\Delta CT}$, where $\Delta CT$ = CT of gene of interest – CT reference gene. CT values that were equal to or higher than 35 were considered non-quantifiable/not detectable.

### *4.3.5 Statistical analysis*

The results were analyzed by one-way ANOVA for repeated measures followed by the protected least-squares means adjusted for multiple comparisons by Tukey (SAS OnDemand for Academics, SAS Institute Inc., NC, USA). Normality was assessed by the Shapiro Wilk test.

When data were not normal, they were log transformed to reach normality where possible. Data that was not normal even when log transformed were analyzed by the non-parametric Kruskal-Wallis test. Outliers were removed when they were above the mean ± 3 SD. Data is reported as mean ± SE (or mean ± SD in **Supplemental Figures 1-5**) and statistical significance was set at $p < 0.05$.

## *4.4 Results*

### *4.4.1 Phospholipase $A_2$ inhibition in the rat heart*

First, specific inhibitors of $PLA_2$ isoforms $cPLA_2\alpha$ (PYR), $iPLA_2\beta$ (S-BEL), $iPLA_2\gamma$ (R-BEL), and $sPLA_2$ IIA, V, and X (VAR, LY311727) were tested on hearts of rats of differing ages and sexes. Using doses reported to be effective for each inhibitor (Dietrich et al., 2010; Flamand et al., 2006; Moon et al., 2018; Quach et al., 2014; Yun et al., 2016), PYR, S-BEL, R-BEL did not inhibit PUFA release and oxylipin formation (Supplemental Figure 2), but VAR and LY311727 exhibited dose dependent inhibition towards the release and formation of some PUFA and oxylipins (Supplemental Figures 3 and 4). Since we did not detect activity of $cPLA_2\alpha$, $iPLA_2\beta$, and $iPLA_2\gamma$ by the inhibitors PYR, S-BEL, and R-BEL, no other specific $iPLA_2\gamma$ inhibitors were commercially available, and $iPLA_2\beta$ is thought to have low activity in healthy hearts (Jenkins et al., 2009), a non-specific inhibitor for $cPLA_2$ and $iPLA_2$ (MAFP) was tested, in an approach similar to Moon *et al* (Moon et al., 2018). Chelation of Ca by EDTA was used to distinguish between $cPLA_2$ and $iPLA_2$ activities, so along with MAFP and VAR, this covered the activities of all relevant $PLA_2$ isoforms. EDTA was used at a concentration of 5mM, in excess of $CaCl_2$ concentration in Tyrode's, and also based on literature values (Lucas & Dennis, 2005; Moon et al., 2012). As shown in Supplemental Figure 5, dose-dependent effects on PUFA release and oxylipin formation were observed for MAFP. Confirmation of the dose dependent effects of

MAFP and VAR are presented in Supplemental Tables 3 and 4. Based on the dose studies, 0.1μM VAR and 10μM MAFP were used in the studies reported herein.

### *4.4.2 Non-esterified polyunsaturated fatty acids*

The most abundant PUFA detected in rat heart homogenates was ARA, followed by DHA, and then much lower levels of α-linolenic acid (ALA) and eicosapentaenoic acid (EPA). After 20 min of incubation at 37°C, ARA and DHA levels increased similarly and to a greater extent than ALA and EPA, resulting in much higher (~50–200 times) $PLA_2$ activity towards ARA and DHA compared to ALA and EPA release (**Figure 1**). LA, GLA, and DGLA are not included since these PUFA are not quantified in our system.

When heart homogenates were incubated for 20 min without and with VAR, MAFP or EDTA, ARA release was inhibited ~50-60% by each of VAR, MAFP and EDTA (Figure 2). EDTA inhibits Ca-dependent PUFA release; thus, the similar extent of inhibition of ARA by EDTA and VAR indicates that the Ca-dependent ARA release was primarily due to the $sPLA_2$ IIA, V, and/or X, and not the remaining Ca-dependent isoforms, which includes $cPLA_2$ and other $sPLA_2$ isoforms. Therefore, this also indicates that $iPLA_2$ isoforms, and not $cPLA_2$ isoforms, were mostly responsible for the ARA released that is inhibited by MAFP.

DHA release was inhibited ~80% by each of VAR and EDTA, and ~40% by MAFP (**Figure 2**). Following the rationale above, this indicates that a greater proportion of DHA release was via $sPLA_2$ IIA, V, and/or X rather than $iPLA_2$, with minimal $cPLA_2$ activity. The fact that these numbers sum to greater than 100% also suggests that there is redundancy in $PLA_2$ activity towards DHA.

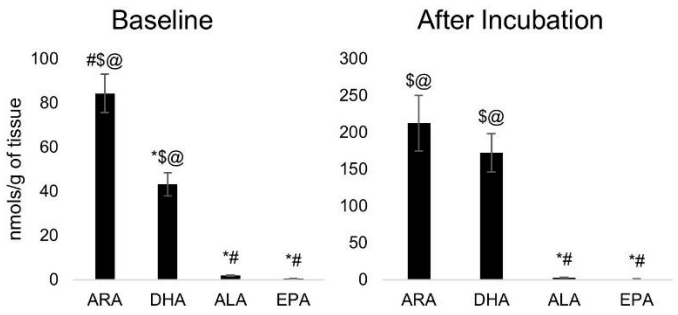


**Figure 4-1.** PUFA levels in rat heart homogenates at baseline and after 20 min incubation (n=6; mean±SE).
Statistical difference is shown when $p<0.05$. Difference from ARA is indicated by *; difference from DHA is indicated by #; difference from ALA is indicated by $; difference from EPA is indicated by @.

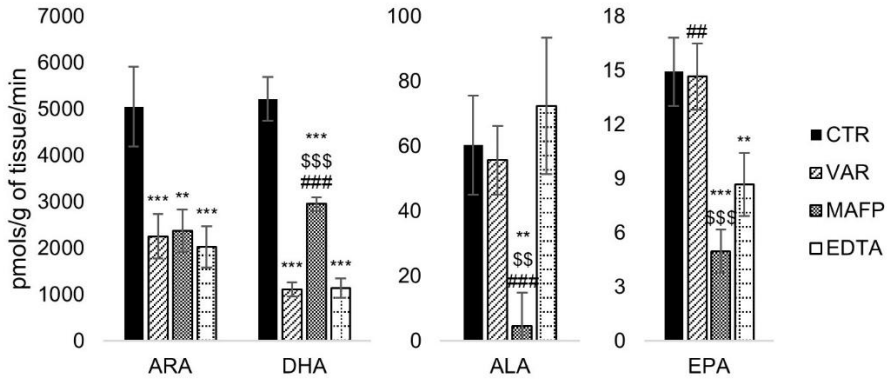


**Figure 4-2.** Effects of MAFP, VAR and EDTA on PUFA release (n=6; mean±SE).
CTR – control samples without inhibitor, VAR - 0.1μM of VAR (sPLA$_2$ IIA, V, and X inhibitor), MAFP – 10μM of MAFP (cPLA$_2$ and iPLA$_2$ inhibitor), EDTA – 5mM (Ca-dependent PLA$_2$ inhibitor). Difference from CTR is indicated by *; difference between inhibitors VAR and MAFP is indicated by $; difference from EDTA is indicated by #. One symbol $p<0.05$; two symbols $p<0.01$; three symbols $p<0.001$.

With respect to other omega-3 PUFA, ALA release was inhibited >90% by MAFP, with no effect of VAR or EDTA (Figure 2). The lack of inhibition by VAR and EDTA excludes roles for Ca-dependent $PLA_2$ isoforms, indicating that the ALA release is due primarily to $iPLA_2$ activity. EPA release was inhibited ~40% by EDTA and ~70% by MAFP, but not by VAR (Figure 2). This indicates that ~40% of EPA is formed via Ca-dependent isoforms and ~60% of EPA release is due to $iPLA_2$ isoform activities. Since VAR has no effect on EPA release, and there was still about 40% of Ca-dependent EPA release that was not inhibited with MAFP, it is likely that this remaining activity is due to other $sPLA_2$ isoforms not inhibited by VAR.

### *4.4.3 Non-esterified oxylipins*

ARA and linoleic acid (LA) oxylipins were the most abundant oxylipins in rat heart homogenates (**Figure 3**), followed by DHA, ALA, and EPA oxylipins. The relative amounts of ARA, ALA and EPA oxylipins generally reflected the relative abundance of their precursor PUFA, while DHA oxylipins were underrepresented relative to the proportion of PUFA (Figures 1 and 3). LA oxylipins could not be compared to LA since our method does not quantify LA. The relative amounts of the increased oxylipins were similar after incubation (Figure 3), except that now the difference between DHA oxylipins and ALA and EPA oxylipins was significant.

We then examined the effect of VAR and MAFP inhibitors on oxylipin formation in order to determine the $PLA_2$ isoforms associated with the formation of oxylipins. EDTA was not tested since $cPLA_2$ activity was minimal, as determined from PUFA analysis (Figure 2). Further, interpretation of results with EDTA treatment on oxylipins would be limited because in addition to inhibiting Ca-dependent $PLA_2$ isoforms, it also inhibits oxylipin synthesizing enzymes that are Ca-dependent, such as 5- and 15-LOX (Brinckmann et al., 1998; S. Kulkarni et al., 2002; Rouzer & Samuelsson, 1987). More than half of individual ARA oxylipins derived via the LOX and

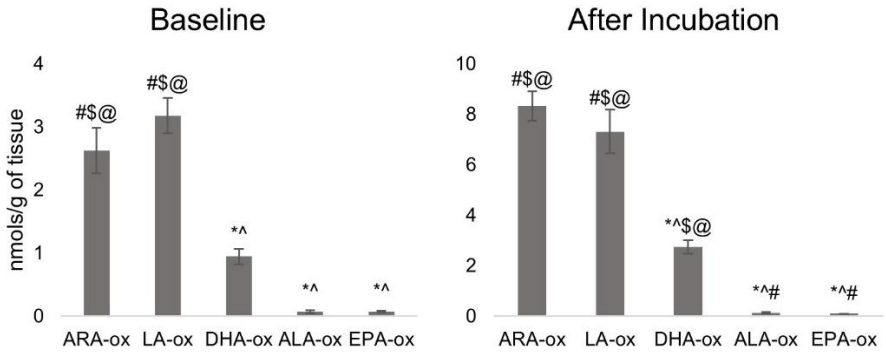


**Figure 4-3.** Total oxylipin levels by PUFA precursor in rat heart homogenates at baseline and after incubation for 20 min (n=6; mean±SE).
'Ox' is used to indicate oxylipin after the abbreviation for each PUFA precursor. Statistical difference is shown when $p<0.05$. Difference from ARA is indicated by *; difference from LA is indicated by ^; difference from DHA is indicated by #; difference from ALA is indicated by $; difference from EPA is indicated by @.

CYP pathways were inhibited by MAFP, which is similar to its inhibitory effect on ARA. In contrast, very few ARA oxylipins were inhibited by VAR, despite it inhibiting ARA release by >50% (Figures 2, **4A-B**). Since VAR had minimal effect on ARA oxylipins and $cPLA_2$ did not contribute to ARA release, this indicates that ARA oxylipins derived via the LOX and CYP pathways are associated primarily with $iPLA_2$ activity. When the oxylipins for the LOX and CYP pathway were summed, the totals reflected most individual oxylipins, except for the 12-LOX product 12-hydroxyeicosatetraenoic acid (12-HETE) (Figure 4A-B).

Similar to ARA, most LOX and CYP oxylipins derived from other omega-6 PUFA, namely LA and dihomo-γ-linoleic acid (DGLA), were also inhibited by MAFP, but not VAR, indicating that their formation is associated with $iPLA_2$ and/or $cPLA_2$ activity (**Figure 5**). Activity of $cPLA_2$ cannot be ruled out for these omega-6 PUFA derived oxylipins, since their precursor PUFA were not quantified and the $PLA_2$ isoforms associated with their release were not identified. Further, like ARA COX oxylipins, total DGLA COX oxylipins also were not affected either by VAR or MAFP, and effects on individual oxylipins were minor (less than 15% different than control) and did not follow a discernable pattern (Figures 4C and 5D).

With respect to DHA oxylipins, total and several individual DHA oxylipins derived via the LOX pathway were inhibited more by VAR than MAFP (**Figure 6A**). This pattern was like that of DHA release and indicated that DHA oxylipin formation also was associated more with $sPLA_2$ IIA, V and/or X activity than $iPLA_2$ activity. Notably, the only DHA LOX oxylipin that was not significantly inhibited by either VAR or MAFP was the 12-LOX metabolite, 14-hydroxy-docosahexaenoic acid (14-HDoHE). With respect to DHA CYP oxylipins, 3 out of 4 were inhibited by VAR, and only 1 out of 4 was inhibited by MAFP. Total DHA CYP oxylipins

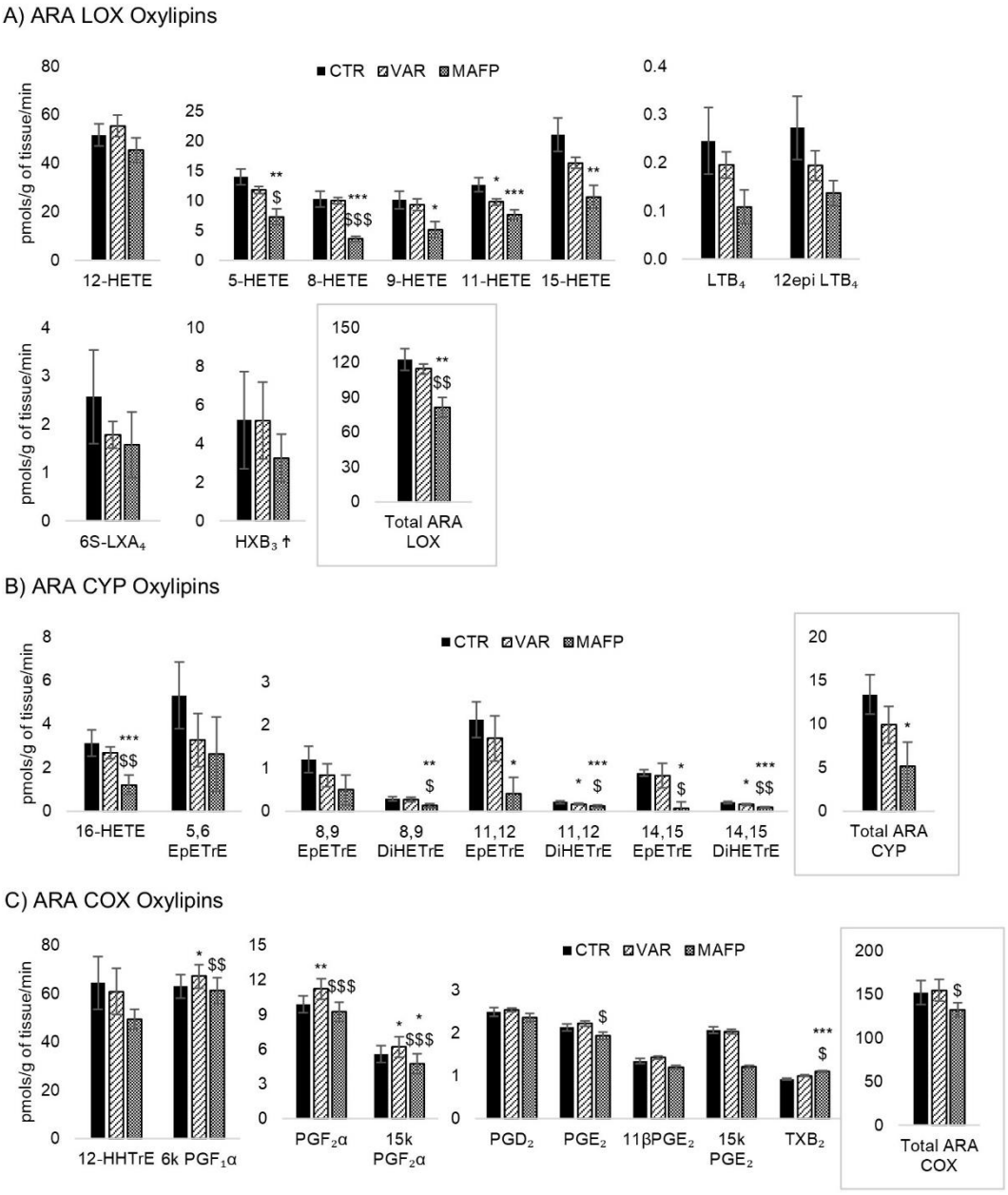


**Figure 4-4.** Effects of VAR and MAFP on ARA oxylipin formation (n=6; mean±SE). A) LOX oxylipins, B) CYP oxylipins, C) COX oxylipins. CTR – control samples without inhibitor, VAR – 0.1μM of VAR (sPLA$_2$ IIA, V, and X inhibitor), MAFP – 10μM of MAFP (cPLA$_2$ and iPLA$_2$ inhibitor). Difference from CTR is indicated by *; difference between inhibitors VAR and MAFP is indicated by $. One symbol $p<0.05$; two symbols $p<0.01$; three symbols $p<0.001$. The symbol † indicates a non-quantifiable oxylipin (so units are arbitrary and not included in corresponding total).

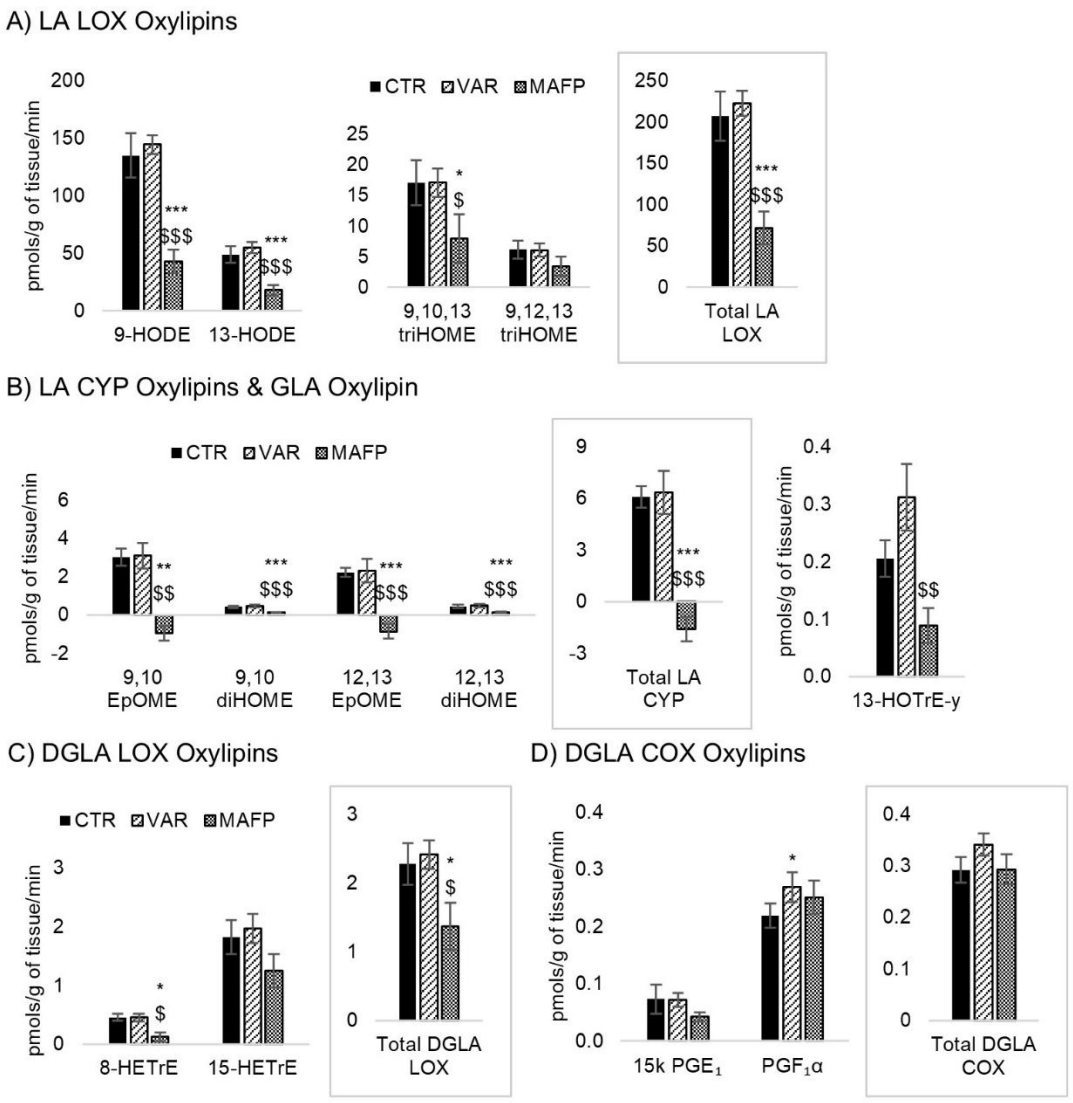


**Figure 4-5.** Effects of VAR and MAFP on LA, GLA, and DGLA oxylipin formation (n=6; mean±SE).
A) LA LOX oxylipins, B) LA CYP oxylipins & GLA oxylipin (13-HOTrE-γ), C) DGLA LOX oxylipins, D) DGLA COX oxylipins. CTR – control samples without inhibitor, VAR – 0.1μM of VAR (sPLA$_2$ IIA, V, and X inhibitor), MAFP – 10μM of MAFP (cPLA$_2$ and iPLA$_2$ inhibitor). Difference from CTR is indicated by *; difference between inhibitors VAR and MAFP is indicated by $. One symbol $p<0.05$; two symbols $p<0.01$; three symbols $p<0.001$.

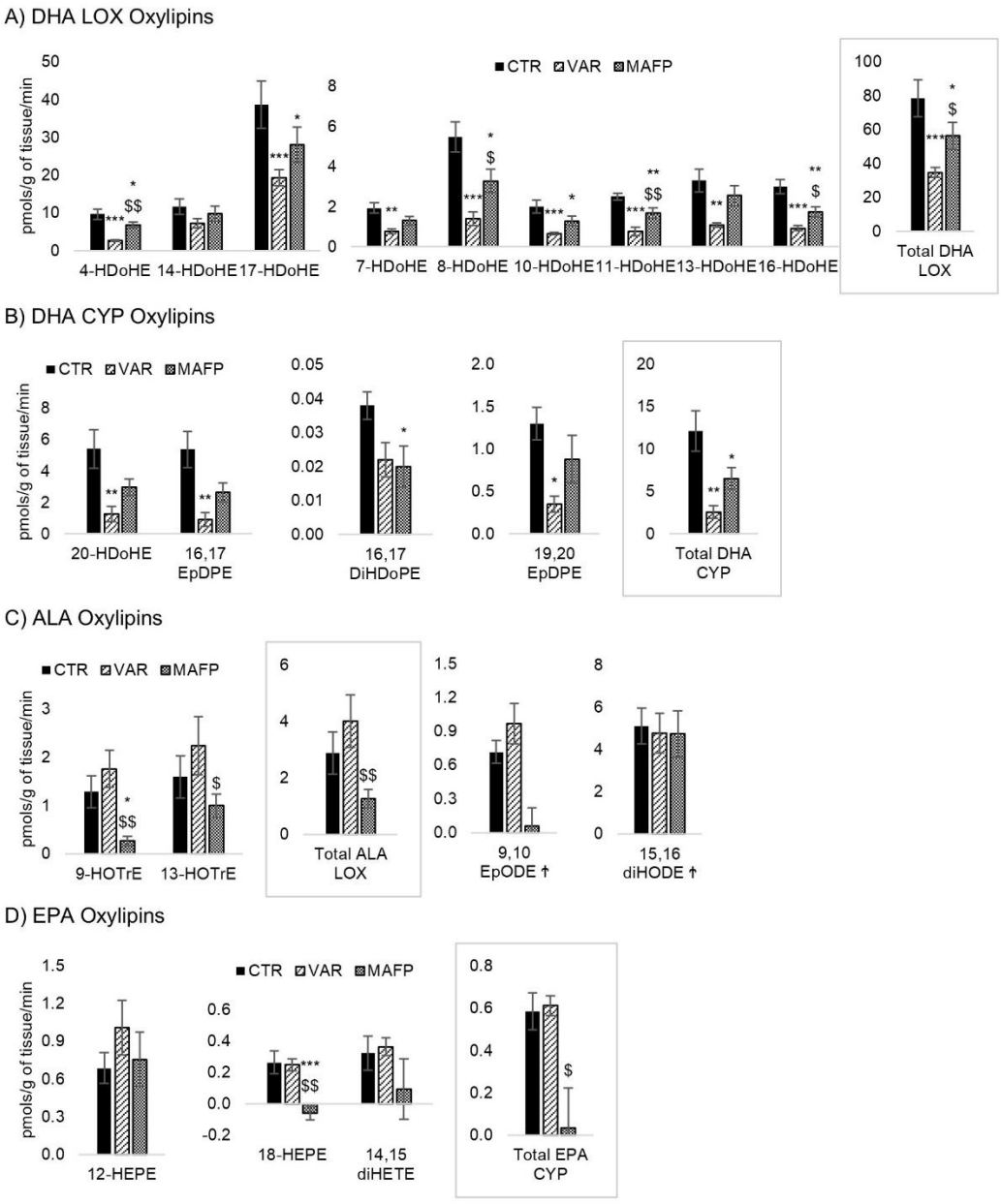


**Figure 4-6.** Effects of VAR and MAFP on DHA, EPA, and ALA oxylipin formation (n=6; mean±SE).
A) DHA LOX metabolites, B) DHA CYP metabolites, C) ALA LOX and CYP metabolites, D) EPA LOX and CYP metabolites. CTR – control samples without inhibitor, VAR - 0.1μM of VAR ($sPLA_2$ IIA, V, and X inhibitor), MAFP – 10μM of MAFP ($cPLA_2$ and $iPLA_2$ inhibitor). Difference from CTR is indicated by *; difference between inhibitors VAR and MAFP is indicated by $. One symbol $p<0.05$; two symbols $p<0.01$; three symbols $p<0.001$. The symbol † indicates a non-quantifiable oxylipin (so units are arbitrary and not included in corresponding total).

were inhibited more by VAR than MAFP, although there was no significant difference between the two inhibitors (Figure 6B).

Individual ALA oxylipins were generally lower only with MAFP incubation, although most did not reach statistical significance (Figure 6C). This effect by MAFP on ALA oxylipins is similar to its effect on ALA release (Figure 2), indicating that ALA oxylipin formation also is primarily associated with iPLA$_2$ activity. Consistent with the small amount of EPA released, less than 1.5 pmol/g tissue/min of EPA oxylipins were formed in CTR conditions (Figure 6D). Formation of 18-hydroxy-eicosapentaenoic acid (18-HEPE) was significantly reduced by MAFP, and 14,15-dihydroxy-eicosatetraenoic acid (14,15-diHETE) followed a similar, but non-significant, pattern. This suggests that iPLA$_2$ activity is associated with both EPA release and oxylipin formation for CYP derived EPA oxylipins.

A distinct pattern was noted for 12-LOX oxylipins, as the formation of the direct products, 12-HETE, 12-HEPE and 14-HDoHE, were inhibited neither by MAFP nor VAR (Figures 4A, 6A and 6D). The combined sum of these 12-LOX products also was not significantly inhibited by either VAR or MAFP. In comparison, formation of total 5-LOX (5-HETE, 4-HDoHE, 7-HDoHE) and 15-LOX (15-HETE, 17-HDoHE, 15-hydroxy-eicosatrienoic acid (15-HETrE)) oxylipins were inhibited by both inhibitors (**Figure 7**), indicating that priority of synthesis goes via the 12-LOX pathway when substrate levels are limited.

### *4.4.4 PLA$_2$ isoform expression*

To determine the expression of PLA$_2$ isoforms in the rat, primers were designed for all possible and relevant isoforms, totalling 25 PLA$_2$ isoforms (Supplemental Table 2). We detected mRNA for sPLA$_2$ IB, IIA, IID, V, and XIIA, cPLA$_2$ α, β, and ζ, all iPLA$_2$ isoforms (β-η), lipoprotein-associated Lp-PLA$_2$, platelet activating factor acetyl hydrolase-2 (PAF-AH2), and

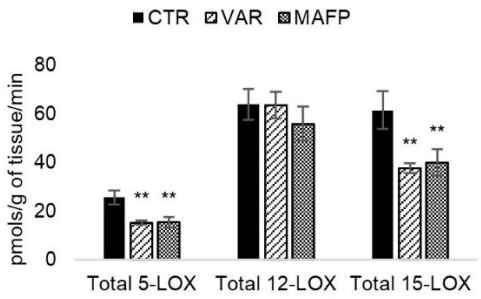


**Figure 4-7.** Effects of VAR and MAFP on 5-LOX, 12-LOX, and 15-LOX oxylipin formation (n=6; mean±SE).
CTR – control samples without inhibitor, VAR - 0.1μM of VAR (sPLA$_2$ IIA, V, and X inhibitor), MAFP – 10μM of MAFP (cPLA$_2$ and iPLA$_2$ inhibitor). Difference from CTR is indicated by *; difference between inhibitors VAR and MAFP is indicated by $. One symbol $p<0.05$; two symbols $p<0.01$; three symbols $p<0.001$. Sums for each enzyme pathway consisted of the following oxylipins: 5-LOX: 5-HETE, 4-HDoHE, 7-HDoHE; 12-LOX: 12-HETE, 12-HEPE and 14-HDoHE; and 15-LOX: 15-HETE, 17-HDoHE, 15-HETrE.

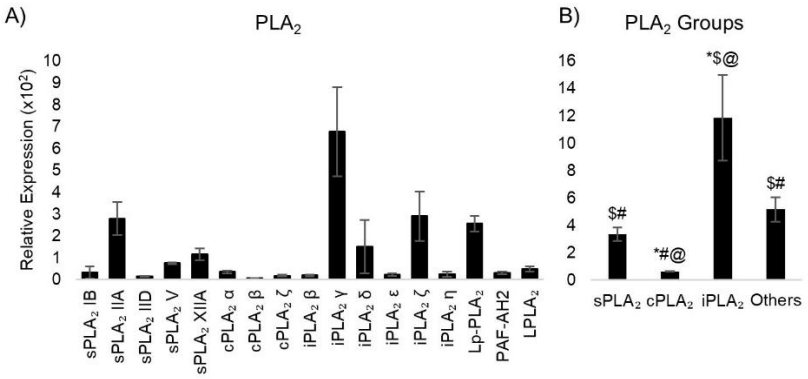


**Figure 4-8.** Relative expression of $PLA_2$ isoforms to GAPDH in the A) heart and B) sum of $PLA_2$ expression by $PLA_2$ group (n=4; mean±SE).
Group "other" includes Lp-$PLA_2$, PAH-AH2, and $LPLA_2$. Different when compared to $sPLA_2$ indicated by *, $cPLA_2$ indicated by $, $iPLA_2$ indicated by #, Other indicated by @ ($p<0.05$).

lysosomal $LPLA_2$ (**Figure 8A**). Primers for $PLA_2$ isoforms that were undetectable in the heart ($sPLA_2$ IIC, IIE, IIF, X, and XIIB, $cPLA_2$ δ, and ε) were verified in tissues known to have these isoforms (**Supplemental Figure 6**). Among the $PLA_2$ isoforms that VAR inhibits, only $sPLA_2$ IIA, and V were detected, and among the $PLA_2$ isoforms that MAFP inhibits, $cPLA_2$ α, β, and ζ and all $iPLA_2$ isoforms (β-η) were detected. In agreement with activity results, expression of $iPLA_2$ isoforms were highest, followed by $sPLA_2$ and other isoforms (Lp-$PLA_2$, PAF-AH2, and $LPLA_2$), and lowest expression was detected for $cPLA_2$ isoforms (Figure 8B).

### *4.5 Discussion*

Using a whole heart homogenate approach to examine $PLA_2$ activity with the complete array of intrinsic $PLA_2$ isoforms and substrate lipids present in rat hearts, the current findings indicate that $sPLA_2$ IIA and/or V release both ARA and DHA but only DHA oxylipins are formed because of this $sPLA_2$ activity. $sPLA_2$ X expression was not detected in the rat hearts, indicating that the $sPLA_2$ activity herein is due to the $sPLA_2$ IIA and V isoforms. These $sPLA_2$ isoforms are associated with the majority of DHA release and formation of DHA oxylipins. The current results also indicate that $iPLA_2$ also mediates ARA and DHA release, is primarily responsible for ALA and EPA release and for the formation oxylipins derived from ARA, LA, DGLA, ALA and EPA, as well as for the formation of some DHA oxylipins. Interestingly, although ARA release and ARA oxylipin formation are both inhibited similarly by MAFP, the effect on oxylipin formation is due to lower LOX and CYP oxylipins, while COX and 12-LOX oxylipins are not inhibited. The discrepancy between non-esterified PUFA and oxylipins may be possible because of the much lower levels of oxylipins compared to fatty acids or could be due to the incomplete inhibition of non-esterified PUFA release, thus allowing for preferential oxylipin synthesis.

These findings reinforce the importance of determining the actual oxylipins formed, as they do not always follow the pattern of PUFA release.

The presence of $sPLA_2$ activity that was inhibited by VAR and the high expression levels of $sPLA_2$ isoforms in rat hearts is consistent with previous findings in mice that suggested the heart to be a major source of $sPLA_2$ (Berry et al., 2015; Hernandez-Anzaldo et al., 2015). VAR inhibited more DHA than ARA release but did not affect release of EPA and ALA or formation of their oxylipins. By comparison, it only inhibited the formation of DHA oxylipins, and did not block ARA oxylipin formation. This is consistent with previous studies showing that $sPLA_2V$, when compared to $iPLA_2\beta$ and $cPLA_2\alpha$, released more DHA (Hayashi et al., 2021; Mouchlis et al., 2018). $sPLA_2V$ also was shown to have a preference for LA, but little activity towards ARA (Mouchlis et al., 2018). Although we could not evaluate the effects on LA release, the differences in ARA release could be because VAR also inhibits $sPLA_2$ IIA. Our results are also similar to studies using an $sPLA_2$ IID lymph node knockout and a transgenic model of spleen $sPLA_2$ X that showed that these $sPLA_2$ isoforms release ARA, DHA, and EPA, but are associated with the formation of oxylipins derived only from DHA and EPA, and not from ARA (including prostaglandins, thromboxane $B_2$, and 5-HETE) (Miki et al., 2013; Murase et al., 2016). Although these studies involved different tissues and $sPLA_2$ isoforms, together these findings indicate that the ARA released by $sPLA_2$ is not available for ARA oxylipin formation. This could be due to $sPLA_2$ being the only group of $PLA_2$ that can act on extracellular membranes (Mouchlis & Dennis, 2019; Murakami et al., 2011). With respect to the EPA release and oxylipin formation observed in the $sPLA_2$ knockout model (Miki et al., 2013; Murase et al., 2016), it is possible that $sPLA_2$ IID plays a role in EPA release in the rat heart, as this $PLA_2$ was expressed at low levels

in the rat heart, but is not inhibited by VAR. Although EPA levels herein were low, this isoform could be important in rats provided high levels of dietary omega-3 PUFA.

These results may help elucidate why VAR has failed phase III clinical trials, which have shown an increased risk of myocardial infarction, stroke and cardiovascular mortality (Nicholls et al., 2014). Collectively with previous findings (Kuksis & Pruzanski, 2022; Miki et al., 2013; Murase et al., 2016) our results suggest that $sPLA_2$ would more likely have anti-inflammatory activity, as DHA oxylipins have been shown to have anti-inflammatory and protective functions (Capó et al., 2018; Gabbs et al., 2015; Motta et al., 2019). In addition, VAR inhibited very few ARA oxylipins, which tend to be the oxylipins with more pro-inflammatory effects (Caligiuri et al., 2017). The association of higher serum $sPLA_2$ levels that have been observed with cardiovascular disease and mortality have been interpreted as a causal link, but our results provide further evidence that it may be an adaptive response to the increased inflammation (Akinkuolie et al., 2019; Boekholdt et al., 2005; Lind et al., 2012; Mallat et al., 2005, 2007). In this regard, higher levels of $sPLA_2$ IIA in severe and deceased COVID-19 patients (Snider et al., 2021) may also be adaptive, so higher $sPLA_2$ levels should be interpreted with caution.

While $sPLA_2$ is responsible for the majority of DHA release and DHA oxylipin formation in rat hearts, the remaining DHA released and DHA oxylipins in the rat heart are formed via $iPLA_2$. Further, the current findings indicate that the ARA released by $iPLA_2$ is primarily responsible for the formation of ARA oxylipins, as shown by treatment with MAFP. MAFP also blocks $cPLA_2$ activity, but the similar effects of EDTA and VAR on PUFA release, and the low expression of $cPLA_2$ isoforms, indicates that there is minimal $cPLA_2$ activity in the rat heart, consistent with other reports (Cedars et al., 2009; Jenkins et al., 2009; S. J. Liu & McHowat, 1998). In addition to LA oxylipin formation, we also could examine the effect of MAFP on ALA

release and ALA, EPA, γ-linoleic acid (GLA) and DGLA oxylipin formation, which have not previously been reported. These were similarly inhibited by MAFP, implicating $iPLA_2$ as the major $PLA_2$ group also responsible for the formation of these oxylipins. These results suggest that inhibiting $iPLA_2$ may not be as anti-inflammatory as might be suggested solely by its effects on reducing ARA, since $iPLA_2$ also mediates the formation of omega-3 PUFA derived oxylipins, which tend to be more anti-inflammatory (Gabbs et al., 2015; Hecker et al., 2018; Pauls et al., 2018). In addition, while those derived from omega-6 PUFA are generally, but not always (e.g. epoxy-eicosatrienoic acid (EpETrE)) more pro-inflammatory, many of the pro-inflammatory oxylipins derived from ARA (e.g. COX and 12-LOX products) (Caligiuri et al., 2017; Gross et al., 2005) were not affected by MAFP.

The findings herein on rat heart homogenates are similar to many studies that have employed diverse methods. The greater release of DHA by $sPLA_2$ isoforms and of ARA and EPA by $iPLA_2$ is consistent with studies using human enzymes and phospholipid mixtures *in vitro* (Hayashi et al., 2021; Mouchlis et al., 2018). Further, our findings ex vivo that DHA oxylipins appear to be formed predominantly by $sPLA_2$ isoforms was also reported in a mouse lymph node $sPLA_2$ IID knockout (Miki et al., 2013) and in mice overexpressing $sPLA_2$ X (Murase et al., 2016). Similarly, the formation of ARA and LA oxylipins by $iPLA_2$ indicated herein is consistent with findings in mouse $iPLA_2\gamma$ cardiac knockouts (Moon et al., 2016), although that study did not observe the resistance to COX and 12-HETE inhibition that was observed in our analysis. This ex vivo assay therefore can add to the knowledge gained from other methods such as those that use single enzyme knockouts, assays using recombinant enzymes, and methods designed with specific assay solutions and phospholipid substrates to optimize their activity. This ex vivo

assay will be useful to investigate the effects of interventions that alter in vivo isoforms and lipid compositions (e.g. dietary treatments).

There were also differences between our findings and of those utilizing other methods and species. The main difference was the apparent lack of $cPLA_2$ activity in the healthy rat heart ex vivo compared to studies with recombinant human enzymes (Hayashi et al., 2021; Mouchlis et al., 2018) and human heart mitochondria (Moon et al., 2018). This may reflect species differences in enzyme isoforms, which have been well-documented between human and mouse (Astudillo et al., 2019; Dennis et al., 2011; Mancuso et al., 2003; Murakami et al., 2011), and even differences between strains of mice (Kennedy et al., 1995). There is little information on $PLA_2$ isoforms in the rat heart, but one study in rat ventricular myocytes indicated the presence of $iPLA_2$, but little $cPLA_2$ activity (S. J. Liu & McHowat, 1998), consistent with our findings. Another difference in the literature from our studies is the finding of significant levels of $iPLA_2$ γ and β isoform activity in other studies, which in addition to possibly being due to species differences (Moon et al., 2016), could also be due to differences in disease state (Bonetti et al., 2020; Mancuso et al., 2003; Moon et al., 2018). On the other hand, despite differences in assay parameters, species and disease state, Moon et al (2018) also reported that inhibitors of these $iPLA_2$ isoforms (S-BEL and R-BEL) did not inhibit ARA release in healthy human heart mitochondria, while MAFP did (Moon et al., 2018). Similarly, Liu & McHowat (1998) found that MAFP but not BEL inhibited $PLA_2$ activity in rat ventricular myocytes (S. J. Liu & McHowat, 1998).

The results from the rat heart ex vivo assay herein therefore provides important information that adds to previous literature reports in other species and assay conditions. However, all studies still have limitations, such as when one isoform is inhibited or knocked out,

other isoforms can increase in activity to compensate, making it appear that the inhibited isoform is less important (Murakami et al., 2011; Offer et al., 2005). The current ex vivo assay, however, may better reflect the in vivo compensation or redundancy that would occur with the other isoforms present in whole tissue. In addition, the specificity or off-target effects of inhibitors is not always clear. For example, VAR has been reported to be inactive against COX (Snyder et al., 1999), but other oxylipin synthesizing enzymes have not been tested. Also, MAFP inhibits PAF-AH (Kell et al., 2003), a $PLA_2$ enzyme that cleaves short chain or oxidized fatty acids (Kono & Arai, 2019), and is involved in the production of omega-3 epoxides in mast cells (Shimanaka et al., 2017). However, its effects on the oxylipin profile are not entirely understood and we are unaware of studies that indicate a major role of this enzyme on the oxylipin profile. Further, another limitation is that the original compartmental separation of organelles and membranes is not represented in homogenized tissues from ex vivo or knockout studies or in *in vitro* studies that use detergents to mimic membrane conditions (Murakami et al., 2011; Offer et al., 2005). It also is important to note that these findings in the heart may not translate to other tissues, since they have differing fatty acid and $PLA_2$ isoform compositions.

In conclusion, the results of this ex vivo analysis indicate that inhibition of activity of $sPLA_2$ IIA and/or V by VAR led to a reduction in ARA and DHA release, but only DHA oxylipin formation was inhibited. Inhibition by MAFP led to a reduction in the release of ARA, DHA, EPA and ALA, and an inhibition of ARA, DGLA, LA, DHA, ALA and EPA oxylipin formation. $PLA_2$ inhibition by EDTA indicated that there was little $cPLA_2$ activity, which was consistent with low expression of $cPLA_2$ in the rat heart. Therefore, these data indicate that there is a preference of $sPLA_2$ IIA and/or V for DHA release and oxylipin formation, while $iPLA_2$ is likely the major isoform responsible for the release of ARA, ALA, EPA, and the formation of ARA,

LA, DGLA, ALA, and EPA oxylipins in healthy rat hearts. Some oxylipins such as those produced via the COX and 12-LOX pathways were not inhibited herein, indicating that these oxylipins may be preferentially synthesized when substrate availability is limited. These findings reinforce the importance of analyzing the oxylipin profile to achieve a more comprehensive understanding of $PLA_2$ activities.

### *4.6 Chapter transition*

After the method was developed for assessment of $PLA_2$ activity towards PUFA and oxylipins in rat hearts, as presented in this chapter, this allowed us to pursue our ultimate objective to determine if sex differences in oxylipins that are dependent on diet fatty acid composition would be driven by $PLA_2$ activity. This was achieved using samples from the study presented in chapter 3.

In chapter 3, the bioequivalence between dietary DHA and the ALA requirement was determined. In this study the data indicated differences in sex effects according to diet. Further analysis indicated the highest DHA dose of 1.3g DHA/100g of diet to be the DHA group that had the greatest sex difference in DHA oxylipins, whereas the group with an equivalent dose of ALA of 1.3g of ALA/100g of diet showed no sex differences in DHA oxylipins. These groups reflected the general trend observed with the data, as dietary ALA did not show a dose by sex response (difference in regression slope according to sex), whereas the DHA groups did. Thus, we decided to proceed with $PLA_2$ activity analysis using male and female samples from the groups containing 1.3g of ALA and DHA/100g of diet. This also allowed comparison between groups that had an equivalent concentration of ALA and DHA. Thus, in the next chapter we aimed to determine how sex and dietary ALA and DHA impacted $PLA_2$ activity towards fatty

acid release and oxylipin formation in rat hearts, and whether $PLA_2$ mediates the different sex effects according to diet, as previously observed in chapter 3.

# Chapter 5: Sex and dietary ALA and DHA effects on rat heart phospholipase $A_2$ activity mediating fatty acid release and oxylipin formation have cardiovascular implications

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*5.1 Abstract*

The ways in which diet and sex influence heart phospholipase $A_2$ ($PLA_2$) activities are underexplored, particularly with respect to effects on oxylipin formation. Therefore, male and female Sprague-Dawley rats were provided diets with 1.3% α-linolenic acid (ALA) or docosahexaenoic acid (DHA) for 6 weeks, and heart homogenates were incubated with inhibitors for secreted (s)$PLA_2$ (Varespladib) or calcium-independent (i)$PLA_2$ (methyl arachidonyl fluorophosphonate). Non-esterified polyunsaturated fatty acids (PUFA) and oxylipins were determined by HPLC-MS/MS and data were analyzed using 3-way repeated measures ANOVA. $sPLA_2$ activity was selective for DHA release and DHA oxylipin formation, contributing to ~50% of arachidonic acid (ARA) release, but only ~30% of ARA oxylipin formation. Eicosapentaenoic acid (EPA) release and oxylipin formation was minor but primarily mediated by $iPLA_2$. $iPLA_2$ activity was less selective, with activity towards all measured PUFA and oxylipins, with the primary PUFA released depending on diet. For both $sPLA_2$ and $iPLA_2$, there was more activity towards DHA and its oxylipins with the DHA diet, and towards ALA and ARA and their oxylipins with the ALA diet. Sex effects on fatty acids and oxylipins differed, as DHA, EPA and ALA release were higher in females, while DGLA and cyclooxygenase-derived ARA oxylipin formation were higher in males. Interestingly, the sex effect on cyclooxygenase ARA oxylipins was attenuated with dietary DHA. These findings provide further rationale for the simultaneous measurement of PUFA and oxylipins since they are not always congruent, and shed new light on diet and sex effects on $PLA_2$ types in cardiovascular oxylipin biology.

**Keywords**: phospholipase $A_2$; oxylipins; polyunsaturated fatty acids; α-linolenic acid; docosahexaenoic acid.

## *5.2 Introduction*

Phospholipase $A_2$ ($PLA_2$) is a superfamily of enzymes that hydrolyse fatty acids from membrane phospholipids, typically at the *sn*-2 position, which preferentially contains a polyunsaturated fatty acid (PUFA). The released PUFA are then converted by cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) enzymes into a variety of bioactive lipid mediators, also known as oxylipins (Astudillo et al., 2023; Dennis et al., 2011; Mouchlis & Dennis, 2019; Murakami, 2023). There is also a recent suggestion that $PLA_2$ can release esterified oxylipins directly from cell membrane phospholipids (Annevelink et al., 2022). Oxylipins are hormone-like molecules that mediate the functions of fatty acids and are influenced by diet and sex, as well as many other factors such as disease, exposure to air pollution, household chemicals, food contaminants and pharmaceuticals (Aukema & Ravandi, 2023; L. G. J. Cayer et al., 2024; Ferdouse et al., 2019a; Gabbs et al., 2015). They are implicated in the physiological roles and pathophysiological functions of several diseases associated with $PLA_2$ activity, including cardiovascular disease (Benjamin et al., 2019; Caligiuri et al., 2017; Murakami, 2023).

Much of the focus from $PLA_2$ research has been on release of arachidonic acid (ARA), which leads to production of generally pro-inflammatory oxylipins (Gabbs et al., 2015; Khan & Ilies, 2023; Y. Zhang et al., 2023). Based on their effects on ARA, $PLA_2$ inhibitors are widely explored as potential drug targets in several diseases, including cardiovascular diseases (Hidalgo et al., 2024; Jantan et al., 2014; Shukla et al., 2015; Y. Zhang et al., 2023). Although some $PLA_2$ inhibitors have reached clinical trials, including for treating cardiovascular disease, none have succeeded (Batsika et al., 2021; Kokotou et al., 2017). The gaps in $PLA_2$ research regarding oxylipins, particularly with respect to inhibitor use, is partly due to making assumptions

concerning oxylipins impacted by $PLA_2$ inhibition solely by analysis of fatty acids, even though oxylipins do not entirely reflect fatty acid findings in $PLA_2$ research (Manson et al., 2023; Miki et al., 2013; Moon et al., 2016; Murase et al., 2016). In addition, PUFA other than ARA are underexplored, even though certain $PLA_2$ enzymes have important roles in the release of other fatty acids, including omega-3 fatty acids that are converted to oxylipins that have generally anti-inflammatory and protective functions (Hayashi et al., 2021; Manson et al., 2023; Miki et al., 2013; Moon et al., 2016; Murase et al., 2016).

There is also a lack of knowledge on how diet and sex influence $PLA_2$ activity. This is important since diet and sex influence phospholipid composition, which is expected to influence $PLA_2$ activity (Rao et al., 2007; Smesny et al., 2014). Several studies support diet (Y. Liu et al., 2014; Rao et al., 2007; Rudkowska et al., 2013; Smesny et al., 2014; K.-P. Su et al., 2018; Vincentini et al., 2011) and sex (Boekholdt et al., 2005; Keeting et al., 1998; Mallat et al., 2007; Pop et al., 2013; Stone et al., 2019; Ward et al., 2018; Xu et al., 2017) effects on $PLA_2$ expression, protein levels, and overall activity (based on a single product), and we recently showed that there are differences in sex effects between dietary omega-3 α-linolenic acid (ALA) and docosahexaenoic acid (DHA) on oxylipins in rat hearts (Ferdouse et al., 2019a). Such diet and sex effects are not entirely explained by their effects on phospholipid fatty acid composition (Cayer et al., 2019; Ferdouse et al., 2019a; Leng et al., 2017, 2018; Mendonça et al., 2018), which suggests more regulation in the conversion of phospholipid fatty acid to oxylipins. Oxylipins produced by different downstream enzymatic pathways (COX, LOX, and CYP) were affected similarly in our study (Ferdouse et al., 2019a), leading to the hypothesis that $PLA_2$ activity could be the key regulatory step mediating these effects.

We previously developed a method (Manson et al., 2023) to measure rat heart $PLA_2$ activity ex vivo from the three main $PLA_2$ types believed to be involved in PUFA release and oxylipin synthesis: secreted (s)$PLA_2$, cytosolic (c)$PLA_2$, and calcium-independent (i)$PLA_2$ (Dennis & Norris, 2015; Mouchlis & Dennis, 2019; Ramanadham et al., 2015). This method allows us to examine diet and sex effects using the endogenous phospholipid composition, which is a strong advantage of this method (Mouchlis et al., 2019), since traditional *in vitro* studies may not reflect the situation *in vivo* (Astudillo et al., 2023) and assessment of substrate specificity is difficult using traditional radioactive assays (Mouchlis et al., 2019). Among the main $PLA_2$ types, $sPLA_2$ and $iPLA_2$ have been shown to have primary activity in rat hearts, with very limited $cPLA_2$ activity (Cedars et al., 2009; Jenkins et al., 2009; S. J. Liu & McHowat, 1998; Manson et al., 2023). Therefore, the objective of this study was to assess the contributions of $sPLA_2$ and $iPLA_2$ activities in rat hearts on the sex and dietary ALA and DHA effects on oxylipins in rat hearts.

## *5.3 Methods*

### *5.3.1 Animal study*

Rat hearts were obtained from 12 male and 12 female 10-week-old Sprague Dawley rats that were randomized to receive either 1.32g ALA/100g of diet or 1.35g DHA/100g of diet for 6 weeks starting at 4 weeks of age, as part of a separate experiment (Manson et al. unpublished). The diets followed the AIN93G guidelines (Reeves et al., 1993) except that soybean oil was replaced by a mix of oils (flaxseed, safflower, coconut, and olive oils) with an equal total oil level (7g/100g of diet) to achieve 1.3g ALA/100g of diet in the ALA diet, and 0.005g of ALA, and 1.3g of DHA/100g of diet in the DHA diet. DHA was provided in purified form containing 98% DHA in ethyl ester form (Maxomega DHA 95 EE, BASF, Germany). Both diets contained

similar concentrations of linoleic acid (LA), monounsaturated fatty acids and saturated fatty acids (**Supplemental Table 1**).

After the intervention period animals were anesthetized with isoflurane and terminated by decapitation. After exsanguination, hearts were removed, rinsed in PBS, immediately frozen in liquid nitrogen, and stored at -80°C until analyses. All procedures were approved by the University of Manitoba Animal Care Committee and conducted in accordance with the Canadian Council for Animal Care and ARRIVE guidelines (Percie du Sert et al., 2020).

### *5.3.2 Diet fatty acid analysis*

Fatty acid compositions of the diets were analyzed by gas chromatography with flame ionization detection (GC-FID), as described (Gabbs et al., 2021). Briefly, 25mg of diet was extracted via liquid-liquid extraction based on the Folch method (Folch et al., 1957) for total lipids with chloroform/methanol (2:1/vol:vol), 0.01% BHT, and 0.73% sodium chloride. Phosphatidylcholine C15:0 (Avanti Polar Lipids, AL, USA) was used as an internal standard. Lipid extracts were dried down and fatty acids were transmethylated using methanolic $H_2SO_4$ (Aukema & Holub, 1989). The resulting methylated fatty acids were extracted using hexane and ultrapure water, dried down and re-suspended in hexane. Samples were separated on a 30m DB-225MS column (Agilent Technologies, CA, USA) using a Bruker 450 GC-FID.

### *5.3.3 $PLA_2$ activity*

$PLA_2$ activity was assessed as previously described (Manson et al., 2023). Briefly, frozen hearts were thawed on ice, weighed, and homogenized in 5.7x vol of Tyrode's buffer (pH7.6, 1.8mM $CaCl_2$, 1.05 mM $MgCl_2$, 2.68 mM KCl, 137 mM NaCl, 0.417 mM $NaH_2PO_4$, 11.9 mM $NaHCO_3$, and 5.55 mM D-Glucose; Sigma-Aldrich, MA, USA) and frozen at -80°C until analysis. For the assay, heart homogenates were thawed on ice and 200µL aliquots were

incubated for 20min in a shaking water bath at 37°C without or with inhibitor [methyl arachidonyl fluorophosphonate (MAFP) for $cPLA_2$ and $iPLA_2$, or Varespladib - LY315920 (VAR) for $sPLA_2$ (non-specific pan-$sPLA_2$ (Batsika et al., 2021; Nicholls et al., 2014)) (Cayman Chemical, MI, USA)]. For each heart an aliquot was taken immediately without incubation to serve as baseline. All inhibitors were diluted in dimethyl sulfoxide (DMSO) and subsequently in Tyrodes, with all sample containing a final concentration of 0.1% DMSO, including control and baseline. Reactions were stopped by the addition of 500 µL of methanol formic acid (100:1/v:v).

### *5.3.4 Non-esterified fatty acid and oxylipin analysis*

As previously described (Manson et al., 2023), the following were then added to each sample: 800 µL pH3 water, 90 µL ethanol, 10 µL antioxidant cocktail [0.2mg/ml BHT, 0.2mg/ml EDTA, 100µM indomethacin, 100µM trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (t-AUCB) in methanol/water (50/50, vol/vol) (Rund et al., 2018)] and deuterated internal standards (Cayman Chemical, MI, USA). A complete list of standards and their concentrations was previously described (Manson et al., 2023). All samples were adjusted to pH3 prior to solid phase extraction with 33 µm polymeric reverse-phase Strata-X solid phase extraction columns (Phenomenex, CA, USA). Non-esterified oxylipins and fatty acids were eluted with methanol, filtered through a Pasteur glass pipette with glass wool and sodium sulfate, and rinsed with methanol. Samples were dried down under a nitrogen stream and resuspended in the mobile phase (water/acetonitrile/acetic acid, 70/30/0.02, vol/vol/vol). Analysis was conducted using HPLC-MS/MS (HPLC: Nexera-XR LC-20AD XR, Shimadzu, Japan; mass spectrometer: QTRAP 6500; AB Sciex, ON) with a reverse phase column: Luna 5u C18(2) 100A; 250 x 2.00 mm (Phenomenex, CA, USA). The software used for the instrument was Analyst 1.6.2 (AB Sciex, ON) and for data analysis MultiQuant 3.0.5373.0 (AB Sciex, ON).

Quantitation was based on the stable isotope dilution method (Hall & Murphy, 1998). The lower limit of quantification (LLOQ) was set at peak heights of 5 times baseline and an oxylipin was considered quantifiable if this criterion was met for at least 5 samples in any group. A list of all oxylipins scanned for, detector response factors, and the lowest limit of quantitation (LLOQ) and detection (LLOD) can be found in (Ferdouse et al., 2019b; Monirujjaman et al., 2017). Sample preparation was conducted in random order and analyses were blinded during sample preparation and peak selection.

### *5.3.5 Statistical analysis*

The amount of each fatty acid released or oxylipin formed during the incubation time was calculated as follows: sample incubated (with or without inhibitor) minus the sample at baseline [i.e. incubated sample with (MAFP or VAR) or without inhibitor (Control) minus baseline (BL)]. Oxylipin data were analyzed by using the sum of all oxylipins from each fatty acid precursor. Data were inspected for the presence of influential oxylipins that changed the pattern of the total oxylipin results, and to ensure the total reflected the individual oxylipin pattern. Full statistical analysis results for individual PUFA and oxylipins are reported in **Supplemental Tables 2-3**.

All statistical analysis were conducted using R Studio (2024.12.1+563, 2009-2025 Posit Software, PBC). Normality was tested using the Shapiro-Wilk test at alpha >0.01 combined with a QQ-plot analysis. Non-normal data were log-transformed and if data contained zeroes or negative numbers log (variable+1) was used for data transformation. Data analyses were conducted using a 3-way repeated measures ANOVA (diet, sex, and treatment), followed by post-hoc testing using the Benjamini-Hochberg (BH) test. If 2 or more 2-way interactions were significant, the post-hoc analysis consisted of all relevant pairwise comparisons at 3-levels (as would be conducted for a 3-way interaction) to eliminate overlap in results from two or more 2-

way interaction analyses. Relevant comparisons when interactions were present were those with the same diet and sex at different treatment categories, same diet and treatment at different sex categories, and same sex and treatment at different diet categories. Ratios of male/female were calculated for the mean of each ARA COX oxylipin (n=8), which were then log transformed on base 2 to generate fold differences, and analyzed by two-way (diet, treatment) ANOVA for repeated measures, followed by BH post hoc tests for treatments. Significance was set at $p<0.05$.

## *5.4 Results*

### *5.4.1 Inhibitor effects on $PLA_2$*

The inhibitors used in this study are MAFP which inhibits both $cPLA_2$ and $iPLA_2$ isoforms and VAR which inhibits $sPLA_2$ isoforms. While specific inhibitors would have been ideal, this was proven not to be possible in previous analysis (Manson et al., 2023). When MAFP is used, the remaining activity is expected to be solely of $sPLA_2$ isoforms (Lucas & Dennis, 2005). When VAR is used, the remaining activity is expected to be that of $cPLA_2$ and $iPLA_2$. This aligns with our findings since the MAFP and VAR inhibition are complementary, resulting in nearly 100% inhibition when considering both separate inhibition results. In combination with our previous findings, using an identical study design without diet and sex (Manson et al., 2023), and those of others that $cPLA_2$ activity is minimal in the heart (Cedars et al., 2009; Jenkins et al., 2009; S. J. Liu & McHowat, 1998), we can say with fair confidence that the activity remaining with VAR inhibition, or the inhibition achieved with MAFP, is primarily of $iPLA_2$ activity. However, it should be noted that we cannot rule out the contribution of $cPLA_2$ activity with absolute certainty as it was not directly tested in this study and may have been influenced by diet and/or sex. For simplicity, we will refer to remaining activity after inhibition by VAR as $iPLA_2$ activity and to the remaining activity after inhibition by MAFP as $sPLA_2$ activity.

*5.4.1.1 PUFA*

DHA release was mostly inhibited by VAR (58-76%) but also by MAFP (33-36%) (**Figure 1A**). This selectivity of $sPLA_2$ resulted in DHA being 73-93% of the total fatty acid mass quantified [DHA, eicosapentaenoic acid (EPA), ALA, and ARA] that was released by $sPLA_2$ activity (**Figure 2A**). The mass of EPA release was minor (~1% as much as DHA or ALA) and was inhibited more by MAFP (59-64%) than VAR (**Figure 1B**), indicating that the primary activity towards EPA is mediated via $iPLA_2$. The mass of ALA release was similar to that of DHA in the hearts of rats provided the ALA diet, but much lower in those provided the DHA diet. ALA release was only inhibited by MAFP (73-77%) (**Figure 1C**), indicating ALA release is primarily due to $iPLA_2$ activity. The mass of ARA release was ~half that of DHA or ALA and was inhibited similarly by MAFP (39-53%) and VAR (22-49%) (**Figure 1D**), indicating that ARA release is similarly mediated by $iPLA_2$ and $sPLA_2$ activity in rat hearts. Both $iPLA_2$ and $sPLA_2$ mediated PUFA release resulted in patterns that were similar to the baseline patterns (Supplemental Figure 1).

*5.4.1.2 Oxylipins*

$PLA_2$ inhibition of oxylipin formation generally reflected what was observed with their precursor PUFA, with several exceptions. DHA oxylipins were mostly inhibited by VAR (74-88%), but there was some inhibition by MAFP (36-44%) (**Figure 3A**), indicating higher $sPLA_2$ than $iPLA_2$ activity towards DHA oxylipin formation, similar to the effect observed for DHA. Like EPA, the mass of oxylipins derived from EPA was minor (only ~3% as much as DHA or ALA) and was only inhibited by MAFP (47-101%), (**Figure 3B**). Unlike EPA, inhibition of EPA oxylipin formation with VAR was not observed, indicating that EPA oxylipin formation is primarily mediated via $iPLA_2$ activity. Both the mass and the number of oxylipins formed from

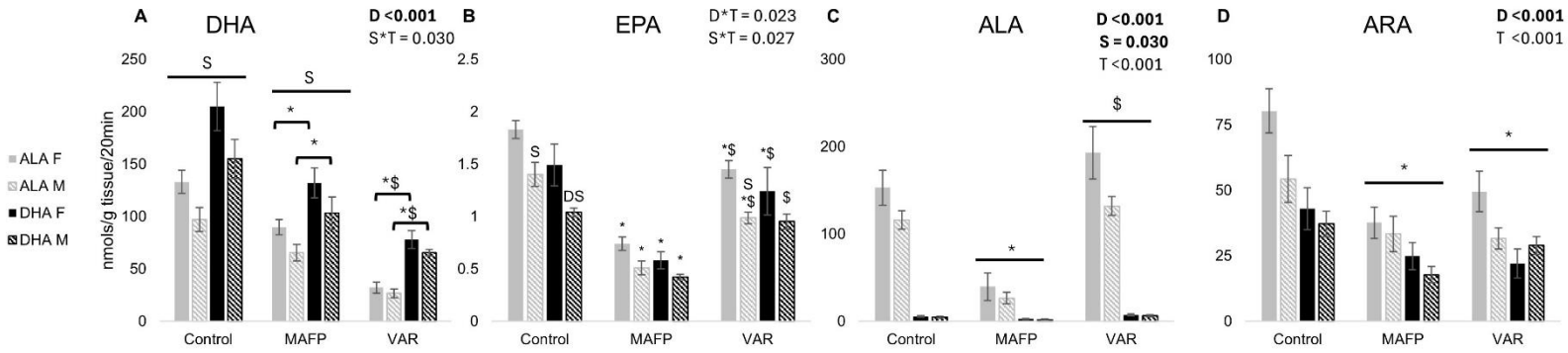


**Figure 5-1.** Effects of sex and dietary ALA and DHA on $PLA_2$ activity towards non-esterified A) DHA; B) EPA; C) ALA and D) ARA release in rat hearts (n=6; mean ± SE).
F, females; M, males. Treatments: Control – incubation without inhibitor, VAR – 0.1 μM of VAR ($sPLA_2$ inhibitor – remaining activity is primarily $iPLA_2$ derived), MAFP – 10 μM of MAFP ($iPLA_2$ inhibitor – remaining activity is $sPLA_2$ derived). Significant results of 3-way repeated measures ANOVA are shown on the upper right corner, with main effects in bold. D, S, and T indicate diet, sex and treatment effects, respectively. For interaction effects, relevant differences as determined by BH post hoc tests were denoted with a * to indicate differences from Control and a $ to indicate differences between inhibitors. When differences are shown above a straight line, the effect was applicable for that treatment group; when above a bracket, the effect was applicable to the groups directly below the ends of the bracket; when above an individual bar the difference was from the same sex or diet group within that treatment.

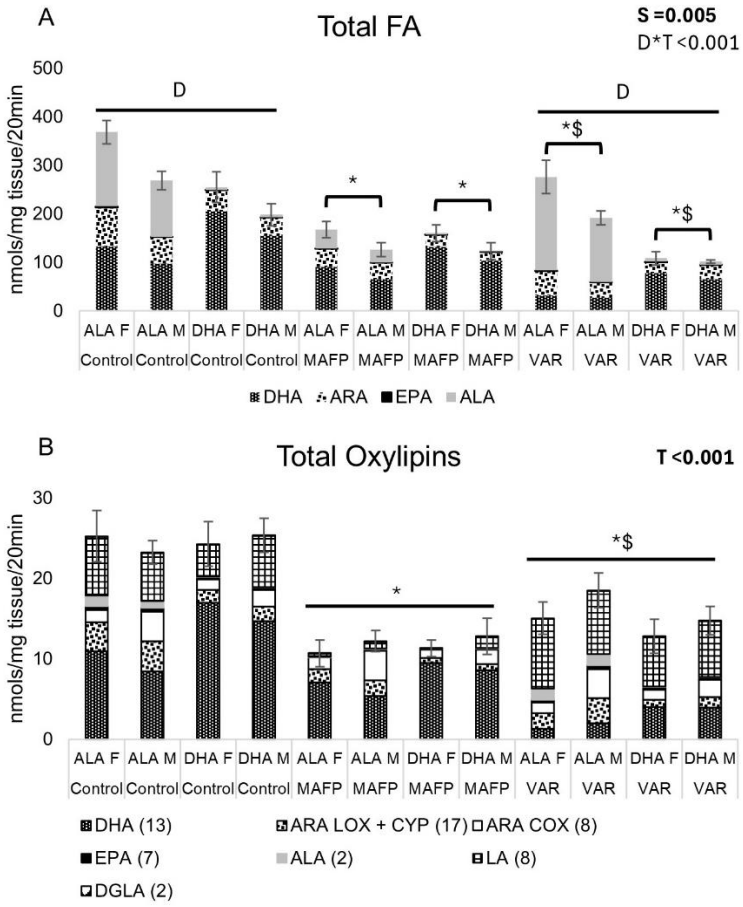


**Figure 5-2.** Effects of sex and dietary ALA and DHA on $PLA_2$ activity towards non-esterified A) total measured PUFA (DHA, EPA, ALA, ARA) and B) total oxylipins in rat hearts (n=6; mean ± SE).
F, females; M, males. Treatments: Control – incubation without inhibitor, VAR – 0.1 μM of VAR ($sPLA_2$ inhibitor – remaining activity is primarily $iPLA_2$ derived), MAFP – 10 μM of MAFP ($iPLA_2$ inhibitor – remaining activity is $sPLA_2$ derived). Significant results of 3-way repeated measures ANOVA are shown on the upper right corner, with main effects in bold. D, S, and T indicate diet, sex and treatment effects, respectively. For interaction effects, relevant differences as determined by BH post hoc tests were denoted with a * to indicate differences from Control and a $ to indicate differences between inhibitors. When differences are shown above a straight line, the effect was applicable for that treatment group; when above a bracket, the effect was applicable to the groups directly below the ends of the bracket. The numbers in parentheses indicate the number of oxylipins included in the total.

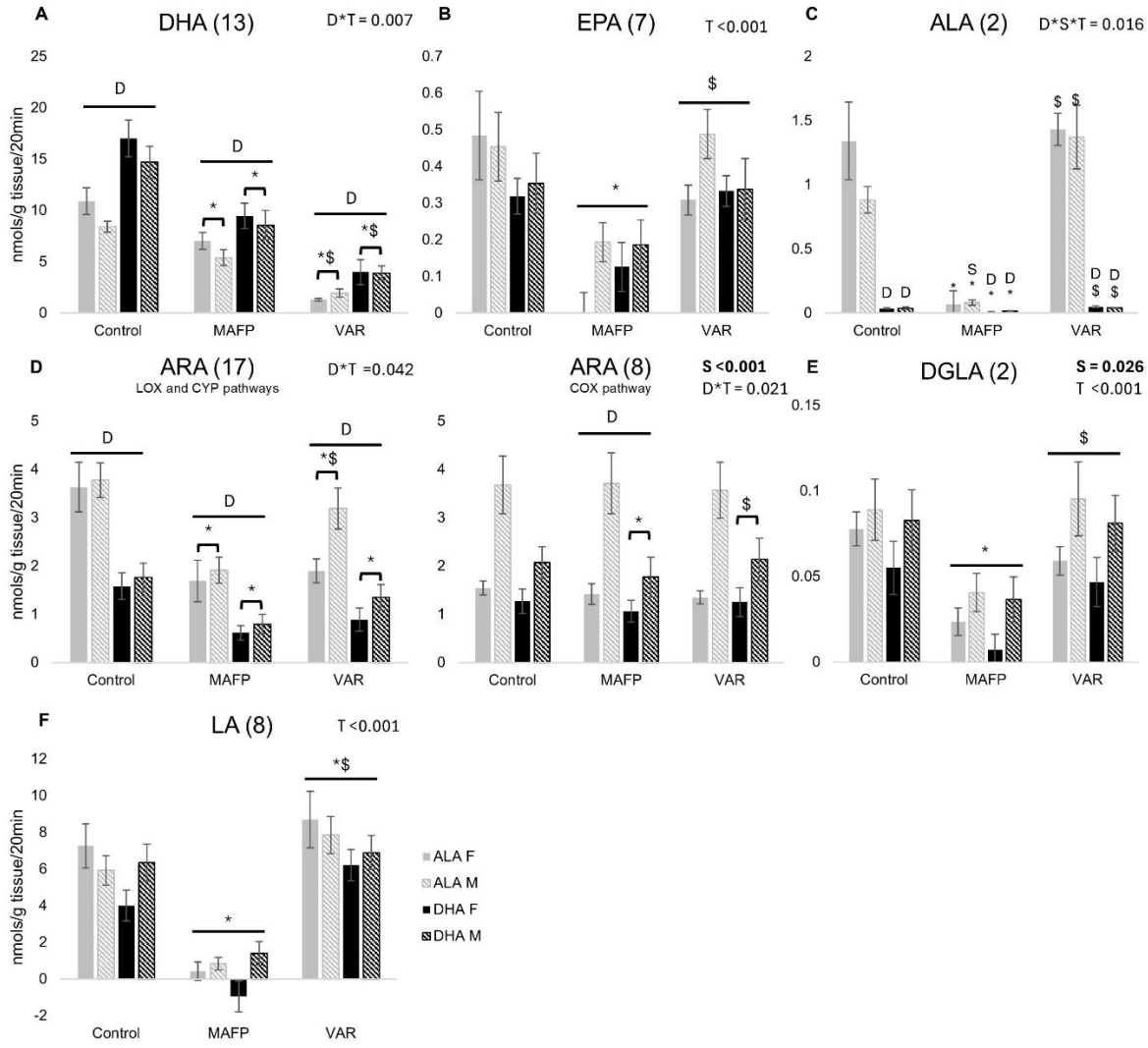


**Figure 5-3.** Effects of sex and dietary ALA and DHA on $PLA_2$ activity towards non-esterified total A) DHA oxylipins; B) EPA oxylipins; C) ALA oxylipins; D) ARA oxylipins; E) DGLA oxylipins; and F) LA oxylipins in rat hearts (n=6; mean ± SE).
F, females; M, males. Treatments: Control – incubation without inhibitor, VAR – 0.1 μM of VAR ($sPLA_2$ inhibitor – remaining activity is primarily $iPLA_2$ derived), MAFP – 10 μM of MAFP ($iPLA_2$ inhibitor – remaining activity is $sPLA_2$ derived). Significant results of 3-way repeated measures ANOVA are shown on the upper right corner, with main effects in bold. D, S, and T indicate diet, sex and treatment effects, respectively. For interaction effects, relevant differences as determined by BH post hoc tests were denoted with a * to indicate differences from Control and a $ to indicate differences between inhibitors. When differences are shown above a straight line, the effect was applicable for that treatment group; when above a bracket, the effect was applicable to the groups directly below the ends of the bracket; when above an individual bar the difference was from the same sex or diet group within that treatment. The numbers in parentheses indicate the number of oxylipins included in the total.

ALA were much lower than from DHA, even in the hearts of rats provided the ALA diet that had similar amounts of ALA and DHA released (Figure 2). ALA oxylipin formation was only inhibited by MAFP (60-95%), similarly to the effect observed with ALA (**Figure 3C**), indicating that $iPLA_2$ is primarily involved in ALA oxylipin formation.

Like ARA compared to DHA, the oxylipin mass formed from ARA was ~half that of DHA but was contributed by ~2 times the number of ARA oxylipins detected, which was mostly because DHA mediates oxylipin formation only via LOX and CYP but not COX. While ARA was similarly inhibited by MAFP and VAR, inhibition of ARA LOX and CYP oxylipins by MAFP (49-61%) was more effective than by VAR (17-48%) (**Figure 3D**), indicating that $iPLA_2$ compared to $sPLA_2$ contributes to more ARA LOX and CYP oxylipin formation. ARA COX oxylipins were analyzed separately as they followed a different pattern and were only significantly inhibited by MAFP (15-16%) and only in the DHA groups (Figure 3D). Dihomo-γ-linolenic acid (DGLA) and LA are not quantifiable using our HPLC-MS/MS method, so no comparisons to their oxylipins are possible. While DGLA oxylipin mass is low, LA oxylipin mass is similar to ARA oxylipins. DGLA (56-87%) (**Figure 3E**) and LA (78-124%) (**Figure 3F**) oxylipins were only inhibited by MAPF, indicating that $iPLA_2$ activity is primarily responsible for these oxylipins being formed.

In our previous investigation we noted that 12-LOX oxylipins were more resistant to inhibition by both MAFP and VAR, so we examined this in the current study. Herein we found that of the 12-LOX products, 12-hydroxy-eicosatetraenoic acid (12-HETE), 12-hydroxy-eicosapentaenoic acid (12-HEPE) and 14-hydroxy-docosahexaenoic acid (14-HDoHE), only the formation of 14-HDoHE was blocked by either inhibitor to some extent (Supplemental Table 2). As these oxylipins did not alter total oxylipin results, they were included in our analysis

described above. Both $iPLA_2$ and $sPLA_2$ mediated oxylipin formation resulted in patterns that were similar to those observed at baseline (Supplemental Figure 2).

### *5.4.2 Diet effects on $PLA_2$*

#### *5.4.2.1 PUFA*

There were main effects of diet on the release of all PUFA except EPA, but the effect differed for each fatty acid. DHA release was higher in DHA groups regardless of treatment or sex (Figure 1A), indicating higher activity of both $iPLA_2$ and $sPLA_2$ towards DHA release with dietary DHA. Interestingly, while DHA release accounted for 73-78% in ALA groups and 87-93% in DHA groups of the total $sPLA_2$ activity (Figure 2A), for $iPLA_2$ activity it made up 21-22% in ALA groups and 67-76% in DHA groups (Figure 2A). For EPA, the only diet effect was in the Control condition, where less EPA was released in male rats provided the DHA diet (Figure 1B). Like for DHA release in the DHA diets, ALA release was higher in ALA groups (Figure 1C), indicating higher activity of $iPLA_2$ towards ALA with dietary ALA (as $sPLA_2$ did not have significant activity towards ALA). ALA release comprised 57-65% of total $iPLA_2$ activity in ALA groups, while it only accounted for 3-6% activity in DHA groups (Figure 2A). The release of ARA also was greater in ALA groups (Figure 1D), indicating both $sPLA_2$ and $iPLA_2$ activities towards ARA are higher in ALA compared to DHA groups. These dietary effects on $PLA_2$ activities for individual PUFA are reflected in the higher total $iPLA_2$ activity in hearts from rats provided ALA diets, which was primarily driven by the much higher ALA released by $iPLA_2$ for rats on the ALA diet, while no overall dietary effects on $sPLA_2$ activity were observed (Figure 2A). These diet effects on PUFA release due to $PLA_2$ activity were similar to the baseline effects (Supplemental Figure 1).

#### *5.4.2.2 Oxylipins*

Although diet effects for oxylipins interacted with sex and/or treatment, the diet effects on oxylipins were similar to the effects on their precursor PUFA. For DHA, there was a diet by treatment interaction, but DHA oxylipin formation was higher with DHA diets in each treatment, in both males and females, indicating that $sPLA_2$ and $iPLA_2$ activity towards DHA oxylipin formation are higher in DHA groups (Figure 3A). As with their PUFA precursors, EPA oxylipin formation was not affected by diet (Figure 3B), and ALA oxylipin formation was higher in ALA groups for all treatments and sex, indicating higher $iPLA_2$ activity towards ALA oxylipin formation in ALA fed rats (as $sPLA_2$ did not have a significant effect on ALA oxylipin formation) (Figure 3C). Dietary effects on LOX and CYP mediated ARA oxylipin formation also reflected ARA release, as ARA oxylipin formation was higher in ALA groups for both treatment groups, regardless of sex (Figure 3D), indicating higher $sPLA_2$ and $iPLA_2$ activity towards LOX and CYP ARA oxylipin formation in ALA fed rats. On the other hand, dietary ALA resulted in higher formation of ARA COX oxylipins only for the MAFP treatment group (Figure 3D). There were no diet effects on $PLA_2$ for DGLA or LA oxylipin formation (Figure 3E-F). In contrast with total PUFA, no $PLA_2$ activities towards total oxylipins were influenced by diet (Figure 2B), reflecting the smaller proportion of ALA oxylipins formed compared to the greater amount of ALA released by $iPLA_2$. As with the PUFA, these diet effects on oxylipin formation due to $PLA_2$ activity were similar to the baseline effects (Supplemental Figure 2).

### *5.4.3 Sex effects on $PLA_2$*

#### *5.4.3.1 PUFA*

$sPLA_2$ but not $iPLA_2$ activity towards DHA release was higher in females than males, indicating that $sPLA_2$ activity is more responsible for the sex difference in total (Control) DHA

release (Figure 1A). EPA release was higher in females than males for the Control group, as well as for iPLA$_2$ activity in ALA groups (Figure 1B). ALA release was higher in females regardless of treatment or diet (Figure 1C). No sex differences were observed for ARA release (Figure 1D). Overall PLA$_2$ activity from all PUFA analyzed (DHA, EPA, ALA and ARA) reflects the individual PUFA, showing that PLA$_2$ activity is higher in females than males (Figure 2A). These sex effects on oxylipin formation due to PLA$_2$ activity were similar to the baseline effects, except for DHA. For baseline DHA, the levels of DHA were only lower in hearts from males provided the DHA diet, while the DHA release by PLA$_2$ activity was significantly lower in males provided both diets but only for sPLA$_2$ activity, further demonstrating the preference for DHA release by sPLA$_2$ in females (Supplemental Figure 1).

*5.4.3.2 Oxylipins*

In contrast to PUFA, there were fewer effects of sex on PLA$_2$ activity towards oxylipin formation, and when they were present, oxylipin formation was higher in males. Notably, ARA COX oxylipins in males were higher than in females (Figure 3D) and when further examining individual ARA COX oxylipins, it was noted that prostaglandin D$_2$ (PGD$_2$) and 15-keto-PGE$_2$ were higher in males only when consuming the ALA, but not the DHA diet (Supplemental Table 2). For 3 other ARA COX oxylipins, 6-keto-PGF$_{1\alpha}$, PGE$_2$, 11β-PGE$_2$, as well as the total ARA COX oxylipins, a similar trend (P=0.08 – 0.10) towards such an interaction was observed. To further investigate this finding, male/female ratios of ARA COX oxylipins were calculated; this analysis indicated a significant diet effect with log$_2$ male/female ratios of 1.1-1.4 when provided the ALA diet, compared to 0.5-0.6 while on the DHA diet (log$_2$ ratios greater than 0 indicate higher ARA COX oxylipins in males), confirming a diet/sex interaction (**Figure 4**). The only other sex effect on oxylipin formation was observed in DGLA oxylipins, which also were higher

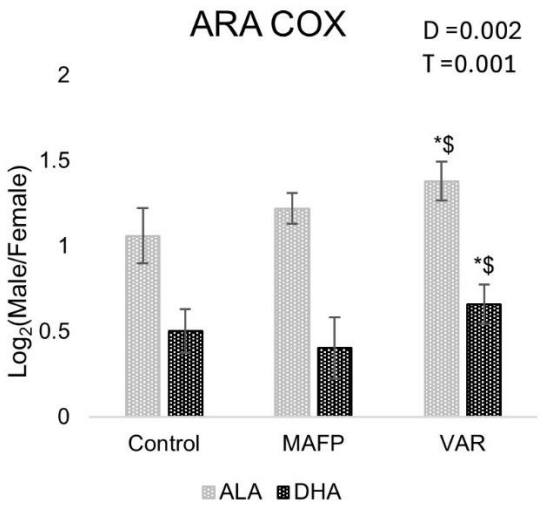


**Figure 5-4.** Effect of dietary ALA and DHA on the fold change in male/female ARA COX oxylipins in rat hearts (n=8, mean ± SE).
Treatments: Control – incubation without inhibitor, VAR – 0.1 μM of VAR ($sPLA_2$ inhibitor – remaining activity is primarily $iPLA_2$ derived), MAFP – 10 μM of MAFP ($iPLA_2$ inhibitor – remaining activity is $sPLA_2$ derived). The fold change was calculated for each oxylipin (n=8). Significant results of 2-way repeated measures ANOVA are shown on the upper right corner. D and T indicate diet and treatment effects, respectively. For treatment effects, relevant differences as determined by BH post hoc tests were denoted with a * to indicate differences from Control and a $ to indicate differences between inhibitors.

in males than females (Figure 3E). Overall, in contrast to the overall higher PUFA release in females, there were no sex differences in $PLA_2$ related total oxylipin formation (Figure 2B).

Interestingly, there were many differences in these sex effects on oxylipin formation (Figure 3) when compared to baseline oxylipin data (Supplemental Figure 2). DHA, EPA and LA oxylipins at baseline were higher in females, compared to no sex effects for $PLA_2$ mediated oxylipin formation. ALA oxylipins at baseline and formed via $PLA_2$ activity were both higher in females, and ARA LOX and CYP oxylipins at baseline and formed after stimulation similarly had no sex effects. ARA COX and DGLA oxylipins at baseline were higher in females, but formation of these oxylipins was higher in males.

### *5.5 Discussion*

The findings in this study indicate that $sPLA_2$ activity is selective towards DHA and its oxylipins, with some activity towards EPA, ARA and ARA oxylipins. In contrast, $iPLA_2$ causes the release of all PUFA and oxylipins that were analyzed, with the primary PUFA released being highly influenced by diet. Diet impacted activities of both $sPLA_2$ and $iPLA_2$, as the DHA diet resulted in more activity towards DHA and its oxylipins, and the ALA diet resulted in more activity towards ALA and its oxylipins (only for $iPLA_2$) and ARA and their oxylipins. While PUFA release was generally higher in females, there were fewer sex differences in oxylipin formation associated with $PLA_2$ activity, and in contrast to PUFA, oxylipins with sex differences were higher in males. Interestingly, the higher ARA COX oxylipin formation in males was modulated by dietary DHA compared to ALA. These findings may help explain dietary effects and sex-based differences in the incidence and prevalence of cardiovascular disease and may have implications for sex-based dietary guidelines and the development of pharmaceuticals in prevention and treatment of cardiovascular disease.

The increase in $PLA_2$ activity towards DHA and its oxylipins with a DHA diet, and the increased activity towards ALA and ARA and their oxylipins with an ALA diet are novel findings. These results are consistent with previously observed changes in phospholipid fatty acid and oxylipin compositions in response to dietary ALA compared to DHA (Ferdouse et al., 2019a; Leng et al., 2018; Mendonça et al., 2018), and suggest that these effects are at least partly driven by $PLA_2$ activity. The reduced $iPLA_2$ activity in the DHA diet herein is consistent with a previous study showing that fish oil decreases $iPLA_2$ activity (Smesny et al., 2014). Fish oil also increased gene expression of most $sPLA_2$ isoforms but decreased $sPLA_2$ IIA (Rudkowska et al., 2013). This is potentially consistent with an overall no effect of diet on total $sPLA_2$ activity, as $sPLA_2$ IIA is highly expressed in the rat heart (Manson et al., 2023). In addition, an ALA deficient diet was shown to decrease $iPLA_2$ and increase $sPLA_2$ activity, protein, and/or mRNA in rat brain when compared to a diet with adequate ALA (Rao et al., 2007) or a diet with primarily DHA (Tyagi et al., 2014). Since we do not have an ALA deficient diet, these findings cannot be directly compared with those herein, but our data suggest a decrease in $iPLA_2$ activity could be due to low levels of ALA and EPA, which are primarily released by $iPLA_2$. The increase in $sPLA_2$ activity may be an adaptive mechanism, given its high selectivity for DHA, or may reflect an increase in activity towards ARA in that scenario. Thus, activity depends on the fatty acid substrate(s) analyzed, and our study highlights the nuances of different $PLA_2$ activities in relation to dietary effects on endogenous phospholipid fatty acids. Further studies are necessary to evaluate the effects of other dietary fatty acids such as EPA and LA.

Our findings indicate that $sPLA_2$ and $iPLA_2$ activity towards several individual and total PUFA are higher in females, which was different than the lack of sex effects on total oxylipins and the higher ARA COX and DGLA oxylipins in males. Although, to the best of our knowledge,

there are no studies of sex differences in $iPLA_2$, a previous study reported higher $sPLA_2$ protein in females in one study (Boekholdt et al., 2005), but not in another (Mallat et al., 2007), which may be due to differences in age (Ward et al., 2018) or disease state (Pop et al., 2013). Since discrepancies in sex effects (including diet by sex interactions) in esterified PUFA and non-esterified oxylipins are known (L. G. J. Cayer et al., 2019; Ferdouse et al., 2019a; Gabbs et al., 2021; Leng et al., 2017, 2018; Mendonça et al., 2018), we hypothesized that non-esterified PUFA would better reflect oxylipin data, and $PLA_2$ activity to be potentially responsible for such discrepancies. However, this does not appear to be the case, and may be due to differences in oxylipin degradation in males and females (Pace, Sautebin, et al., 2017), which warrants further investigation. While a strength of the current analysis is that release of 4 major PUFA and the formation of 6 groups of oxylipins derived from PUFA were accounted for, there are even more fatty acids and oxylipins that could influence total activity. Given that sex differences in $PLA_2$ activity vary depending on which fatty acid or oxylipin is analyzed, more research is needed in this understudied topic of sex differences in $PLA_2$ activity.

A notable sex difference herein was the higher ARA COX metabolite production in stimulated male hearts. This is consistent with previous research showing higher production of ARA COX metabolites in stimulated human male neutrophils (Pace, Rossi, et al., 2017), higher $PGE_2$ production by male stimulated macrophages (Upmacis et al., 2022), and the possibly greater effectiveness of aspirin, a cyclooxygenase inhibitor, in males than females (Pace, Sautebin, et al., 2017). Interestingly, dietary DHA compared to ALA resulted in lower male/female ARA COX oxylipins in the stimulated heart, suggesting that DHA has a stronger effect on reducing ARA COX oxylipins in males. Similarly, variations in dietary DHA effects according to sex have also been shown as DHA supplementation upregulated wound healing and

PPARα activation genes only in men's monocytes (So et al., 2024), and EPA reduced platelet aggregation in men whereas only DHA did in women (Phang et al., 2013). Due to the roles of ARA COX metabolites in the pathogenesis of cardiovascular disease (Gerges & El-Kadi, 2022; Pace, Sautebin, et al., 2017), these findings may be relevant to the complex sex-based differences in incidence, prevalence and prognosis of cardiovascular disease, for which sex differences vary according to specific diseases (Bergami et al., 2022; Regitz-Zagrosek & Gebhard, 2023; Roeters van Lennep et al., 2023; Suman et al., 2023). Therefore, our results suggest DHA may be a more effective therapeutic in acute cardiovascular events in males compared to females and this warrants further investigation. Notably, this study was conducted with minimally stressed and healthy animals, which should be considered in further analysis.

The current results and previous studies (Manson et al., 2023; Miki et al., 2013; Moon et al., 2016; Murase et al., 2016) show $PLA_2$ mediated oxylipin formation does not always reflect PUFA release. This is important because there is a general belief that $PLA_2$ enzymes could be potential drug targets (Hidalgo et al., 2024; Jantan et al., 2014; Shukla et al., 2015; Y. Zhang et al., 2023) due to their ability to reduce ARA release and consequently their pro-inflammatory oxylipins (Gabbs et al., 2015; Khan & Ilies, 2023; Y. Zhang et al., 2023). However, we show herein that Varespladib decreases DHA and its oxylipins (which are thought to have protective anti-inflammatory effects (Cambiaggi et al., 2023; Duan et al., 2021; Gabbs et al., 2015), has no effect on ARA COX oxylipins and only causes a small reduction in some LOX and CYP ARA oxylipins, despite a 50% reduction in ARA release. This may help explain why Varespladib failed phase-III clinical trials (Nicholls et al., 2014), reenforcing the importance of considering the impact of inhibitors on both oxylipin formation and fatty acid release.

The findings regarding $sPLA_2$ activity and impact on fatty acid release and oxylipin formation in the heart closely align with several previous studies and also may help explain some discrepancies in the literature. First, human $sPLA_2$ V (in-vitro) (Hayashi et al., 2021), mouse lymphoid tissue $sPLA_2$ IID (Miki et al., 2013), mouse recombinant $sPLA_2$ IIF (Yamamoto et al., 2015), mouse colon and sperm $sPLA_2$ X (Murase et al., 2016) and our previous study on rat heart $sPLA_2$ activity (Manson et al., 2023) all showed a preference for DHA release with an impact on DHA oxylipin formation (Manson et al., 2023; Miki et al., 2013; Murase et al., 2016). While most studies support a role of $sPLA_2$ in ARA release (Hayashi et al., 2021; Manson et al., 2023; Miki et al., 2013; Murase et al., 2016), ARA oxylipins are not always impacted (Manson et al., 2023; Miki et al., 2013; Murase et al., 2016), also consistent with current findings. Second, while some studies support a role of $sPLA_2$ enzymes on EPA release and their oxylipin formation (Hayashi et al., 2021; Miki et al., 2013; Murase et al., 2016), others do not (Manson et al., 2023). The current study provides evidence this could be due to variations in the substrate lipid composition, as endogenous EPA and its oxylipin concentrations are typically very low. Third, while there is a suggestion that human $sPLA_2$ V has *in vitro* activity towards LA (Hayashi et al., 2021; Mouchlis et al., 2018), our results on LA oxylipins do not suggest this, although fatty acid and oxylipin data are not always congruent, so this remains to be elucidated.

Our findings on $iPLA_2$ having activity towards all PUFA and the formation of their oxylipins closely matches a cardiac myocyte specific $iPLA_2\gamma$ knockout study, where ARA, LA, and DHA oxylipins were affected (other oxylipin classes were not analyzed) (Moon et al., 2016). Human $iPLA_2$ has a preference for EPA release *in vitro* (Hayashi et al., 2021), but the data herein highlight the very low mass of EPA relative to other PUFA, placing the importance of EPA *in vivo* in context. Our findings are similar to our previous study, which, to the best of our

knowledge, is the only one to demonstrate the importance of $iPLA_2$ activity towards ALA release and oxylipin formation (Manson et al., 2023). Some studies suggest that $iPLA_2$ is a major mediator of DHA release in brain (Cheon et al., 2012; Strokin et al., 2007) and liver (Deng et al., 2016). This difference from the current findings of DHA as a less important fatty acid released by $iPLA_2$ could be due to lack of comparison between $sPLA_2$ and $iPLA_2$, as well as tissue differences in $sPLA_2$ expression and DHA concentrations. Indeed, most $iPLA_2$ activity was directed towards DHA in the DHA group, whereas ALA was the major fatty acid released in the ALA group, indicating that the diet and/or substrate availability are an important consideration in determining the primary fatty acid released by $iPLA_2$.

In conclusion, a DHA diet resulted in more activity of $sPLA_2$ and $iPLA_2$ towards DHA and its oxylipins, whereas an ALA diet resulted in more activity of $iPLA_2$ towards ALA and its oxylipins, and more activity of both $iPLA_2$ and $sPLA_2$ towards ARA and its oxylipins. $sPLA_2$ activity was selective towards DHA and its oxylipins regardless of diet and sex, with some activity towards ARA and possibly EPA, with relatively less impact on their derived oxylipins. $iPLA_2$ appears to be less selective, and more dependent on diet/fatty acid composition. PUFA release by $sPLA_2$ and $iPLA_2$ were both generally higher in females, while sex differences in oxylipin formation was only higher in males for ARA COX and DGLA oxylipins. These sex differences and the attenuation of the sex difference in ARA COX oxylipins by dietary DHA may have implications in the development of sex-based dietary omega-3 guidelines in prevention and treatment of cardiovascular disease.

# Chapter 6: Conclusion

## *6.1 Overall thesis discussion*

This thesis highlights and confirms that oxylipins can be used to develop new methods and demonstrates their application to answer key questions in lipid metabolism. These findings confirm the overarching hypothesis that oxylipins can be used to provide novel insights to the field. Examples include guiding optimal dietary omega-3 fatty acid intake, such as ALA, with a comprehensive comparison to DHA; aiding in the development of new drugs by providing a thorough assessment of a drug's potential; in addition to answering fundamental questions in the understanding of lipid metabolism. These questions are all visited through the lenses of oxylipin analysis, which was not available until recently, by advances in mass spectrometry analysis, which makes feasible simultaneous analysis of the oxylipin profile. Since oxylipins mediate the effects of fatty acids and do not necessarily reflect fatty acid data (Cayer et al., 2019; Ferdouse et al., 2019a, 2019b; Leng et al., 2017, 2018; Mendonça et al., 2018; Pauls et al., 2020; Penner et al., 2021), this provides an important avenue to better understand and answer questions in lipid metabolism which have important value in physiology and pathophysiology due to the relevance of oxylipins' biological functions (Aukema & Ravandi, 2023; Gabbs et al., 2015).

Chapter 2 shows how non-esterified DHA/ARA fatty acids and oxylipins can be used to determine the dietary ALA requirement, which remains unknown for humans and rodents (European Food Safety Authority, 2010; Hamilton & Hogan, 1995; Institute of Medicine, 2005). Previous studies had indicated a lower ALA requirement by analysis of esterified DHA alone (Bourre et al., 1989; Couëdelo et al., 2022; Domenichiello et al., 2017; Gibson et al., 2013; Tu et al., 2010; Whelan et al., 1991). However, since oxylipins are the biologically active lipid metabolites that mediate the functions of fatty acids, with non-esterified oxylipins and fatty acids

binding to their cognate receptors to elicit a biological response (Aukema & Ravandi, 2023; Gabbs et al., 2015), analysis of these compounds is expected to better reflect their biological functions. In addition, taking ARA into account when assessing the ALA requirement is more encompassing of its overall health benefits, as DHA and ARA have generally opposed effects (Gabbs et al., 2015). Since both DHA and ARA oxylipins are influenced by dietary ALA in a way that these effects reach a plateau with a certain level of dietary ALA intake, this indicates that dietary ALA intake beyond the plateau is unlikely to provide additional health benefits. Analysis of several tissues, including serum, liver, kidney and brain was a strength that allowed for comparison of which tissues would be expected to better reflect the dietary ALA requirement. Among these tissues, brain appears to be prioritized for ALA metabolism, thus, not being a useful marker of dietary ALA intake. Analysis of serum, liver and kidney of male rats and male and female mice generated breakpoints ranging from 0.2-0.75g ALA/100g of diet. Although we had suggested a requirement around the highest and more consistent result of around 0.7g ALA/100g of diet, a better estimate could be achieved with the average of our estimates which would be 0.54 ± 0.04g of ALA/100g of diet 95% CI [0.45, 0.63]. This was concluded after having a more comprehensive picture that also included data from chapter 3. Thus, the objective of determining the dietary ALA requirement was achieved through the development of a new method and the hypothesis that by analyzing oxylipins this estimate is higher than previous estimates was confirmed, although non-esterified fatty acid data also suggested these can be used.

In chapter 3, we further expanded our analysis on the dietary ALA requirement, to understand what dosage of dietary DHA would be bioequivalent to the dietary ALA requirement. Although ALA is the essential omega-3 fatty acid, DHA alone can meet this requirement

(Carlson et al., 2019; De Meijer et al., 2010; Le et al., 2009, 2013; Ling et al., 2012). Even though this is an important topic that is highly debated in the lipid field, only one paper had previously aimed to address bioequivalence of ALA and DHA, and it was by analysis of fetal accretion of DHA in the brain (Greiner et al., 1997). Brain is a tissue we previously showed to be prioritized for ALA metabolism, and in this study, the growing rat would likely have different requirements than the fetus at peak brain development, and the DHA bioequivalence was not compared to the dietary requirement of ALA. The study in chapter 3 was fine tuned for determination of the ALA requirement based on previous findings, and considered the heart, in addition to serum, liver and brain, and females in addition to males. The dietary ALA requirement estimate was based on analysis of liver and serum of males and females. This was since the heart appears to be prioritized, even though it still has a higher ALA requirement than brain, and there were no conclusive sex differences in our studies. The average ALA requirement found was of 0.55g ALA/100g diet (1.3% energy), which is remarkably consistent with the study in chapter 2. In both studies, this requirement is slightly higher than what is provided by the AIN93G (i.e. ~0.5g ALA/100g diet), the standard rodent diet for growing rodents (Reeves et al., 1993). Based on this, the dose of dietary DHA that was bioequivalent to this dietary ALA requirement was 0.12g DHA/100g diet (0.3% energy), indicating that dietary DHA is about 5x more powerful than ALA in meeting omega-3 requirements in the growing rat. This confirmed the hypothesis we could apply this method to estimate the bioequivalence of DHA to the dietary ALA requirement and reached the objective of defining this bioequivalence.

In chapter 4 another method is presented. This is a method for evaluation of $PLA_2$ activity in the rat heart, while simultaneously considering the impact of $iPLA_2$ and $sPLA_2$ activities by inhibitor use on fatty acids and oxylipins. This research highlights the first method

to assess activity of $PLA_2$ ex vivo, with advantages such as better mimicking *in vivo* conditions by use of endogenous lipid composition and enzyme expression, over commonly used in-vitro models with synthetic or labelled substrates and recombinant $PLA_2$ enzymes (Astudillo et al., 2023; Mouchlis et al., 2019). Most importantly, it allows assessment of changes in the oxylipin profile, in addition to PUFA, which even in $PLA_2$ research, do not always mimic the fatty acid results (Manson et al., 2023; Miki et al., 2013; Moon et al., 2016; Murase et al., 2016). This comprehensive assessment of PUFA and oxylipin profiles, allowed better determination of $PLA_2$ specificity for PUFA release, which was very poorly understood, particularly with respect to omega-3 fatty acids, and encompassing the impact on oxylipin classes that were never explored before. These findings raised important concerns for the use of $PLA_2$ inhibitors as potential drug targets. We showed that $sPLA_2$ has specificity towards DHA and its oxylipins, while it does not impact ARA oxylipins to the same extent as it impacts ARA. In addition, $iPLA_2$ was less specific and was primarily involved in EPA and ALA release and their oxylipin formation, which was unknown when this study began. While this manuscript was under production, Dennis and colleagues showed $iPLA_2$ preference for EPA release (Hayashi et al., 2021); however, our work remains the only one to demonstrate $PLA_2$ activity towards ALA and its oxylipins to the best of my knowledge. Therefore, by developing a new method we reached the objective of determining how $PLA_2$ activity impact fatty acid release and oxylipin formation in rat hearts and proved the hypothesis that different $PLA_2$ types would have distinct activities towards PUFA release and oxylipin formation in rat hearts.

In chapter 5 the method presented in chapter 4 was applied to understand the nuances of sex and dietary ALA and DHA on $iPLA_2$ and $sPLA_2$ activity mediating fatty acid release and oxylipin formation in the rat heart. Since our previous esterified fatty acid data did not reflect

oxylipin analysis (Ferdouse et al., 2019a), we hypothesized and tested the theory that $PLA_2$ could be responsible for differences in these profiles, particularly diet by sex interactions found. However, this hypothesis was not proven to be true as $PLA_2$ activity did not reflect the pattern observed in diet by sex interactions in our studies. Although other studies had shown diet (Y. Liu et al., 2014; Rao et al., 2007; Rudkowska et al., 2013; Smesny et al., 2014; K.-P. Su et al., 2018; Vincentini et al., 2011) and sex (Boekholdt et al., 2005; Keeting et al., 1998; Mallat et al., 2007; Pop et al., 2013; Stone et al., 2019; Ward et al., 2018; Xu et al., 2017) to influence $PLA_2$, others had not looked into how diet and sex would impact $PLA_2$ activity by measuring release of specific PUFA and oxylipins, in a comprehensive analysis. Gene expression, protein levels, or commercially available $PLA_2$ activity assays do not provide insights on enzyme preference and specificity. Thus, in addition to further confirming our findings in chapter 4, this work highlights important diet and sex effects on $PLA_2$ activity, which vary according to fatty acid or oxylipin class studied. Diet impacted both $sPLA_2$ and $iPLA_2$ activities, with a DHA rich diet resulting in more activity towards DHA and its oxylipins, while an ALA rich diet resulted in more activity towards ALA and ARA and their oxylipins. Diet impacted whether $sPLA_2$ had significant activity towards EPA or not, which helps understand discrepancies in previous studies in the field. Also, the primary fatty acid released by $iPLA_2$ was highly dependent on diet. In contrast to $PLA_2$ activity towards PUFA release being generally higher in females than males, few sex effects were found on oxylipins, and when they were present, they were higher in males. An unexpected finding of this work was the higher ARA COX oxylipin formation in stimulated male hearts, which was attenuated by dietary DHA. These findings shed new light on diet and sex effects on the role of $PLA_2$ activity in oxylipin biology, contributing meaningfully to the fundamental understanding of lipid metabolism. Although the hypothesis was not confirmed in this study, we

reached our objective of determining sex and dietary ALA and DHA influence on $PLA_2$ activity and if these could explain previous diet by sex interactions observed.

### *6.2 Implications, limitations and future directions*

The findings in this thesis have important implications and expand knowledge in the field in addition to these studies' limitations generating insightful questions for further investigation. First, the studies in chapters 2 and 3 estimate the dietary ALA requirement in growing rats. Collectively, data of both studies suggest an average ALA requirement of 0.54 ± 0.04 g of ALA/100g diet 95% CI [0.47, 0.62]. Since the AIN93G, which is the standard rodent diet contains approximately 0.5 g of ALA/100g diet (Reeves et al., 1993), this suggests that this diet may need reformulation to meet the dietary ALA requirement. Placed in the context of dietary requirements, 0.54 is the average dietary requirement, which means it would be expected to meet the needs of about half of a rodent population (Institute of Medicine, 2005), thus, some animals could have higher requirements that would not be met by the AIN93G, particularly between different animal strains. Our studies suggests that the upper CI of 0.62g of ALA/100g of diet would be sufficient to meet the requirements of most animals, and this could reduce inconsistencies in research findings, such as on the benefits of increasing dietary ALA. The method developed could potentially be applied to human populations to test its feasibility; however, it would need to be in a population with low dietary ALA intake that does not consume dietary EPA and DHA. Importantly, there are some potentially deficient pediatric populations (Almasri et al., 2023; Cardino et al., 2024; Vyncke et al., 2012).

More research is necessary on the topic of understanding the dietary ALA requirement. Although the studies herein contained a high number of animals, this represents a relatively small sample size and only one rat and mouse strain. This comes as a limitation in contrast with

the whole rodent population, requiring further research to generate more precise estimates of the dietary ALA requirement. Further research should include males and females since our results are limited due to the inconclusive sex differences on the ALA requirement. It would be advisable to include more animals, particularly in the groups with dietary ALA intake around the breakpoint for improved estimates. Since we only used one concentration of dietary LA in all the diets, this limits extrapolation of the findings to different doses of LA. Since the concentration of LA could influence the dietary ALA requirement, different doses of LA should be further explored to determine the optimal omega-6/omega-3 ratio for dietary intake. Additionally, since the tissue with the highest ALA requirement would reflect what would be the optimal ALA intake, other tissues should be explored to determine if the requirement may not be underestimating the needs of a tissue with higher ALA requirement. Furthermore, it would be important to assess different life stages, as the research conducted for this thesis was limited to growing rats. Another point is that although the hypothesis that non-esterified DHA/ARA oxylipins generate higher breakpoints than esterified fatty acids appear to be true, we ended up finding that non-esterified fatty acids lead to generally similar results, but generally lower, to oxylipin analysis. This led to a further hypothesis that oxylipins lead to higher breakpoints than non-esterified fatty acids, and non-esterified fatty acids lead to higher breakpoints than esterified fatty acids. Further research is needed to answer this question. Additionally, oxylipin enantiomers can be produced enzymatically and non-enzymatically. Since different enantiomers can have distinct functions, further analysis to test whether non-enzymatic oxylipin production would affect the findings herein would be warranted (Fu et al., 2024).

In chapter 3, in addition to revisiting the dietary ALA requirement, the bioequivalence of DHA to the dietary ALA requirement was determined in growing rats. A DHA dose of 0.12 g of

DHA/100g diet was bioequivalent to the dietary ALA requirement of 0.55g of ALA/100g of diet, suggesting that DHA is roughly 5x more potent in comparison with dietary ALA. In addition, we found that any intake of DHA above the dietary ALA requirement is likely to generate an additional effect on DHA/ARA fatty acids and oxylipins beyond the maximal effect that can be achieved with dietary ALA alone. However, as a limitation we cannot affirm if this would result in additional health benefits, and this should be further investigated. This finding has potential implications as it is consistent with research on the health benefits of dietary ALA, which has either no effect or a small one, while EPA and DHA appear to have an effect on cardiovascular disease risk (Abdelhamid et al., 2020; Jiang et al., 2022; Wang et al., 2006). Importantly, research on health benefits of ALA, EPA, and DHA is inconsistent, and these discrepancies could arise from variation in baseline intake of omega-3 fatty acids (Bae et al., 2023), which should be considered in future studies.

It remains to be clarified if there are any sex differences on the DHA bioequivalence to dietary ALA requirement, as our results are limited in reaching a conclusion, showing a potential difference that was not significant, and this could have been due to variability in research results or lack of power to detect a small difference. In addition, females had a stronger response to DHA dose in increasing DHA oxylipins in the heart. This suggests a difference in DHA dosage according to sex may be important to achieve health benefits in males and females, which remains to be further elucidated. Additionally, and similar to the limitations presented for the ALA requirement research, the DHA bioequivalence should be examined in relation to the dietary LA content, in other tissues and life cycles to determine if these factors would influence the DHA bioequivalence to dietary ALA. The method presented could also be used to answer other questions in lipid metabolism. Examples include determining the bioequivalence of dietary

EPA to ALA and DHA; determining the dietary LA requirement; or establishing requirements in different life cycles.

The results from chapter 4 on $iPLA_2$ and $sPLA_2$ activity for fatty acid release, which were further confirmed in the study on chapter 5, have important implications for drug development. One of the inhibitors used in these studies, Varespladib, an $sPLA_2$ inhibitor, had previously advanced into phase-III clinical trials for cardiovascular disease (Nicholls et al., 2014). Instead of improving cardiovascular disease, Varespladib showed an increased risk of myocardial infarction, stroke and cardiovascular mortality. The rationale for use of Varespladib was due to inhibition of ARA, which would then presumably lead to reduction of ARA oxylipins. In our study we show that ARA oxylipins, particularly pro-inflammatory ones were not altered, while DHA and its oxylipins, which have anti-inflammatory beneficial functions were decreased by Varespladib. Thus, these results provide a likely reason why Varespladib was linked to an increased risk of myocardial infarction, stroke and cardiovascular mortality. This research highlights how assumptions on the oxylipin profile should not be made from fatty acid data. Our results also suggest that either $sPLA_2$ or $iPLA_2$ inhibitors are unlikely to be potential successful drug targets, given their involvement in the release of omega-3 oxylipins. However, since a limitation of the study was that specific inhibitors were unavailable commercially or did not significantly inhibit fatty acid release or oxylipin formation, further research is needed to elucidate the role of specific $PLA_2$ isoforms in relation to our findings.

Lastly, the results from chapter 5 highlight the nuances of diet and sex effects on $iPLA_2$ and $sPLA_2$ activity mediating fatty acid release and oxylipin formation. This has implications in drug development, as discussed above, in the fundamental understanding of lipid metabolism, but also in cardiovascular physiology and pathophysiology. Importantly, the finding that ARA

COX oxylipins upon stimulation are higher in males could be related to the complex sex-based differences in the incidence and prevalence of cardiovascular disease (Bergami et al., 2022; Regitz-Zagrosek & Gebhard, 2023; Roeters van Lennep et al., 2023; Suman et al., 2023). Since DHA attenuated this sex-difference, this also offers potential avenues for the development of sex-based nutrition guidelines in the prevention and treatment of cardiovascular disease. However, since this is an ex vivo study in healthy rat hearts, it should be further elucidated whether these results would reflect effects seen in any specific type of cardiovascular disease and whether DHA would be more beneficial in the treatment of males.

The studies in $PLA_2$ activities also have limitations to be addressed in future studies. Future research should focus on targeting different specific isoforms, which were not explored herein. Individual isoforms can be studied and $cPLA_2$ activity in relation to diet and sex could be evaluated, but this would probably have to be conducted in a tissue that highly expresses $cPLA_2$ enzymes. In addition, further research on the understanding of how sex and dietary fat impact $PLA_2$ activity would be important since we here show it can influence $PLA_2$ activity findings. Further analysis can be conducted to evaluate complete dietary effects on fatty acids, which although contained the main understudied PUFA, it did not include LA and other remaining PUFA, or MUFA and SFA. However, it should be noted that MUFA and SFA do not form oxylipins. It would be important to test other dietary approaches that could have a substantial impact on $PLA_2$ activity, such as a high EPA diet, given the small proportion of EPA *in vivo* to other fatty acids and oxylipins without dietary EPA. Since $PLA_2$ does not seem to be responsible for discrepancies in fatty acid and oxylipin data, particularly with respect to sex effects and diet by sex interactions, further research is required to understand if differences in oxylipin turnover between males and females could explain these research findings.

In summary, the studies presented in this thesis focus on development of methods that aid in the understanding of how diet and sex impact metabolism of oxylipins, contributing to the fundamental understanding of lipid metabolism. The findings highlight how these methods using oxylipins can be effectively applied to advance the development of dietary intake guidelines and drugs targeting oxylipins, achieving this thesis overarching objective. In addition, this research has a potential impact in the development of therapies involving diet and sex for cardiovascular health.

# Chapter 7: References


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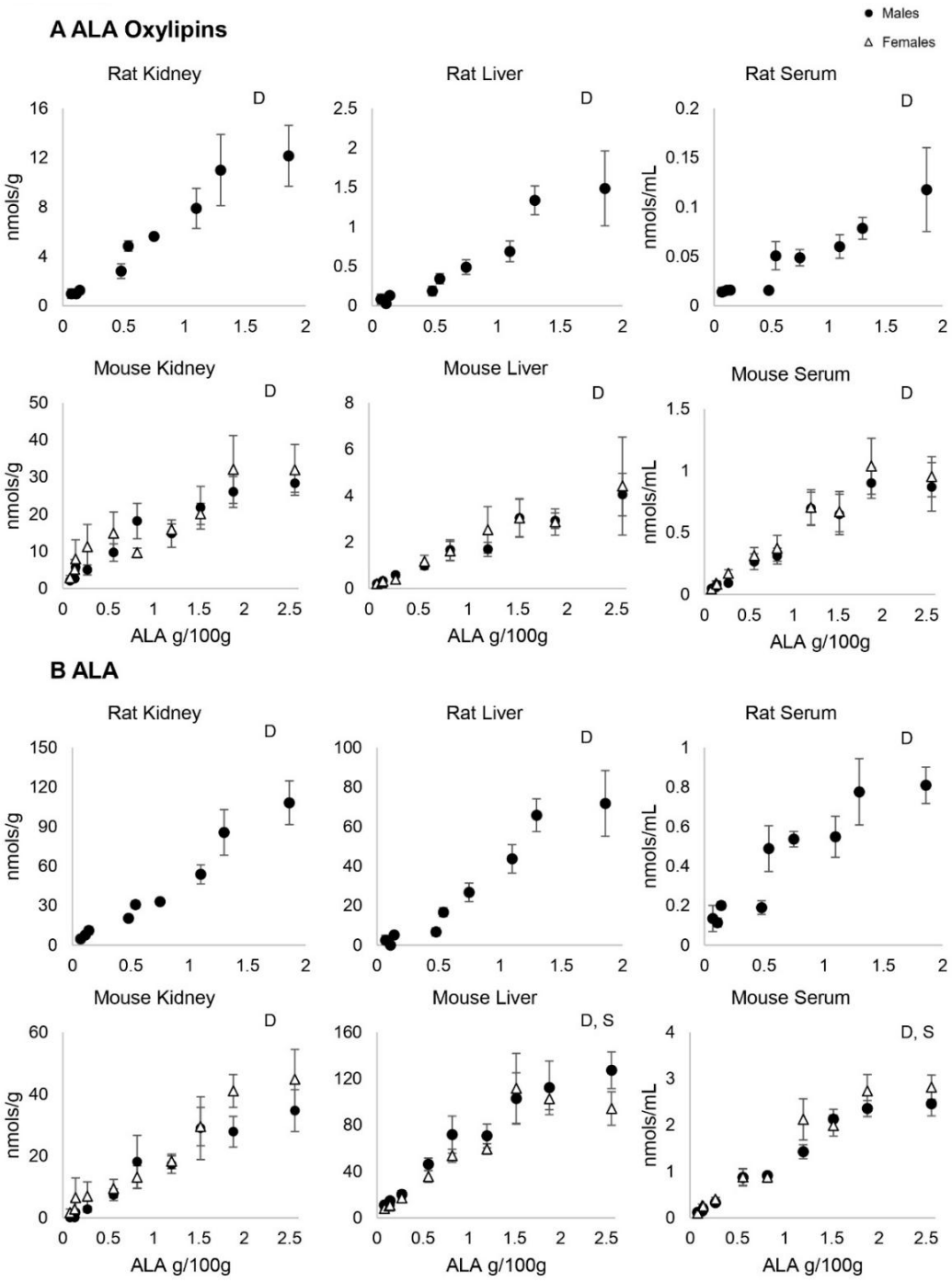
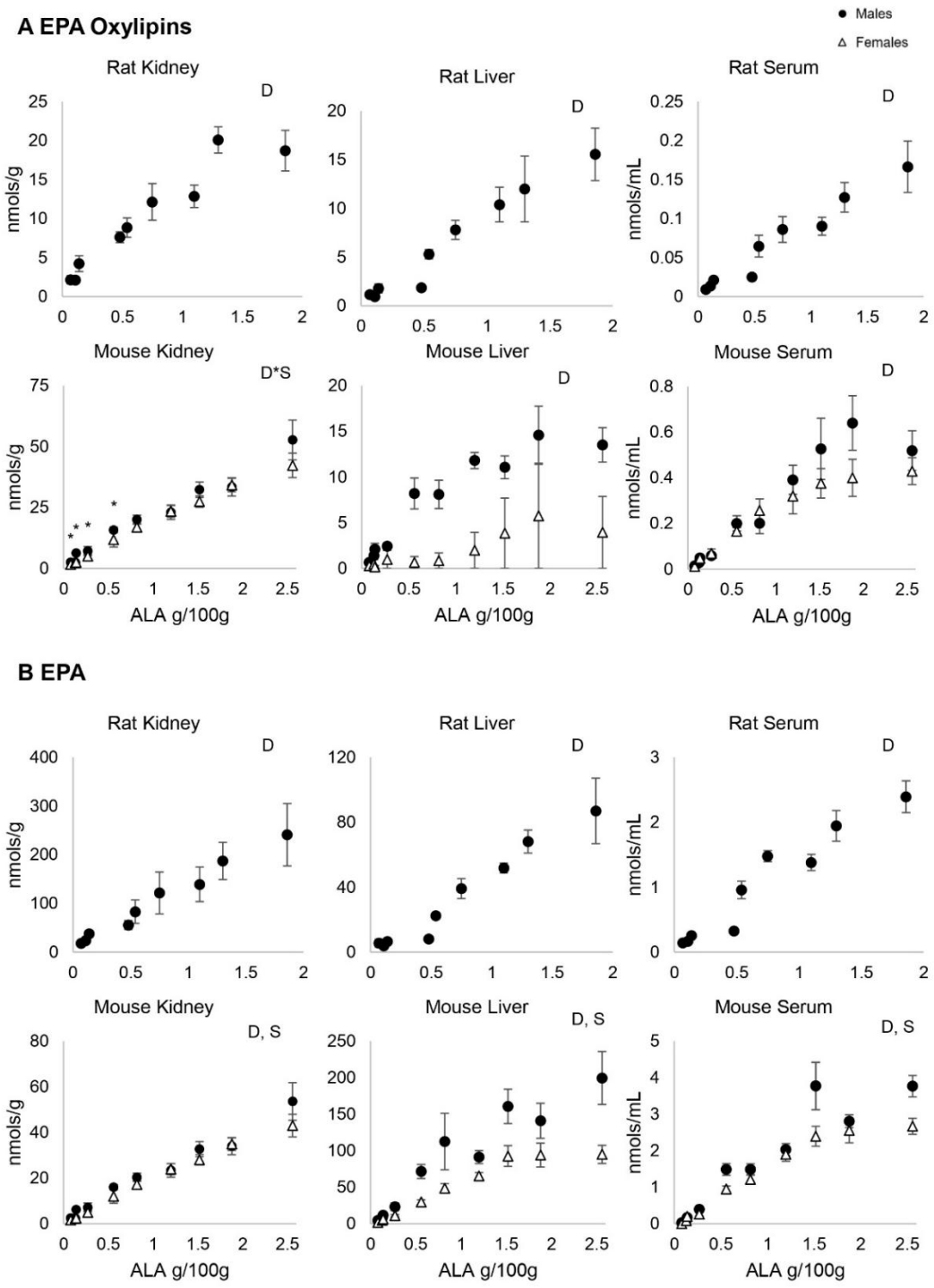
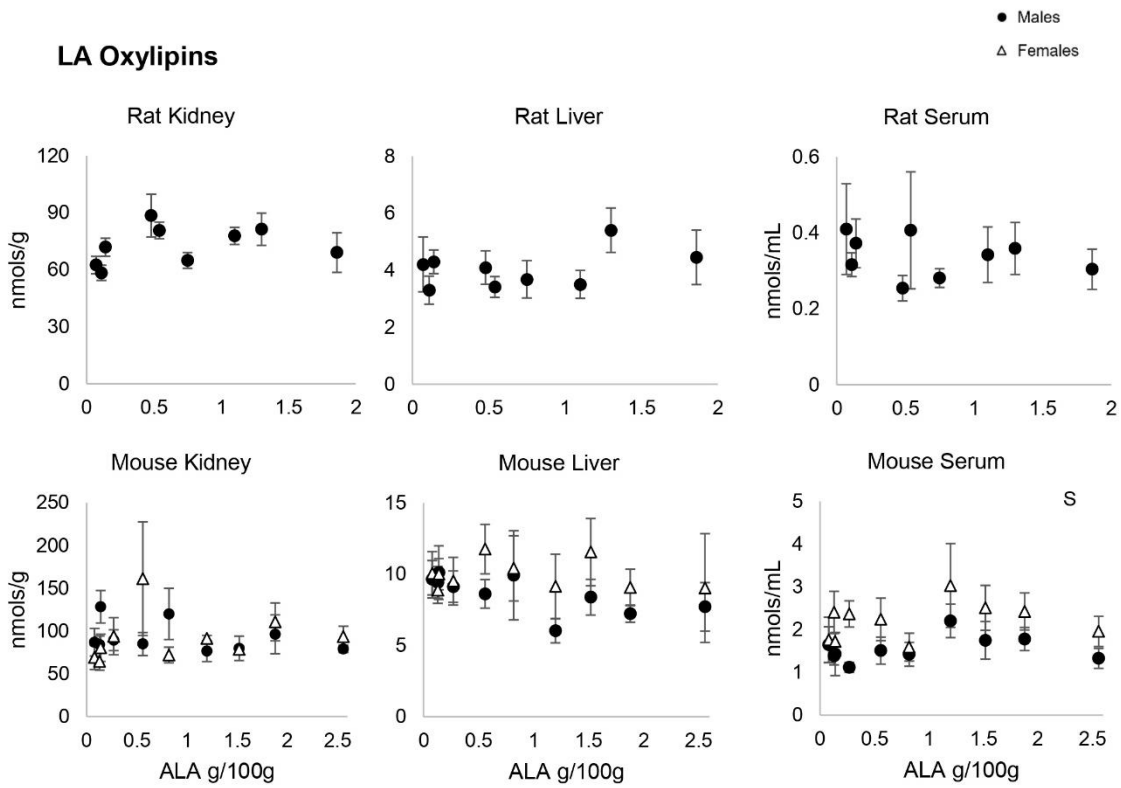
# Appendix 1: Supplemental figures and tables for Chapter 2




**Supplemental Figure 2-1.** Total ALA oxylipins (A) and ALA (B) in response to dietary ALA. Females: white triangles with black outline; Males: black-filled circles. Data shown as mean and SE (N=119-120, n=5-6 mice; N=45, n=5 rats, N total animals, n animals per group). D and S denote main effects of diet and sex, respectively, and diet effects within each sex are reported in Supplemental Tables 4 and 5.




**Supplemental Figure 2-2**. Total EPA oxylipins (A) and EPA (B) in response to dietary ALA. Females: white triangles with black outline; Males: black-filled circles. Data shown as mean and SE (N=119-120, n=5-6 mice; N=45, n=5 rats, N total animals, n animals per group). D and S denote main effects of diet and sex, respectively. D*S denotes diet by sex interaction for which sex differences are denoted by * above each diet group, and diet effects within each sex are reported in Supplemental Tables 4 and 5.




**Supplemental Figure 2-3**. Total LA oxylipins in response to dietary ALA. Females: white triangles with black outline; Males: black-filled circles. Data shown as mean and SE (N=119-120, n=5-6 mice; N=45, n=5 rats, N total animals, n animals per group). S denotes sex main effect. Diet effects within each sex are reported in Supplemental Tables 4 and 5.

**Supplemental Table 2-1**. Oxylipins detected and quantified in serum and each tissue, ordered by FA precursor. Oxylipins included in $DHA_{OH}/ARA_{OH}$ are in bold italics.

| Rat Kidney | Rat Liver | Rat Serum | Mouse Kidney | Mouse Liver | Mouse Serum | Mouse Brain |
|---|---|---|---|---|---|---|
| | | | **ALA** | | | |
| 9-HOTrE | 9-HOTrE | 9-HOTrE | 9-HOTrE | 9-HOTrE | 9-HOTrE | 9-HOTrE |
| 13-HOTrE | 13-HOTrE | 13-HOTrE | 13-HOTrE | 13-HOTrE | 13-HOTrE | 13-HOTrE |
| | 12,13 EpODE | 12,13 EpODE | 12,13 EpODE | 12,13 EpODE | | |
| | | | | 12,13 diHODE | | |
| | | | **EPA** | | | |
| PGE3 | PGE3 | TXB3 | PGE3 | PGF3a | 5-HEPE | PGD3 |
| PGF3a | 5-HEPE | 5-HEPE | TXB3 | 5-HEPE | 8-HEPE | 12-HEPE |
| 5-HEPE | 8-HEPE | 8-HEPE | 5-HEPE | 8-HEPE | 9-HEPE | 18-HEPE |
| 9-HEPE | 9-HEPE | 11-HEPE | 8-HEPE | 11-HEPE | 11-HEPE | |
| 11-HEPE | 11-HEPE | 12-HEPE | 9-HEPE | 12-HEPE | 12-HEPE | |
| 12-HEPE | 12-HEPE | 15-HEPE | 11-HEPE | 15-HEPE | 15-HEPE | |
| 15-HEPE | 15-HEPE | 14,15 EpETE | 12-HEPE | 14,15 diHETE | 14,15 EpETE | |
| 17,18 diHETE | 14,15 EpETE | 14,15 diHETE | 15-HEPE | 17,18 diHETE | 14,15 diHETE | |
| 18-HEPE | 14,15 diHETE | 17,18 EpETE | 14,15 diHETE | 18-HEPE | 17,18 EpETE | |
| | 17,18 diHETE | 18-HEPE | 17,18 diHETE | | 17,18 diHETE | |
| | 18-HEPE | | 18-HEPE | | 18-HEPE | |
| | | | **DHA** | | | |
| ***4-HDoHE*** | ***4-HDoHE*** | ***4-HDoHE*** | ***4-HDoHE*** | ***4-HDoHE*** | ***4-HDoHE*** | ***4-HDoHE*** |
| ***7-HDoHE*** | ***7-HDoHE*** | ***7-HDoHE*** | ***7-HDoHE*** | ***7-HDoHE*** | ***7-HDoHE*** | ***7-HDoHE*** |
| ***8-HDoHE*** | ***8-HDoHE*** | ***8-HDoHE*** | ***8-HDoHE*** | ***10-HDoHE*** | ***8-HDoHE*** | ***8-HDoHE*** |
| ***10-HDoHE*** | ***10-HDoHE*** | ***10-HDoHE*** | ***10-HDoHE*** | ***11-HDoHE*** | ***10-HDoHE*** | ***10-HDoHE*** |
| ***11-HDoHE*** | ***11-HDoHE*** | ***13-HDoHE*** | ***11-HDoHE*** | ***13-HDoHE*** | ***11-HDoHE*** | ***11-HDoHE*** |
| ***13-HDoHE*** | ***13-HDoHE*** | ***14-HDoHE*** | ***13-HDoHE*** | ***14-HDoHE*** | ***13-HDoHE*** | ***13-HDoHE*** |

| | | | | | | |
|---|---|---|---|---|---|---|
| ***14-HDoHE*** | ***14-HDoHE*** | ***16-HDoHE*** | ***14-HDoHE*** | ***16-HDoHE*** | ***14-HDoHE*** | ***14-HDoHE*** |
| ***16-HDoHE*** | ***16-HDoHE*** | ***17-HDoHE*** | ***16-HDoHE*** | ***17-HDoHE*** | ***16-HDoHE*** | ***16-HDoHE*** |
| ***17-HDoHE*** | ***17-HDoHE*** | 16,17 EpDoPE | ***17-HDoHE*** | 16,17 DiHDoPE | ***17-HDoHE*** | ***17-HDoHE*** |
| 16,17 DiHDoPE | 16,17 DiHDoPE | 16,17 DiHDoPE | 10S,17S-DiHDoHE | 19,20 DiHDoPE | 16,17 EpDoPE | 16,17 EpDoPE |
| 19,20 DiHDoPE | 19,20 DiHDoPE | 19,20 EpDoPE | 16,17 EpDoPE | ***20-HDoHE*** | 16,17 DiHDoPE | 16,17 DiHDoPE |
| ***20-HDoHE*** | ***20-HDoHE*** | 19,20 DiHDoPE | 16,17 DiHDoPE | | 19,20 EpDoPE | 19,20 DiHDoPE |
| | | ***20-HDoHE*** | 19,20 EpDoPE | | 19,20 DiHDoPE | ***20-HDoHE*** |
| | | | 19,20 DiHDoPE | | ***20-HDoHE*** | |
| | | | ***20-HDoHE*** | | | |

**ARA**

| | | | | | | |
|---|---|---|---|---|---|---|
| PGD2 | PGD2 | PGD2 | PGD2 | PGD2 | PGE2 | PGD2 |
| 15d PGD2 | PGE2 | PGE2 | PGE2 | PGE2 | 11b PGE2 | 15d PGD2 |
| PGE2 | 11b PGE2 | PGA2 | 11b PGE2 | 11b PGE2 | 15k PGE2 | dhk PGD2 |
| 11b PGE2 | 6k PGF1a | 11b PGE2 | 15k PGE2 | 15k PGE2 | TXB2 | PGJ2 |
| 15k PGE2 | PGF2a | PGF2a | 6k PGF1a | 6k PGF1a | 12-HHTrE | PGE2 |
| 6k PGF1a | 12-HHTrE | TXB2 | PGF2a | PGF2a | ***5-HETE*** | PGA2 |
| PGF2a | ***5-HETE*** | 12-HHTrE | TXB2 | TXB2 | ***8-HETE*** | PGB2 |
| TXB2 | ***8-HETE*** | ***5-HETE*** | 12-HHTrE | 12-HHTrE | ***9-HETE*** | 11b PGE2 |
| 12-HHTrE | ***9-HETE*** | ***8-HETE*** | ***5-HETE*** | ***5-HETE*** | ***11-HETE*** | 15k PGE2 |
| ***5-HETE*** | ***11-HETE*** | ***11-HETE*** | ***8-HETE*** | ***8-HETE*** | ***12-HETE*** | 6k PGF1a |
| 5,15 diHETE | ***12-HETE*** | ***12-HETE*** | ***9-HETE*** | ***11-HETE*** | tetranor 12-HETE | PGF2a |
| ***8-HETE*** | ***15-HETE*** | tetranor 12-HETE | ***11-HETE*** | ***12-HETE*** | ***15-HETE*** | 15k PGF2a |
| ***9-HETE*** | 5,6 DiHETrE | ***15-HETE*** | ***12-HETE*** | tetranor 12-HETE | 6t LTB4 | TXB2 |
| ***11-HETE*** | 8,9 EpETrE | 6t LTB4 | tetranor 12-HETE | ***15-HETE*** | 6t, 12epi LTB4 | 12-HHTrE |
| ***12-HETE*** | 8,9 DiHETrE | 6t, 12epi LTB4 | ***15-HETE*** | 5,6 EpETrE | 5,6 EpETrE | ***5-HETE*** |
| ***15-HETE*** | 11,12 DiHETrE | LTB4 | LTB4 | 5,6 DiHETrE | 5,6 DiHETrE | ***8-HETE*** |
| 8,15 diHETE | 14,15 DiHETrE | 6S-LXA4 | 12epi LTB4 | 8,9 EpETrE | 8,9 EpETrE | ***9-HETE*** |
| 6t LTB4 | ***16-HETE*** | 5,6 EpETrE | 6S-LXA4 | 8,9 DiHETrE | 8,9 DiHETrE | ***11-HETE*** |
| 6t, 12epi LTB4 | ***18-HETE*** | 8,9 EpETrE | 5,6 EpETrE | 11,12 EpETrE | 11,12 DiHETrE | ***12-HETE*** |
| 5,6 DiHETrE | ***20-HETE*** | 8,9 DiHETrE | 5,6 DiHETrE | 11,12 DiHETrE | 14,15 EpETrE | ***15-HETE*** |
| 8,9 EpETrE | | 11,12 DiHETrE | 8,9 EpETrE | 14,15 DiHETrE | 14,15 DiHETrE | 6S-LXA4 |
| 8,9 DiHETrE | | 14,15 EpETrE | 8,9 DiHETrE | | ***16-HETE*** | 11,12 EpETrE |

| | | | | | | |
|---|---|---|---|---|---|---|
| 11,12 EpETrE | | 14,15 DiHETrE | 11,12 DiHETrE | | ***17-HETE*** | 11,12 DiHETrE |
| 11,12 DiHETrE | | ***16-HETE*** | 14,15 EpETrE | | ***18-HETE*** | 14,15 EpETrE |
| 14,15 EpETrE | | ***18-HETE*** | 14,15 DiHETrE | | | 14,15 DiHETrE |
| 14,15 DiHETrE | | | ***16-HETE*** | | | |
| ***16-HETE*** | | | ***18-HETE*** | | | |
| ***18-HETE*** | | | | | | |
| ***20-HETE*** | | | | | | |
| | | | **LA** | | | |
| 9-HODE | 9-HODE | 9-HODE | 9-HODE | 9-HODE | 9-HODE | 9-HODE |
| 13-HODE | 13-HODE | 13-HODE | 13-HODE | 13-HODE | 13-HODE | 13-HODE |
| 9,10,13 triHOME | 9,10,13 triHOME | 9,10,13 triHOME | 9,10,13 triHOME | 9,10,13 triHOME | 9,10,13 triHOME | 9,10,13 triHOME |
| 9,12,13 triHOME | 9,12,13 triHOME | 9,12,13 triHOME | 9,12,13 triHOME | 9,12,13 triHOME | 9,12,13 triHOME | 9,12,13 triHOME |
| 9,10 diHOME | 9,10 diHOME | 9,10 EpOME | 9,10 EpOME | 9,10 EpOME | 9,10 EpOME | 9,10 EpOME |
| 12,13 diHOME | 12,13 EpOME | 9,10 diHOME | 9,10 diHOME | 9,10 diHOME | 9,10 diHOME | 9,10 diHOME |
| | 12,13 diHOME | 12,13 EpOME | 12,13 EpOME | 12,13 EpOME | 12,13 EpOME | 12,13 EpOME |
| | | 12,13 diHOME | 12,13 diHOME | 12,13 diHOME | 12,13 diHOME | |

Abbreviations: ARA, arachidonic acid; ALA, α-linolenic acid; DiHDoPE, dihydroxy-docosapentaenoic acid; DiHDoHE, dihydroxy-docosahexaenoic acid; DiHETE, dihydroxy-eicosatetraenoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; DiHODE, dihydroxy-octadecadienoic acid; diHOME, dihydroxy-octadecenoic acid; EpDoPE, epoxy-docosapentaenoic acid; EpETrE, epoxy-eicosatrienoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; FA, fatty 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; LA, linoleic acid; LT, leukotriene; PG, prostaglandin; triHOME, trihydroxy-octadecenoic acid; TX, thromboxane.

**Supplemental Table 2-2.** Rat body and organ weights at the end of the study period.

| Diet Group | 0.1 | 0.15 | 0.2 | 0.3 | 0.6 | 0.9 | 1.2 | 1.5 | 2 | Diet (P) |
|---|---|---|---|---|---|---|---|---|---|---|
| **Body Weight** | 469 ± 18.0 | 487 ± 14.4 | 452 ± 29.6 | 462 ± 16.7 | 465 ± 19.7 | 482 ± 26.4 | 437 ± 13.5 | 445 ± 14.6 | 454 ± 12.3 | 0.6963 |
| **Right kidney** | 1.54 ± 0.077 | 1.64 ± 0.055 | 1.57 ± 0.096 | 1.56 ± 0.059 | 1.55 ± 0.080 | 1.60 ± 0.093 | 1.51 ± 0.060 | 1.56 ± 0.076 | 1.59 ± 0.058 | 0.9401 |
| **Left kidney** | 1.53 ± 0.090 | 1.66 ± 0.046 | 1.52 ± 0.082 | 1.55 ± 0.063 | 1.56 ± 0.080 | 1.58 ± 0.094 | 1.5 ± 0.049 | 1.52 ± 0.050 | 1.58 ± 0.039 | 0.8114 |
| **Liver** | 19.4 ± 1.50 | 23.8 ± 1.20 | 20.2 ± 2.34 | 21.5 ± 1.43 | 19.2 ± 1.80 | 22.1 ± 2.23 | 18.3 ± 0.916 | 18.7 ± 1.69 | 19.0 ± 1.08 | 0.3187 |

Data are shown in grams as means ± SEs (n=5).

**Supplemental Table 2-3.** Mouse body and organ weights at the end of the study period.

| Diet Group | | 0.1 | 0.15 | 0.2 | 0.3 | 0.6 | 0.9 | 1.2 | 1.5 | 2 | 2.5 | Diet (P) | Sex (P) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Body weight** | Males | 44.6 ± 1.73 | 41.1 ± 1.73 | 43.3 ± 1.62 | 42.6 ± 1.44 | 44.4 ± 1.12 | 42.9 ± 1.45 | 43 ± 1.44 | 45 ± 2.15 | 45.9 ± 2.26 | 47.9 ± 1.24 | 0.7783 | <0.0001 |
| | Females | 34.2 ± 1.84 | 32.4 ± 1.74 | 35.5 ± 0.722 | 34 ± 2.17 | 32 ± 1.30 | 33.7 ± 2.53 | 35.5 ± 1.86 | 34.6 ± 3.30 | 32.6 ± 1.68 | 32.6 ± 1.22 | | |
| **Right Kidney** | Males | 0.293 ± 0.014 | 0.332 ± 0.010 | 0.295 ± 0.014 | 0.333 ± 0.026 | 0.315 ± 0.016 | 0.327 ± 0.009 | 0.332 ± 0.009 | 0.333 ± 0.010 | 0.323 ± 0.012 | 0.33 ± 0.022 | 0.4796 | <0.0001 |
| | Females | 0.212 ± 0.014 | 0.213 ± 0.014 | 0.215 ± 0.004 | 0.220 ± 0.008 | 0.205 ± 0.012 | 0.187 ± 0.013 | 0.208 ± 0.010 | 0.218 ± 0.015 | 0.182 ± 0.004 | 0.193 ± 0.008 | | |
| **Left Kidney** | Males | 0.290 ± 0.018 | 0.315 ± 0.017 | 0.282 ± 0.011 | 0.322 ± 0.022 | 0.312 ± 0.013 | 0.318 ± 0.008 | 0.308 ± 0.008 | 0.317 ± 0.004 | 0.317 ± 0.010 | 0.308 ± 0.010 | 0.5922 | <0.0001 |
| | Females | 0.208 ± 0.014 | 0.202 ± 0.011 | 0.205 ± 0.006 | 0.223 ± 0.009 | 0.195 ± 0.006 | 0.192 ± 0.010 | 0.205 ± 0.007 | 0.202 ± 0.015 | 0.188 ± 0.009 | 0.192 ± 0.007 | | |
| **Liver** | Males | 2.57 ± 0.086 | 2.28 ± 0.117 | 2.34 ± 0.095 | 2.17 ± 0.086 | 2.39 ± 0.101 | 2.40 ± 0.126 | 2.18 ± 0.112 | 2.25 ± 0.246 | 2.25 ± 0.104 | 2.42 ± 0.078 | 0.6526 | <0.0001 |
| | Females | 1.82 ± 0.149 | 1.61 ± 0.108 | 1.93 ± 0.101 | 1.92 ± 0.226 | 1.59 ± 0.092 | 1.83 ± 0.178 | 1.71 ± 0.102 | 1.83 ± 0.289 | 1.64 ± 0.131 | 1.65 ± 0.051 | | |
| **Brain** | Males | 0.466 ± 0.013 | 0.477 ± 0.013 | 0.457 ± 0.018 | 0.467 ± 0.013 | 0.465 ± 0.057 | 0.452 ± 0.011 | 0.509 ± 0.014 | 0.492 ± 0.015 | 0.487 ± 0.010 | 0.488 ± 0.007 | 0.8089 | 0.2618 |
| | Females | 0.477 ± 0.015 | 0.487 ± 0.025 | 0.479 ± 0.009 | 0.532 ± 0.012 | 0.474 ± 0.020 | 0.488 ± 0.012 | 0.469 ± 0.012 | 0.480 ± 0.015 | 0.491 ± 0.020 | 0.480 ± 0.015 | | |

Data are shown in grams as means ± SEs (n=6).

**Supplemental Table 2-4**. ANOVA results for total oxylipins by FA precursor and for FA in rat kidney (A), liver (B), serum (C).

**A. Rat Kidney**

| Diet Group | 0.07 | 0.11 | 0.14 | 0.48 | 0.54 | 0.75 | 1.1 | 1.3 | 1.86 | SW (P) | TSW (P) | Diet (P) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *NE Oxylipins (nmols/g)* | | | | | | |
| **ALA** | 0.989 ± 0.384 d | 0.989 ± 0.078 d | 1.27 ± 0.188 d | 2.81 ± 0.593 c | 4.85 ± 0.417 b | 5.63 ± 0.216 b | 7.90 ± 1.63 ab | 11.0 ± 2.88 a | 12.2 ± 2.47 a | <0.0001 | 0.1048 | <0.0001 |
| **EPA** | 2.16 ± 0.529 f | 2.12 ± 0.197 f | 4.22 ± 1.01 e | 7.62 ± 0.687 d | 8.85 ± 1.25 cd | 12.2 ± 2.35 cd | 12.9 ± 1.44 bc | 20.1 ± 1.69 a | 18.7 ± 2.61 ab | 0.0007 | 0.0877 | <0.0001 |
| **DHA** | 18.6 ± 2.26 d | 20.2 ± 2.14 cd | 24.0 ± 2.79 bcd | 39.8 ± 8.45 a | 28.5 ± 1.5 abc | 28.8 ± 3.95 abc | 24.8 ± 1.33 abcd | 33.1 ± 3.16 ab | 22.3 ± 4.58 cd | 0.0089 | 0.1953 | 0.0121 |
| **ARA** | 131 ± 19.3 abc | 119 ± 24.7 abcd | 149 ± 28.8 ab | 185 ± 41.5 a | 104 ± 23.9 bcd | 77.7 ± 14.8 cd | 76.8 ± 15.9 cd | 95.0 ± 18.1 bcd | 54.4 ± 13.6 d | 0.1324 | NA | 0.0129 |
| **LA** | 62.5 ± 4.61 | 58.4 ± 4.00 | 71.9 ± 4.73 | 88.5 ± 11.2 | 80.7 ± 4.39 | 64.9 ± 4.12 | 77.8 ± 4.54 | 81.3 ± 8.48 | 69.0 ± 10.4 | 0.4704 | NA | 0.0588 |
| | | | | | | *NE Fatty Acids (nmols/g)* | | | | | | |
| **ALA** | 4.86 ± 2.13 d | 7.78 ± 1.05 d | 11.1 ± 2.90 d | 20.4 ± 1.55 c | 30.9 ± 2.82 bc | 33.1 ± 2.05 bc | 53.8 ± 7.26 ab | 85.6 ± 17.3 a | 108.1 ± 16.6 a | 0.0001 | <0.0001 | <0.0001 |
| **EPA** | 18.2 ± 4.27 g | 23.6 ± 5.27 fg | 37.5 ± 6.18 ef | 55.6 ± 9.23 de | 82.9 ± 24.0 cd | 121.4 ± 42.9 bcd | 139.1 ± 35.5 abc | 187.3 ± 38.1 ab | 240.9 ± 64.1 a | 0.0054 | 0.2311 | <0.0001 |
| **DHA** | 587 ± 104 | 653 ± 104 | 991 ± 172 | 1451 ± 365 | 1069 ± 309 | 1126 ± 282 | 1298 ± 260 | 1787 ± 401 | 1531 ± 287 | 0.2090 | NA | 0.0603 |
| **ARA** | 1324 ± 163 | 1179 ± 280 | 1353 ± 304 | 1889 ± 597 | 2121 ± 863 | 1556 ± 464 | 1230 ± 445 | 1504 ± 333 | 1491 ± 464 | 0.0485 | 0.3522 | 0.9867 |

**B. Rat Liver**

| Diet Group | 0.07 | 0.11 | 0.14 | 0.48 | 0.54 | 0.75 | 1.1 | 1.3 | 1.86 | SW (P) | TSW (P) | Diet (P) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *NE Oxylipins (nmols/g)* | | | | | | |
| **ALA** | 0.085 ± 0.064 de | 0.031 ± 0.015 e | 0.132 ± 0.034 de | 0.189 ± 0.059 cde | 0.344 ± 0.063 bcd | 0.491 ± 0.092 bc | 0.69 ± 0.131 b | 1.34 ± 0.182 a | 1.49 ± 0.474 a | <0.0001 | 0.1147 | <0.0001 |
| **EPA** | 1.16 ± 0.108 de | 0.943 ± 0.169 e | 1.79 ± 0.456 de | 1.85 ± 0.283 d | 5.31 ± 0.473 c | 7.8 ± 0.983 bc | 10.4 ± 1.79 ab | 12 ± 3.39 ab | 15.6 ± 2.69 a | <0.0001 | 0.2795 | <0.0001 |
| **DHA** | 2.40 ± 0.901 | 2.61 ± 0.678 | 2.12 ± 0.430 | 3.43 ± 0.670 | 3.71 ± 0.531 | 4.14 ± 0.760 | 3.04 ± 0.572 | 4.99 ± 1.08 | 3.82 ± 0.615 | 0.7815 | NA | 0.1511 |
| **ARA** | 9.33 ± 1.90 a | 6.96 ± 0.882 ab | 8.58 ± 1.62 a | 6.67 ± 0.922 abc | 5.96 ± 0.403 abcd | 4.52 ± 0.593 cde | 3.95 ± 0.394 de | 4.86 ± 0.683 bcde | 3.59 ± 0.518 e | 0.0016 | 0.775 | 0.0004 |
| **LA** | 4.21 ± 0.963 | 3.30 ± 0.490 | 4.29 ± 0.414 | 4.09 ± 0.590 | 3.41 ± 0.363 | 3.68 ± 0.659 | 3.5 ± 0.491 | 5.41 ± 0.780 | 4.45 ± 0.958 | 0.0266 | 0.8935 | 0.4860 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *NE Fatty Acids (nmols/g)* | | | | | | | |
| **ALA** | 2.44 ± 2.44 | ND | 5.21 ± 1.42 | 6.73 ± 2.02 | 16.7 ± 2.24 | 26.7 ± 4.70 | 43.7 ± 7.17 | 65.8 ± 8.32 | 71.8 ± 16.6 | 0.0008 | 0.002 | <0.0001 |
| | e | e | d | d | c | bc | ab | a | a | | | |
| **EPA** | 5.48 ± 2.48 | 3.84 ± 0.467 | 6.50 ± 0.503 | 8.07 ± 0.697 | 22.4 ± 1.39 | 39.2 ± 6.15 | 51.8 ± 3.09 | 68.1 ± 7.11 | 87.0 ± 20.1 | <0.0001 | 0.0006 | <0.0001 |
| | ef | f | de | d | c | b | ab | a | a | | | |
| **DHA** | 395 ± 73.5 | 520 ± 33.4 | 483 ± 31.4 | 651 ± 35.3 | 675 ± 59.0 | 830 ± 73.9 | 749 ± 60.6 | 893 ± 98.8 | 742 ± 108 | 0.4507 | NA | <0.0001 |
| | d | cd | cd | bc | bc | ab | ab | a | ab | | | |
| **ARA** | 526 ± 117 | 451 ± 54.6 | 416 ± 29.3 | 436 ± 35.3 | 345 ± 16.6 | 339 ± 47.6 | 301 ± 31.1 | 386 ± 47.1 | 266 ± 42.5 | 0.0018 | 0.6517 | 0.0207 |
| | a | ab | abc | ab | abcd | bcd | cd | abc | d | | | |

**C. Rat Serum**

| **Diet Group** | **0.07** | **0.11** | **0.14** | **0.48** | **0.54** | **0.75** | **1.1** | **1.3** | **1.86** | **SW (P)** | **TSW (P)** | **Diet (P)** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *NE Oxylipins (nmols/mL)* | | | | | | | |
| **ALA** | 0.014 ± 0.005 | 0.016 ± 0.004 | 0.016 ± 0.004 | 0.016 ± 0.002 | 0.051 ± 0.014 | 0.049 ± 0.008 | 0.06 ± 0.012 | 0.079 ± 0.011 | 0.118 ± 0.042 | <0.0001 | 0.8722 | <0.0001 |
| | c | c | c | c | b | b | ab | ab | a | | | |
| **EPA** | 0.009 ± 0.002 | 0.014 ± 0.004 | 0.021 ± 0.003 | 0.025 ± 0.003 | 0.065 ± 0.014 | 0.086 ± 0.017 | 0.09 ± 0.011 | 0.127 ± 0.019 | 0.167 ± 0.033 | 0.0054 | 0.4616 | <0.0001 |
| | f | ef | de | d | c | bc | bc | ab | a | | | |
| **DHA** | 0.074 ± 0.014 | 0.076 ± 0.012 | 0.072 ± 0.012 | 0.085 ± 0.009 | 0.139 ± 0.017 | 0.145 ± 0.024 | 0.128 ± 0.014 | 0.149 ± 0.014 | 0.162 ± 0.043 | 0.0834 | NA | 0.0062 |
| | c | c | c | bc | ab | a | abc | a | a | | | |
| **ARA** | 2.81 ± 0.429 | 3.01 ± 0.513 | 2.60 ± 0.289 | 1.95 ± 0.197 | 1.78 ± 0.240 | 1.51 ± 0.306 | 1.20 ± 0.130 | 1.52 ± 0.425 | 1.11 ± 0.161 | 0.4791 | NA | 0.0005 |
| | ab | a | abc | bcd | cd | d | d | d | d | | | |
| **LA** | 0.410 ± 0.120 | 0.316 ± 0.031 | 0.372 ± 0.064 | 0.254 ± 0.034 | 0.406 ± 0.154 | 0.281 ± 0.025 | 0.342 ± 0.073 | 0.359 ± 0.068 | 0.304 ± 0.053 | 0.0116 | 0.7789 | 0.9737 |
| | | | | | *NE Fatty Acids (nmols/mL)* | | | | | | | |
| **ALA** | 0.135 ± 0.066 | 0.114 ± 0.022 | 0.201 ± 0.019 | 0.190 ± 0.034 | 0.489 ± 0.116 | 0.537 ± 0.039 | 0.549 ± 0.104 | 0.776 ± 0.168 | 0.809 ± 0.092 | 0.0192 | 0.1605 | <0.0001 |
| | d | d | d | d | c | bc | bc | ab | a | | | |
| **EPA** | 0.144 ± 0.034 | 0.165 ± 0.024 | 0.255 ± 0.037 | 0.323 ± 0.029 | 0.957 ± 0.135 | 1.48 ± 0.084 | 1.38 ± 0.124 | 1.94 ± 0.237 | 2.39 ± 0.245 | 0.0165 | 0.6680 | <0.0001 |
| | e | e | d | d | c | b | bc | ab | a | | | |
| **DHA** | 9.14 ± 2.02 | 9.95 ± 0.888 | 11.3 ± 1.75 | 12.6 ± 1.45 | 18.3 ± 1.50 | 22.3 ± 2.61 | 17.5 ± 1.77 | 20.4 ± 2.89 | 19.2 ± 2.39 | 0.2079 | <0.0001 | <0.0001 |
| | c | c | c | bc | ab | a | ab | a | a | | | |
| **ARA** | 15.4 ± 1.67 | 14.7 ± 1.14 | 13.9 ± 0.931 | 12.1 ± 0.633 | 13.3 ± 0.731 | 12.8 ± 1.09 | 10.9 ± 0.702 | 12 ± 0.898 | 10.1 ± 0.854 | 0.8354 | <0.0001 | 0.0125 |
| | a | ab | ab | bcd | abc | abcd | cd | bcd | d | | | |

Data reported as means ± SEs (n=5; N=45). The SW column shows the shapiro wilk test p value results and the TSW column shows shapiro-wilk p values for log transformed data. Data that were normal was analyzed using a one-way ANOVA for diet effects shown in the diet (P) column. If data were not normal even following log-transformation, ANOVA was applied to data that were closest to normality. Diet group means within each row with differing letters below the means indicate significant differences between diet groups as determined by Fisher's Protected LSD test ($p<0.05$). ND: not detected.

**Supplemental Table 2-5**. ANOVA results for total oxylipins by FA precursor and for FA in mouse kidney (A), liver (B), serum (C), and brain (D).

**A. Mouse Kidney**

| Diet Group | | 0.08 | 0.13 | 0.14 | 0.27 | 0.56 | 0.82 | 1.2 | 1.52 | 1.88 | 2.56 | SW (P) | TSW (P) | Diet (P) | Sex (P) | Diet*Sex (P) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | *NE Oxylipins (nmols/g)* | | | | | | | | | |
| **ALA** | F | 2.80 ± 0.701 | 5.12 ± 2.67 | 7.91 ± 5.23 | 11.3 ± 5.99 | 14.8 ± 5.76 | 9.58 ± 1.30 | 15.9 ± 1.52 | 20.1 ± 2.84 | 32.1 ± 9.14 | 32.0 ± 6.87 | <0.0001 | 0.0027 | <0.0001 | 0.5587 | 0.8253 |
| | M | 2.08 ± 0.476 | 2.60 ± 0.552 | 5.68 ± 1.04 | 4.97 ± 1.37 | 9.65 ± 2.34 | 18.2 ± 4.77 | 14.8 ± 3.70 | 21.8 ± 5.74 | 26.0 ± 4.17 | 28.4 ± 2.42 | | | | | |
| | | f | ef | de | d | c | bc | bc | ab | a | a | | | | | |
| **EPA** | F | 1.63 ± 0.194 | 2.30 ± 0.105 | 2.44 ± 0.201 | 4.96 ± 0.817 | 12.0 ± 3.02 | 17.2 ± 1.54 | 23.9 ± 2.40 | 28.0 ± 2.28 | 34.9 ± 2.73 | 43.0 ± 4.94 | <0.0001 | 0.2636 | <0.0001 | <0.0001 | 0.0040 |
| | | l | k | k | j | h | fg | def | cde | bc | ab | | | | | |
| | M | 2.56 ± 0.311 | 2.56 ± 0.536 | 6.25 ± 0.731 | 7.16 ± 1.91 | 16.0 ± 1.13 | 20.3 ± 1.84 | 23.4 ± 2.97 | 32.7 ± 3.27 | 34.0 ± 3.77 | 53.6 ± 8.23 | | | | | |
| | | k | k | ij | i | g | efg | ef | bcd | bcd | a | | | | | |
| **DHA** | F | 96.8 ± 24.6 | 87.4 ± 8.85 | 69.8 ± 8.44 | 116.4 ± 11.2 | 128.7 ± 32.2 | 123.9 ± 22.5 | 139 ± 22.9 | 137 ± 10.8 | 138.2 ± 20 | 123.7 ± 25.2 | 0.1149 | NA | 0.0011 | 0.0002 | 0.7747 |
| | M | 93.5 ± 15.2 | 94.1 ± 18.7 | 149 ± 13.1 | 153 ± 17.4 | 161 ± 17.2 | 151 ± 19.7 | 174 ± 26.4 | 183 ± 18.2 | 171 ± 24.7 | 168 ± 29.6 | | | | | |
| | | c | c | bc | ab | ab | ab | a | a | a | ab | | | | | |
| **ARA** | F | 208 ± 34.4 | 179 ± 17.7 | 145 ± 21.0 | 183 ± 20.9 | 164 ± 28.7 | 158 ± 24.1 | 154.6 ± 24.0 | 148 ± 11.9 | 127 ± 15.8 | 114 ± 15.1 | 0.0019 | 0.3702 | <0.0001 | <0.0001 | 0.0812 |
| | M | 169 ± 13.7 | 105 ± 10.2 | 167 ± 15.2 | 122 ± 18.1 | 102 ± 4.50 | 98.5 ± 7.73 | 82.6 ± 10.3 | 79.9 ± 9.61 | 76.8 ± 13.4 | 72.9 ± 14.2 | | | | | |
| | | a | bc | ab | ab | bc | bcd | cde | cde | de | e | | | | | |
| **LA** | F | 69.0 ± 14.0 | 64.4 ± 10.2 | 80.4 ± 15.9 | 93.8 ± 21.7 | 161 ± 66.4 | 71.9 ± 9.15 | 91.1 ± 3.75 | 78.4 ± 4.29 | 111 ± 22.1 | 93.3 ± 12.2 | <0.0001 | 0.0860 | 0.6514 | 0.4858 | 0.2076 |
| | M | 86.9 ± 16.2 | 84 ± 10.2 | 128 ± 19.0 | 89.2 ± 12.2 | 84.8 ± 13.4 | 120 ± 30.0 | 76.4 ± 12.2 | 79.7 ± 14.3 | 96.1 ± 22.8 | 79.0 ± 4.25 | | | | | |
| | | | | | | | *NE Fatty Acids (nmols/g)* | | | | | | | | | |
| **ALA** | F | 1.66 ± 1.14 | 2.74 ± 2.65 | 6.54 ± 6.32 | 6.93 ± 4.68 | 9.43 ± 2.98 | 13.1 ± 3.69 | 18.3 ± 2.36 | 29.5 ± 6.20 | 41.0 ± 5.30 | 44.8 ± 9.66 | <0.0001 | <0.0001 | <0.0001 | 0.1163 | 0.9751 |
| | M | 0.056 ± 0.056 | 0.038 ± 0.715 | 1.74 ± 0.686 | 2.76 ± 0.911 | 7.44 ± 1.91 | 18.1 ± 8.51 | 17.2 ± 2.84 | 29.0 ± 10.2 | 27.8 ± 4.97 | 34.7 ± 6.79 | | | | | |
| | | f | f | f | e | d | cd | bc | ab | a | a | | | | | |
| **EPA** | F | 3.02 ± 1.27 | 3.49 ± 0.451 | 3.32 ± 0.547 | 7.65 ± 1.40 | 14.3 ± 1.80 | 31.5 ± 6.38 | 38.8 ± 7.24 | 60.5 ± 11.0 | 70.3 ± 15.4 | 91.7 ± 26.5 | <0.0001 | 0.7915 | <0.0001 | 0.0008 | 0.8086 |
| | M | 1.94 ± 0.349 | 1.69 ± 0.385 | 3.32 ± 0.661 | 5.81 ± 1.58 | 14.5 ± 3.66 | 15.1 ± 2.79 | 33.3 ± 10.5 | 48.3 ± 12.9 | 43.5 ± 10.3 | 78.3 ± 27.1 | | | | | |
| | | g | fg | f | e | d | c | bc | ab | a | a | | | | | |
| **DHA** | F | 12321 ± 5777 | 9212 ± 1290 | 6676 ± 1070 | 10779 ± 1988.5 | 8380 ± 1037 | 12041 ± 1839 | 10296 ± 1830 | 13833 ± 2205 | 13044 ± 2722 | 15030 ± 4873 | <0.0001 | 0.2562 | 0.0267 | 0.7573 | 0.5998 |
| | M | 8449 ± 1631 | 5810 ± 1740 | 9720 ± 1319 | 10827.1 ± 1569 | 9838 ± 1967 | 8991 ± 948 | 12596 ± 2160 | 15094 ± 3406 | 11427 ± 2310 | 13038 ± 2968 | | | | | |
| | | bcd | d | cd | abc | bcd | abc | abc | a | abc | ab | | | | | |
| **ARA** | F | 7002 ± 2609 | 5377 ± 712 | 3608 ± 502 | 5658 ± 892 | 3896 ± 673 | 4871 ± 894 | 4648 ± 981 | 5353 ± 832 | 5171 ± 1230 | 5651 ± 1939 | <0.0001 | 0.8399 | 0.3184 | <0.0001 | 0.5489 |
| | M | 4304 ± 518 | 2627 ± 650 | 3858 ± 475 | 3553 ± 428 | 2714 ± 485 | 2167 ± 236 | 2926 ± 740 | 3368 ± 697 | 2385 ± 673 | 2613 ± 698 | | | | | |

**B. Mouse Liver**

| Diet Group | | 0.08 | 0.13 | 0.14 | 0.27 | 0.56 | 0.82 | 1.2 | 1.52 | 1.88 | 2.56 | SW (P) | TSW (P) | Diet (P) | Sex (P) | Diet*Sex (P) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | *NE Oxylipins (nmols/g)* | | | | | | | | | |
| **ALA** | F | 0.196 ± 0.081 | 0.234 ± 0.083 | 0.304 ± 0.058 | 0.388 ± 0.097 | 1.16 ± 0.265 | 1.61 ± 0.416 | 2.53 ± 1.01 | 3.04 ± 0.807 | 2.86 ± 0.568 | 4.42 ± 2.11 | <0.0001 | <0.0001 | <0.0001 | 0.8384 | 0.9967 |
| | M | 0.198 ± 0.037 | 0.234 ± 0.040 | 0.319 ± 0.066 | 0.577 ± 0.097 | 0.970 ± 0.148 | 1.66 ± 0.447 | 1.68 ± 0.305 | 3.04 ± 0.831 | 2.92 ± 0.332 | 4.05 ± 0.918 | | | | | |
| | | e | e | e | e | d | cd | bc | ab | a | a | | | | | |
| **EPA** | F | 0.991 ± 0.268 | 1.79 ± 0.479 | 1.54 ± 0.155 | 3.66 ± 0.986 | 5.00 ± 0.647 | 7.48 ± 0.848 | 10.7 ± 1.98 | 11.7 ± 3.85 | 15.4 ± 5.76 | 17.8 ± 3.94 | <0.0001 | 0.0009 | <0.0001 | 0.7941 | 0.9120 |
| | M | 0.656 ± 0.114 | 1.39 ± 0.199 | 2.09 ± 0.655 | 2.42 ± 0.341 | 8.20 ± 1.68 | 8.10 ± 1.55 | 11.8 ± 0.888 | 11.1 ± 1.25 | 14.6 ± 3.17 | 13.5 ± 1.88 | | | | | |
| | | f | e | e | d | c | bc | ab | ab | a | a | | | | | |
| **DHA** | F | 2.65 ± 0.424 | 3.29 ± 0.241 | 3.96 ± 0.397 | 4.18 ± 0.817 | 5.09 ± 0.642 | 4.59 ± 0.545 | 6.03 ± 2.11 | 4.92 ± 0.852 | 4.57 ± 0.297 | 3.62 ± 0.431 | <0.0001 | 0.0274 | <0.0001 | 0.3800 | 0.5074 |
| | M | 1.67 ± 0.171 | 3.01 ± 0.499 | 2.89 ± 0.332 | 3.88 ± 0.403 | 4.78 ± 0.515 | 4.79 ± 1.12 | 5.05 ± 0.178 | 5.42 ± 0.566 | 5.02 ± 0.395 | 4.15 ± 0.583 | | | | | |
| | | d | c | c | abc | ab | ab | a | a | ab | bc | | | | | |
| **ARA** | F | 6.11 ± 0.799 | 4.41 ± 0.348 | 5.16 ± 0.647 | 4.08 ± 0.736 | 3.85 ± 0.503 | 2.75 ± 0.618 | 2.36 ± 0.539 | 2.18 ± 0.291 | 2.01 ± 0.234 | 1.54 ± 0.235 | <0.0001 | 0.4096 | <0.0001 | <0.0001 | 0.4271 |
| | M | 3.78 ± 0.220 | 5.33 ± 0.825 | 3.79 ± 0.403 | 3.54 ± 0.378 | 2.29 ± 0.191 | 1.98 ± 0.277 | 1.32 ± 0.116 | 1.53 ± 0.170 | 1.27 ± 0.258 | 1.15 ± 0.234 | | | | | |
| | | a | a | a | ab | bc | cd | de | de | ef | f | | | | | |
| **LA** | F | 10.1 ± 1.52 | 8.87 ± 0.916 | 10 ± 1.09 | 9.51 ± 1.67 | 11.8 ± 1.73 | 10.4 ± 2.28 | 9.14 ± 2.27 | 11.6 ± 2.36 | 9.08 ± 1.28 | 9.03 ± 3.82 | <0.0001 | 0.1894 | 0.3409 | 0.1146 | 0.9773 |
| | M | 9.65 ± 1.31 | 9.43 ± 1.11 | 10.1 ± 1.88 | 9.13 ± 1.10 | 8.62 ± 1.00 | 9.93 ± 3.13 | 6.04 ± 0.857 | 8.38 ± 1.25 | 7.23 ± 0.607 | 7.71 ± 1.70 | | | | | |
| | | | | | | | *NE Fatty Acids (nmols/g)* | | | | | | | | | |
| **ALA** | F | 7.90 ± 1.14 | 10.3 ± 1.23 | 10.5 ± 1.69 | 16.9 ± 2.96 | 35.6 ± 4.98 | 53.5 ± 5.63 | 59.3 ± 4.56 | 112 ± 30.2 | 103 ± 9.36 | 94.4 ± 14.4 | <0.0001 | 0.7332 | <0.0001 | 0.0115 | 0.9635 |
| | M | 10.9 ± 1.41 | 10.8 ± 2.18 | 14.5 ± 2.51 | 20.1 ± 3.43 | 46.2 ± 5.26 | 71.7 ± 16.0 | 70.7 ± 10.3 | 103 ± 22.1 | 112 ± 23.1 | 127 ± 15.8 | | | | | |
| | | e | e | e | d | c | b | b | a | a | a | | | | | |
| **EPA** | F | 1.74 ± 0.356 | 4.68 ± 0.509 | 6.43 ± 0.677 | 10.9 ± 1.65 | 29.6 ± 3.22 | 48.5 ± 6.33 | 65.2 ± 4.78 | 92.6 ± 14.3 | 94.0 ± 16.3 | 94.9 ± 12.4 | <0.0001 | 0.2001 | <0.0001 | <0.0001 | 0.6580 |
| | M | 4.51 ± 0.799 | 7.68 ± 1.47 | 11.8 ± 3.06 | 23.2 ± 5.12 | 71.5 ± 9.58 | 113 ± 38.6 | 91.3 ± 8.94 | 161 ± 23.5 | 141 ± 24 | 199 ± 36.2 | | | | | |
| | | g | f | e | d | c | b | b | a | a | a | | | | | |
| **DHA** | F | 406 ± 24.6 | 559 ± 46.8 | 538 ± 58.7 | 611 ± 63.6 | 808 ± 80.1 | 754 ± 114 | 854 ± 45.8 | 1016 ± 147 | 940 ± 140 | 648 ± 52.1 | 0.0011 | 0.5634 | <0.0001 | 0.1334 | 0.4647 |
| | M | 341 ± 15.1 | 529 ± 74.5 | 514 ± 49.9 | 787 ± 122 | 904 ± 62.8 | 965 ± 191 | 1001 ± 90.0 | 1155 ± 190 | 840 ± 113 | 920 ± 92 | | | | | |
| | | f | de | e | cd | abc | abc | ab | a | abc | bc | | | | | |
| **ARA** | F | 362 ± 37.2 | 352 ± 31.7 | 293 ± 27.4 | 301 ± 57.5 | 263 ± 31 | 230 ± 35.1 | 215 ± 25.1 | 238 ± 34.5 | 205 ± 24.6 | 132 ± 14.2 | 0.2571 | NA | <0.0001 | 0.7500 | 0.5552 |
| | M | 394 ± 31.4 | 392 ± 46.2 | 362 ± 40 | 301 ± 41.1 | 251 ± 25.4 | 213 ± 36.8 | 163 ± 24.9 | 203 ± 40.7 | 125 ± 22.1 | 140 ± 25.5 | | | | | |
| | | a | a | ab | bc | cd | de | ef | de | ef | f | | | | | |

**C. Mouse Serum**

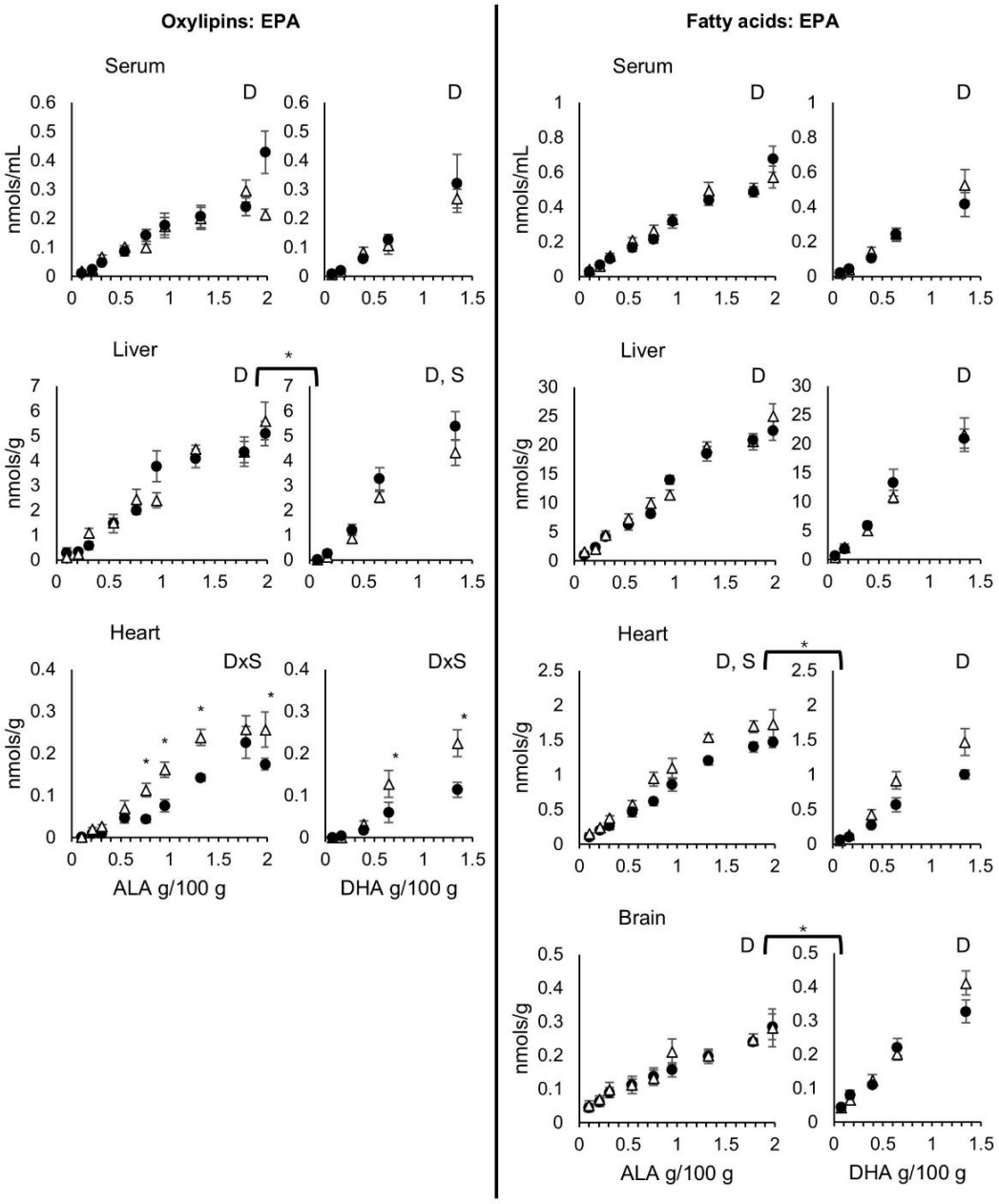
| Diet Group | | 0.08 | 0.13 | 0.14 | 0.27 | 0.56 | 0.82 | 1.2 | 1.52 | 1.88 | 2.56 | SW (P) | TSW (P) | Diet (P) | Sex (P) | Diet*Sex (P) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | *NE Oxylipins (nmols/mL)* | | | | | | | | | |
| **ALA** | F | 0.043 ± 0.015 | 0.089 ± 0.019 | 0.083 ± 0.008 | 0.170 ± 0.030 | 0.312 ± 0.067 | 0.373 ± 0.104 | 0.703 ± 0.144 | 0.669 ± 0.163 | 1.04 ± 0.226 | 0.953 ± 0.162 | <0.0001 | 0.8123 | <0.0001 | 0.1062 | 0.8353 |
| | M | 0.048 ± 0.011 | 0.056 ± 0.007 | 0.079 ± 0.027 | 0.092 ± 0.014 | 0.265 ± 0.064 | 0.310 ± 0.065 | 0.696 ± 0.133 | 0.647 ± 0.164 | 0.902 ± 0.124 | 0.87 ± 0.196 | | | | | |
| | | f | e | e | d | c | c | ab | b | a | ab | | | | | |
| **EPA** | F | 0.012 ± 0.005 | 0.043 ± 0.010 | 0.048 ± 0.006 | 0.069 ± 0.019 | 0.167 ± 0.016 | 0.257 ± 0.05 | 0.320 ± 0.077 | 0.376 ± 0.065 | 0.400 ± 0.081 | 0.429 ± 0.059 | <0.0001 | 0.5251 | <0.0001 | 0.1870 | 0.4305 |
| | M | 0.014 ± 0.003 | 0.029 ± 0.006 | 0.050 ± 0.009 | 0.060 ± 0.007 | 0.201 ± 0.034 | 0.201 ± 0.045 | 0.391 ± 0.064 | 0.527 ± 0.134 | 0.640 ± 0.119 | 0.519 ± 0.087 | | | | | |
| | | e | d | cd | c | b | b | a | a | a | a | | | | | |
| **DHA** | F | 0.213 ± 0.054 | 0.304 ± 0.051 | 0.33 ± 0.026 | 0.429 ± 0.086 | 0.585 ± 0.109 | 0.533 ± 0.118 | 0.695 ± 0.147 | 0.664 ± 0.104 | 0.565 ± 0.098 | 0.501 ± 0.121 | <0.0001 | 0.9570 | <0.0001 | 0.0032 | 0.3130 |
| | M | 0.144 ± 0.02 | 0.145 ± 0.018 | 0.228 ± 0.069 | 0.219 ± 0.027 | 0.406 ± 0.07 | 0.387 ± 0.072 | 0.792 ± 0.087 | 0.739 ± 0.239 | 0.560 ± 0.112 | 0.460 ± 0.084 | | | | | |
| | | e | de | d | cd | b | bc | a | ab | ab | b | | | | | |
| **ARA** | F | 1.11 ± 0.253 | 1.26 ± 0.3 | 0.809 ± 0.128 | 0.86 ± 0.357 | 0.699 ± 0.139 | 0.925 ± 0.191 | 0.523 ± 0.152 | 0.698 ± 0.144 | 0.548 ± 0.217 | 0.431 ± 0.093 | <0.0001 | 0.0401 | 0.0004 | <0.0001 | 0.4086 |
| | M | 0.521 ± 0.052 | 0.470 ± 0.08 | 0.869 ± 0.285 | 0.338 ± 0.036 | 0.339 ± 0.045 | 0.275 ± 0.058 | 0.300 ± 0.057 | 0.406 ± 0.109 | 0.545 ± 0.201 | 0.207 ± 0.018 | | | | | |
| | | a | a | a | ab | ab | ab | bc | ab | bc | c | | | | | |
| **LA** | F | 1.76 ± 0.532 | 2.4 ± 0.493 | 1.72 ± 0.203 | 2.37 ± 0.306 | 2.24 ± 0.497 | 1.59 ± 0.325 | 3.03 ± 0.980 | 2.51 ± 0.527 | 2.42 ± 0.433 | 1.96 ± 0.353 | <0.0001 | 0.9602 | 0.3987 | 0.0006 | 0.8519 |
| | M | 1.65 ± 0.417 | 1.38 ± 0.208 | 1.43 ± 0.506 | 1.12 ± 0.117 | 1.51 ± 0.314 | 1.42 ± 0.279 | 2.2 ± 0.391 | 1.75 ± 0.442 | 1.78 ± 0.271 | 1.33 ± 0.234 | | | | | |
| | | | | | | | *NE Fatty Acids (nmols/mL)* | | | | | | | | | |
| **ALA** | F | 0.099 ± 0.011 | 0.259 ± 0.037 | 0.25 ± 0.021 | 0.401 ± 0.039 | 0.88 ± 0.182 | 0.873 ± 0.104 | 2.12 ± 0.446 | 1.99 ± 0.225 | 2.74 ± 0.352 | 2.82 ± 0.263 | <0.0001 | 0.7262 | <0.0001 | 0.0156 | 0.2182 |
| | M | 0.113 ± 0.015 | 0.146 ± 0.012 | 0.186 ± 0.036 | 0.327 ± 0.030 | 0.871 ± 0.183 | 0.909 ± 0.063 | 1.42 ± 0.150 | 2.13 ± 0.215 | 2.36 ± 0.172 | 2.46 ± 0.261 | | | | | |
| | | f | e | e | d | c | c | b | ab | a | a | | | | | |
| **EPA** | F | ND | 0.083 ± 0.025 | 0.201 ± 0.042 | 0.265 ± 0.05 | 0.951 ± 0.082 | 1.21 ± 0.122 | 1.90 ± 0.190 | 2.39 ± 0.273 | 2.55 ± 0.340 | 2.67 ± 0.218 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | 0.1060 |
| | M | 0.029 ± 0.011 | 0.095 ± 0.015 | 0.170 ± 0.026 | 0.394 ± 0.051 | 1.49 ± 0.158 | 1.49 ± 0.151 | 2.03 ± 0.166 | 3.77 ± 0.648 | 2.80 ± 0.175 | 3.76 ± 0.295 | | | | | |
| | | g | fg | ef | e | d | d | c | ab | b | a | | | | | |
| **DHA** | F | 7.42 ± 0.476 | 11.0 ± 1.43 | 16.4 ± 1.46 | 15.7 ± 1.57 | 24.3 ± 3.34 | 21.5 ± 1.14 | 30.2 ± 2.07 | 31.0 ± 5.1 | 28.9 ± 4.02 | 24.9 ± 2.71 | <0.0001 | 0.2545 | <0.0001 | 0.0627 | 0.0061 |
| | | hi | g | ef | f | bcd | cde | ab | abc | bcd | bcd | | | | | |
| | M | 6.29 ± 0.592 | 6.35 ± 0.547 | 10.1 ± 0.811 | 13.4 ± 1.85 | 24.9 ± 3.48 | 21.3 ± 1.9 | 28.3 ± 2.00 | 41.5 ± 6.39 | 30 ± 3.38 | 28.5 ± 2.46 | | | | | |
| | | i | i | gh | fg | bcd | de | bcd | a | ab | bcd | | | | | |
| **ARA** | F | 7.37 ± 0.455 | 7.48 ± 0.848 | 7.98 ± 0.775 | 6.50 ± 1.01 | 7.03 ± 1.52 | 6.54 ± 0.485 | 6.50 ± 0.545 | 7.53 ± 1.05 | 5.45 ± 1.10 | 4.67 ± 0.524 | 0.0128 | 0.3771 | <0.0001 | <0.0001 | 0.3069 |
| | M | 6.49 ± 0.473 | 3.94 ± 0.532 | 5.84 ± 0.545 | 5.03 ± 0.622 | 5.01 ± 0.979 | 3.51 ± 0.495 | 3.40 ± 0.267 | 5.94 ± 0.704 | 3.89 ± 0.455 | 2.87 ± 0.344 | | | | | |
| | | a | bc | ab | abc | abc | c | c | ab | cd | d | | | | | |

**D. Mouse Brain**

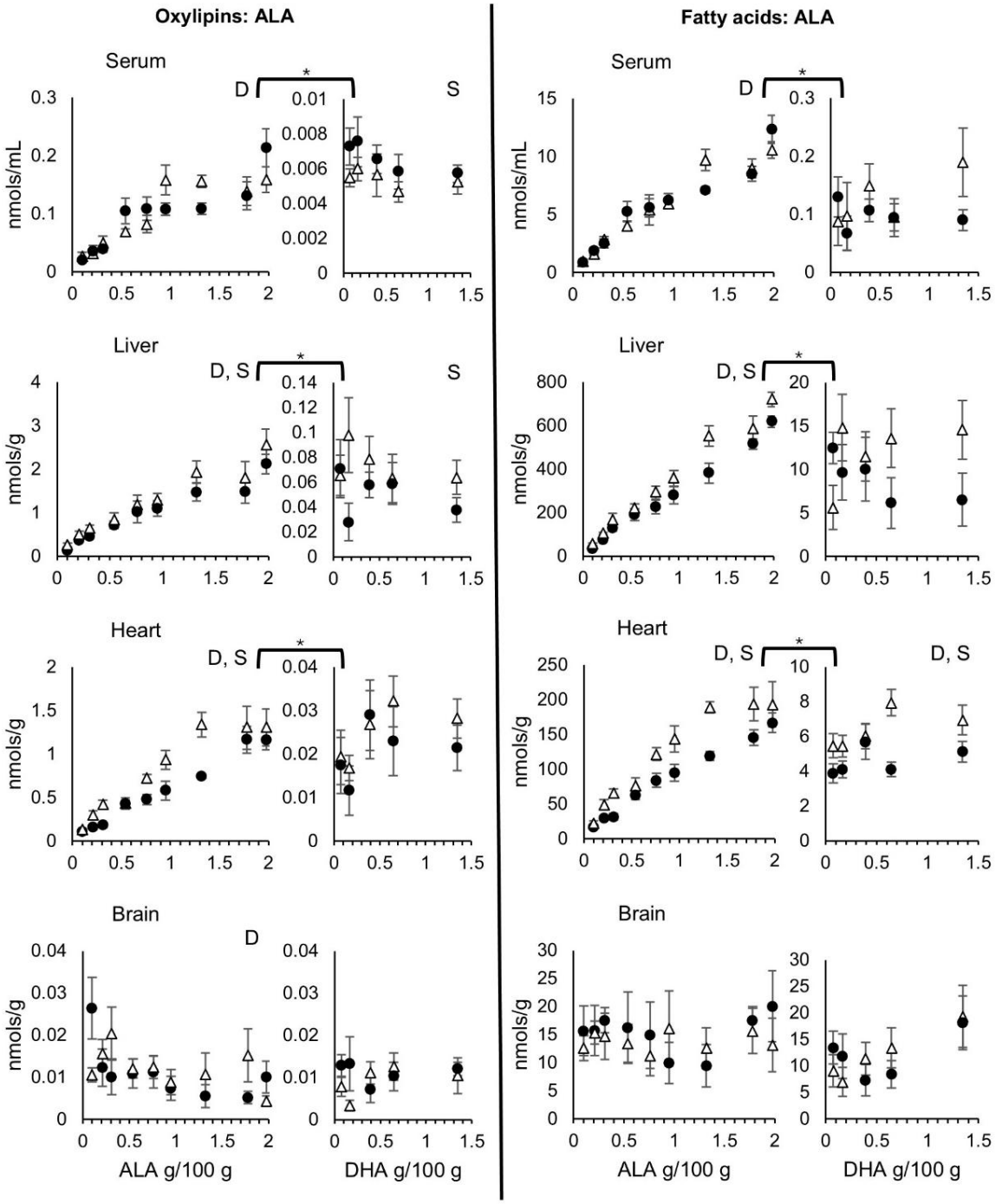
| Diet Group | | 0.08 | 0.13 | 0.14 | 0.27 | 0.56 | 0.82 | 1.2 | 1.52 | 1.88 | 2.56 | SW (P) | TSW (P) | Diet (P) | Sex (P) | Diet*Sex (P) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | *NE Oxylipins (nmols/g)* | | | | | | | | | |
| **ALA** | F | 7.05 ± 5.75 | 2.03 ± 1.11 | 0.214 ± 0.156 | 0.622 ± 0.290 | 1.37 ± 0.747 | 0.668 ± 0.368 | 1.26 ± 0.641 | 1.82 ± 0.807 | 1.61 ± 1.07 | 0.421 ± 0.285 | <0.0001 | 0.0004 | 0.5210 | 0.5973 | 0.6755 |
| | M | 1.09 ± 0.622 | 1.15 ± 0.466 | 1.01 ± 0.432 | 1.17 ± 0.682 | 0.566 ± 0.287 | 3.62 ± 2.38 | 1.11 ± 0.626 | 5.56 ± 3.04 | 0.908 ± 0.469 | 1.01 ± 0.548 | | | | | |
| **EPA** | F | 0.015 ± 0.007 fgh | 0.008 ± 0.006 h | 0.011 ± 0.003 h | 0.012 ± 0.001 h | 0.020 ± 0.004 fgh | 0.030 ± 0.005 fgh | 0.040 ± 0.009 efg | 0.065 ± 0.012 de | 0.059 ± 0.015 de | 0.087 ± 0.016 cd | 0.0010 | 0.0036 | <0.0001 | <0.0001 | <0.0001 |
| | M | 0.008 ± 0.002 h | 0.013 ± 0.002 gh | 0.015 ± 0.003 fgh | 0.026 ± 0.007 fgh | 0.041 ± 0.008 ef | 0.072 ± 0.012 cd | 0.067 ± 0.012 de | 0.100 ± 0.016 bc | 0.126 ± 0.009 b | 0.194 ± 0.023 a | | | | | |
| **DHA** | F | 2.20 ± 0.475 | 1.30 ± 0.156 | 1.57 ± 0.251 | 1.93 ± 0.268 | 1.69 ± 0.294 | 1.63 ± 0.210 | 2.27 ± 0.431 | 2.47 ± 0.260 | 2.13 ± 0.277 | 2.12 ± 0.316 | 0.0409 | 0.8298 | 0.4455 | 0.7872 | 0.2308 |
| | M | 1.45 ± 0.172 | 2.1 ± 0.271 | 1.87 ± 0.428 | 1.82 ± 0.301 | 1.86 ± 0.566 | 2.8 ± 0.512 | 1.97 ± 0.173 | 1.94 ± 0.241 | 1.76 ± 0.188 | 2.07 ± 0.199 | | | | | |
| **ARA** | F | 7.83 ± 0.841 | 6.34 ± 0.508 | 7.54 ± 0.653 | 7.04 ± 0.632 | 6.90 ± 0.516 | 6.75 ± 0.665 | 7.61 ± 0.657 | 8.19 ± 0.740 | 7.69 ± 0.306 | 7.7 ± 0.516 | 0.5180 | NA | 0.8317 | 0.0273 | 0.0730 |
| | M | 7.46 ± 0.298 | 8.67 ± 0.68 | 7.84 ± 0.846 | 8.13 ± 0.296 | 7.25 ± 0.748 | 9.79 ± 0.977 | 8.20 ± 0.474 | 8.00 ± 0.622 | 7.31 ± 0.345 | 7.38 ± 0.539 | | | | | |
| **LA** | F | 4.31 ± 2.90 | 5.95 ± 2.86 | 0.903 ± 0.529 | 2.03 ± 1.09 | 3.88 ± 1.78 | 2.13 ± 0.926 | 2.74 ± 1.50 | 5.48 ± 2.51 | 5.18 ± 3.21 | 1.57 ± 0.807 | <0.0001 | 0.0030 | 0.4689 | 0.6952 | 0.7953 |
| | M | 2.39 ± 1.43 | 2.64 ± 0.941 | 2.79 ± 1.09 | 2.53 ± 1.43 | 1.02 ± 0.356 | 9.07 ± 5.29 | 2.24 ± 1.11 | 14.2 ± 8.36 | 2.08 ± 1.07 | 1.79 ± 0.985 | | | | | |
| | | | | | | | *NE Fatty Acids (nmols/g)* | | | | | | | | | |
| **ALA** | F | 64.4 ± 44.3 | 13.5 ± 9.12 | 0.641 ± 0.641 | 3.72 ± 3.27 | 12.9 ± 9.64 | 3.22 ± 2.55 | 10.7 ± 4.97 | 6.26 ± 4.04 | 20.7 ± 19.7 | 4.11 ± 3.29 | <0.0001 | <0.0001 | 0.8080 | 0.6574 | 0.3522 |
| | M | 8.02 ± 7.67 | 10.4 ± 6.32 | 7.55 ± 4.90 | 13.4 ± 8.56 | 0.974 ± 0.493 | 27.9 ± 16.4 | 8.55 ± 6.54 | 60.0 ± 28.4 | 3.90 ± 2.36 | 7.11 ± 6.28 | | | | | |
| **EPA** | F | 0.487 ± 0.231 | 0.328 ± 0.057 | 0.378 ± 0.036 | 0.533 ± 0.055 | 0.85 ± 0.081 | 1.16 ± 0.029 | 1.83 ± 0.191 | 2.15 ± 0.109 | 2.40 ± 0.158 | 2.49 ± 0.199 | <0.0001 | <0.0001 | <0.0001 | 0.0008 | 0.1193 |
| | M | 0.261 ± 0.043 g | 0.411 ± 0.076 fg | 0.503 ± 0.069 f | 0.868 ± 0.104 e | 1.30 ± 0.213 d | 1.85 ± 0.232 c | 1.82 ± 0.309 c | 2.60 ± 0.170 b | 3.21 ± 0.201 ab | 4.42 ± 0.376 a | | | | | |
| **DHA** | F | 379 ± 51.5 | 341 ± 39.2 | 351 ± 31.8 | 408 ± 28.6 | 425 ± 54.4 | 410 ± 20.3 | 466 ± 46.9 | 490 ± 40.9 | 460 ± 15.1 | 438 ± 32.3 | 0.5423 | NA | 0.0813 | 0.8077 | 0.7390 |
| | M | 358 ± 30.8 | 382 ± 32.3 | 406 ± 43.9 | 466 ± 44.7 | 426 ± 57.6 | 454 ± 31.1 | 436 ± 38.8 | 414 ± 28.9 | 446 ± 30 | 418 ± 39.4 | | | | | |
| **ARA** | F | 368 ± 48.7 | 359 ± 44.6 | 348 ± 34.4 | 415 ± 26.5 | 402 ± 46.5 | 363 ± 30.1 | 420 ± 49.9 | 419 ± 32.9 | 374 ± 17.4 | 394 ± 29.7 | 0.1562 | NA | 0.9728 | 0.2188 | 0.9490 |
| | M | 420 ± 42.1 | 409 ± 39.7 | 411 ± 43.4 | 433 ± 45.9 | 390 ± 55.1 | 409 ± 28.9 | 418 ± 45.9 | 389 ± 28.9 | 417 ± 41.4 | 378 ± 38.3 | | | | | |

Data is reported as means ± SEs (n=5-6; N=119-120). The SW column shows the shapiro wilk test p value results and the TSW column shows shapiro-wilk p values for log transformed data. Data that were normal was analyzed using a two-way ANOVA for diet, sex and diet*sex effects shown in the Diet (P), Sex (P) and Diet*Sex (P) column. If data were not normal even following log-transformation, ANOVA was applied to data that were closest to normality. Post hoc results are shown under each group diet mean, with main effects under both female and male data, and interactions under each male and female group, as determined by Fisher's Protected LSD test ($p<0.05$). Different letters indicate statistical significance between diet groups ($p<0.05$). ND: not detected.

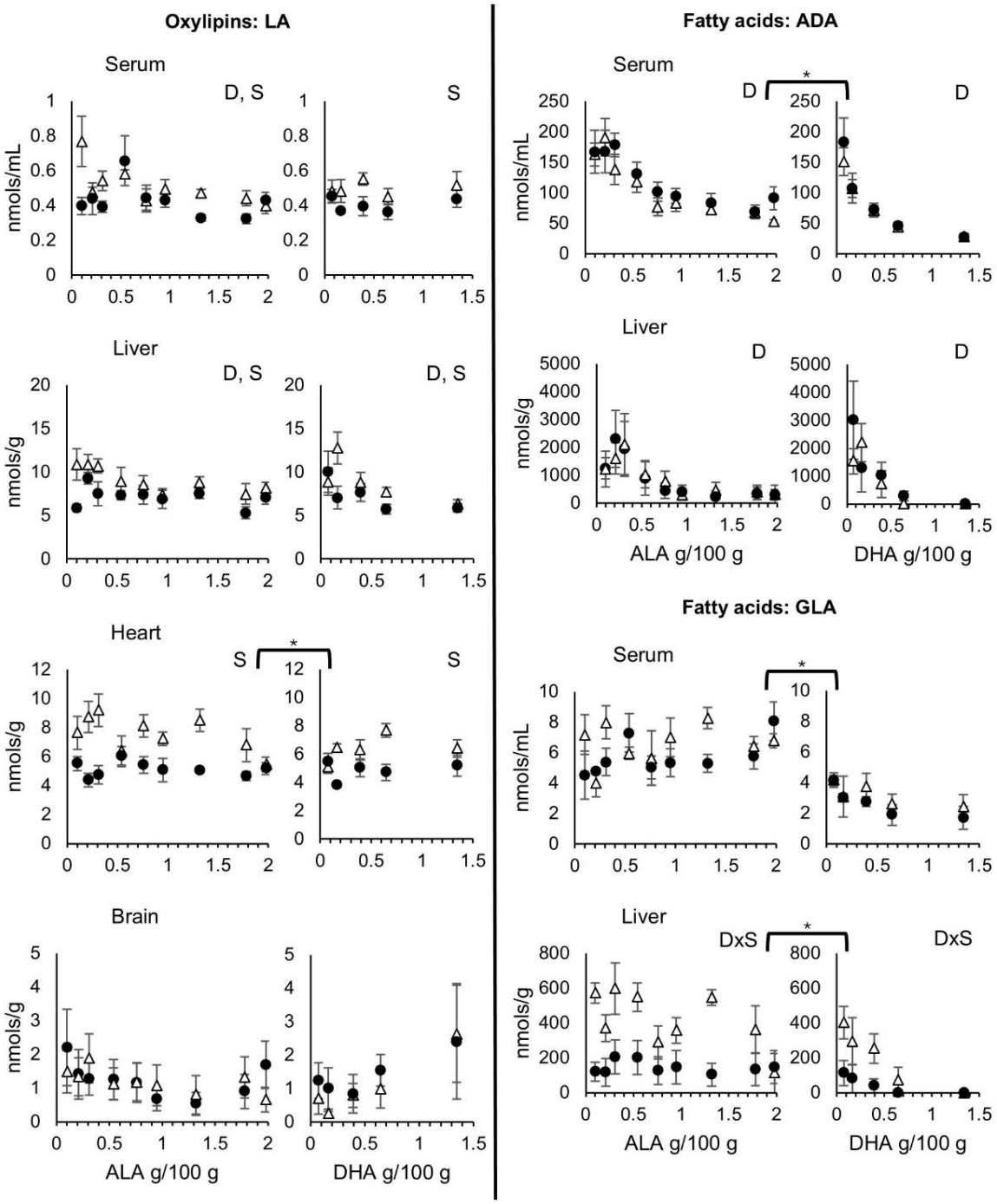
# Appendix 2: Supplemental figures and tables for Chapter 3




**Supplemental Figure 3-1**. Non-esterified total EPA oxylipins (left) and EPA (right) in response to dietary ALA and DHA in serum, liver, heart, and brain (mean±SE; n=6). Multiple linear regression was applied separately to dietary ALA and DHA groups to determine dose and sex effects. Results are shown on the upper right corner (D: Dose; S: Sex; DxS: interaction – for the interaction * denotes significant differences. "Indicates a result that was only significant when not adjusted by run). A separate multiple linear regression analysis was used to determine difference in effect between dietary ALA and DHA while controlling for dose and sex, with significance indicated by * above brackets between the dietary ALA and DHA graphs. ALA: grey; DHA: black; Males: filled circles; Females: outlined triangles. Complete statistical results are reported in Supplemental Tables 6-7.




**Supplemental Figure 3-2**. Non-esterified total ALA oxylipins (left) and ALA (right) in response to dietary ALA and DHA in serum, liver, heart, and brain (mean±SE; n=6). Multiple linear regression was applied separately to dietary ALA and DHA groups to determine dose and sex effects. Results are shown on the upper right corner (D: Dose; S: Sex; "Indicates a result that was only significant when not adjusted by run). A separate multiple linear regression analysis was used to determine difference in effect between dietary ALA and DHA while controlling for dose and sex, with significance indicated by * above brackets between the dietary ALA and DHA graphs. ALA: grey; DHA: black; Males: filled circles; Females: outlined triangles. Complete statistical results are reported in Supplemental Tables 6-7. Graphs for ALA and DHA diets are not on the same scale.




**Supplemental Figure 3-3**. Non-esterified total LA oxylipins (left), ADA (upper right) and GLA (lower right) in response to dietary ALA and DHA in serum, liver, heart, and brain (mean±SE; n=6). Multiple linear regression was applied separately to dietary ALA and DHA groups to determine dose and sex effects. Results are shown on the upper right corner (D: Dose; S: Sex; DxS: interaction – for the interaction * denotes significant differences. "Indicates a result that was only significant when not adjusted by run). A separate multiple linear regression analysis was used to determine difference in effect between dietary ALA and DHA while controlling for dose and sex, with significance indicated by * above brackets between the dietary ALA and DHA graphs. ALA: grey; DHA: black; Males: filled circles; Females: outlined triangles. Complete statistical results are reported in Supplemental Tables 6-7.

**Supplemental Table 3-1**.

DHA oil composition

| | % Total FA Mean ± SE |
|---|---|
| C12:0 | 0.001 ± <0.001 |
| C14:0 | 0.020 ± <0.001 |
| C16:0 | 0.598 ± 0.001 |
| C16:1t | 0.004 ± <0.001 |
| C16:1 | 0.026 ± <0.001 |
| C17:1 | 0.001 ± <0.001 |
| C18:0 | 0.026 ± 0.001 |
| C18:1 | 0.011 ± <0.001 |
| C18:2 | 0.046 ± 0.001 |
| C18:3n6 | 0.012 ± <0.001 |
| C18:3n3 | 0.051 ± 0.003 |
| C20:0 | 0.042 ± 0.001 |
| C20:3n6 | 0.470 ± 0.002 |
| C20:4 | 0.070 ± 0.003 |
| C20:5 | 0.418 ± 0.009 |
| C22:0 | 0.068 ± <0.001 |
| C22:1 | 0.005 ± 0.001 |
| C22:2 | 0.007 ± 0.001 |
| C22:5n3 | 0.069 ± 0.014 |
| C22:6n3 | 98.0 ± 0.020 |
| C24:0 | 0.040 ± 0.006 |

DHA oil analysis was conducted in triplicate and the results are shown as % of total fatty acid mass (mean ± SE).

**Supplemental Table 3-2**. Oxylipins detected and quantified that are included in total oxylipin sums in serum and each tissue, ordered by FA precursor. Oxylipins included in $DHA_{OH}$/$ARA_{OH}$ are in bold italics.

| **Serum** | **Liver** | **Heart** | **Brain** |
|---|---|---|---|
| | | **ALA** | |
| 9-HOTrE | 9-HOTrE | 9-HOTrE | 12,13 EpODE |
| 13-HOTrE | 13-HOTrE | 13-HOTrE | |
| 12,13 EpODE | 12,13 EpODE | 12,13 EpODE | |
| | 12,13 DiHODE | 12,13 DiHODE | |
| | | **EPA** | |
| 5-HEPE | 5-HEPE | 5-HEPE | |
| 8-HEPE | 8-HEPE | 12-HEPE | |
| 9-HEPE | 9-HEPE | 18-HEPE | |
| 11-HEPE | 11-HEPE | 14,15 EpETE | |
| 12-HEPE | 12-HEPE | 17,18 EpETE | |
| 15-HEPE | 15-HEPE | | |
| 18-HEPE | 18-HEPE | | |
| 14,15 DiHETE | 14,15 EpETE | | |
| 17,18 EpETE | 14,15 DiHETE | | |
| 17,18 DiHETE | 17,18 DiHETE | | |
| | | **DHA** | |
| ***4-HDoHE*** | ***4-HDoHE*** | ***4-HDoHE*** | ***4-HDoHE*** |
| ***7-HDoHE*** | ***7-HDoHE*** | ***7-HDoHE*** | ***7-HDoHE*** |
| ***8-HDoHE*** | ***8-HDoHE*** | ***8-HDoHE*** | ***8-HDoHE*** |
| ***10-HDoHE*** | ***10-HDoHE*** | ***10-HDoHE*** | ***10-HDoHE*** |
| ***11-HDoHE*** | ***11-HDoHE*** | ***11-HDoHE*** | ***11-HDoHE*** |
| ***13-HDoHE*** | ***13-HDoHE*** | ***13-HDoHE*** | ***13-HDoHE*** |
| ***14-HDoHE*** | ***14-HDoHE*** | ***14-HDoHE*** | ***14-HDoHE*** |
| ***16-HDoHE*** | ***16-HDoHE*** | ***16-HDoHE*** | ***16-HDoHE*** |
| ***17-HDoHE*** | ***17-HDoHE*** | ***17-HDoHE*** | ***17-HDoHE*** |
| ***20-HDoHE*** | ***20-HDoHE*** | ***20-HDoHE*** | ***20-HDoHE*** |
| 16,17 EpDoPE | 16,17 EpDoPE | 16,17 EpDoPE | |
| 16,17 DiHDoPE | 16,17 DiHDoPE | 19,20 EpDoPE | |
| 19,20 DiHDoPE | 19,20 EpDoPE | | |
| | 19,20 DiHDoPE | | |
| | | **ARA** | |
| $PGD_2$ | $PGD_2$ | $PGD_2$ | $PGD_2$ |
| $PGJ_2$ | $PGE_2$ | $PGE_2$ | $PGE_2$ |
| $PGE_2$ | 11β $PGE_2$ | 15-keto $PGE_2$ | 11β $PGE_2$ |
| 11β $PGE_2$ | 15-keto $PGE_2$ | 6-keto $PGF_{1\alpha}$ | 15-keto $PGE_2$ |
| 15-keto $PGE_2$ | $PGF_{2\alpha}$ | 12-HHTrE | 6-keto $PGF_{1\alpha}$ |
| $PGF_{2\alpha}$ | $TXB_2$ | ***5-HETE*** | $PGF_{2\alpha}$ |
| $TXB_2$ | 12-HHTrE | ***8-HETE*** | $TXB_2$ |
| 12-HHTrE | ***5-HETE*** | ***9-HETE*** | 12-HHTrE |

**Supplemental Table 3-2 (cont)**. Oxylipins detected and quantified that are included in total oxylipin sums in serum and each tissue, ordered by FA precursor. Oxylipins included in $DHA_{OH}$/$ARA_{OH}$ are in bold italics.

| | | | |
|---|---|---|---|
| ***5-HETE*** | ***8-HETE*** | ***11-HETE*** | ***5-HETE*** |
| ***8-HETE*** | ***9-HETE*** | ***12-HETE*** | ***8-HETE*** |
| ***9-HETE*** | ***11-HETE*** | ***15-HETE*** | ***9-HETE*** |
| ***11-HETE*** | ***12-HETE*** | ***16-HETE*** | ***11-HETE*** |
| ***12-HETE*** | ***15-HETE*** | 5,6 EpETrE | ***12-HETE*** |
| tetranor 12-HETE | 6S-LXA4 | 8,9 EpETrE | ***15-HETE*** |
| ***15-HETE*** | ***18-HETE*** | 8,9 DiHETrE | 6-trans $LTB_4$ |
| 6-trans $LTB_4$ | ***20-HETE*** | 11,12 EpETrE | 6-trans, 12epi $LTB_4$ |
| 6-trans, 12epi $LTB_4$ | 5,6 EpETrE | 14,15 EpETrE | 5,6 EpETrE |
| 6S-LXA4 | 5,6 DiHETrE | 14,15 DiHETrE | 5,6 DiHETrE |
| ***16-HETE*** | 8,9 EpETrE | | 8,9 EpETrE |
| ***18-HETE*** | 8,9 DiHETrE | | 11,12 EpETrE |
| 5,6 EpETrE | 11,12 EpETrE | | 14,15 EpETrE |
| 5,6 DiHETrE | 11,12 DiHETrE | | 14,15 DiHETrE |
| 8,9 EpETrE | 14,15 EpETrE | | |
| 8,9 DiHETrE | 14,15 DiHETrE | | |
| 11,12 EpETrE | | | |
| 11,12 DiHETrE | | | |
| 14,15 EpETrE | | | |
| 14,15 DiHETrE | | | |
| 20-COOH AA | | | |
| | | **LA** | |
| 9-HODE | 9-HODE | 9-HODE | 9-HODE |
| 13-HODE | 13-HODE | 13-HODE | 13-HODE |
| 9,10,13 TriHOME | 9,10,13 TriHOME | 9,10,13 TriHOME | 9,10,13 TriHOME |
| 9,12,13 TriHOME | 9,12,13 TriHOME | 9,12,13 TriHOME | 9,12,13 TriHOME |
| 9,10 EpOME | 9,10 EpOME | 9,10 EpOME | 9,10 EpOME |
| 9,10 DiHOME | 9,10 DiHOME | 9,10 DiHOME | 12,13 EpOME |
| 12,13 EpOME | 12,13 EpOME | 12,13 EpOME | |
| 12,13 DiHOME | 12,13 DiHOME | 12,13 DiHOME | |

Abbreviations: ARA, arachidonic acid; ALA, α-linolenic acid; DiHDoPE, dihydroxy-docosapentaenoic acid; diHETE, dihydroxy-eicosatetraenoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; diHODE, dihydroxy-octadecadienoic acid; diHOME, dihydroxy-octadecenoic acid; EpDoPE, epoxy-docosapentaenoic 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; HHTrE, hydroxy-heptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; LA, linoleic acid; LT, leukotriene; LX, lipoxin; PG, prostaglandin; triHOME, trihydroxy-octadecenoic acid; TX, thromboxane.

**Supplemental Table 3-3.** Animal body weight at termination, growth during study period, and tissue weight (liver, heart, brain) for rats in the ALA diets.

| | | *Body and Tissue Weights (g)* | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ALA Dose | | **0.097** | **0.212** | **0.305** | **0.537** | **0.764** | **0.946** | **1.32** | **1.78** | **1.98** | SW | TSW | ALA Dose | Sex |
| Body Weight | F | 292.6 ± 16.1 | 286.4 ± 17.5 | 326.8 ± 10.9 | 291.2 ± 10.5 | 298.6 ± 13.9 | 307.6 ± 15.1 | 303.7 ± 15.1 | 301.7 ± 5.45 | 302.9 ± 6.84 | 0.2614 | NA | 0.8040 | <0.0001 |
| | M | 523.4 ± 14.2 | 509.7 ± 14.3 | 533.0 ± 29.2 | 503.4 ± 19.0 | 504.2 ± 13.9 | 497.6 ± 14.8 | 514.6 ± 9.65 | 513.5 ± 15.8 | 524.6 ± 10.2 | | | | |
| Growth | F | 200.5 ± 13.3 | 198.7 ± 14.3 | 235.9 ± 11.0 | 204.2 ± 8.93 | 207.2 ± 11.6 | 214.4 ± 14.1 | 216.5 ± 12.0 | 209.3 ± 9.34 | 215.2 ± 9.01 | 0.5341 | NA | 0.7510 | <0.0001 |
| | M | 423.7 ± 14.7 | 409.0 ± 11.4 | 428.6 ± 25.8 | 403.7 ± 19.3 | 405.1 ± 13.2 | 395.0 ± 12.0 | 413.8 ± 10.3 | 412.7 ± 12.9 | 423.9 ± 8.71 | | | | |
| Liver | F | 10.1 ± 0.716 | 10.9 ± 0.79 | 12.1 ± 0.525 | 11.1 ± 0.685 | 11.7 ± 0.818 | 12.7 ± 1.33 | 11.3 ± 0.641 | 11.0 ± 0.185 | 12.1 ± 0.288 | 0.0024 | 0.2377 | 0.4490 | <0.0001 |
| | M | 21.6 ± 1.30 | 19.9 ± 0.533 | 21.9 ± 2.08 | 19.9 ± 1.75 | 20.2 ± 1.62 | 19.5 ± 0.950 | 19.9 ± 0.688 | 20.0 ± 0.831 | 20.9 ± 0.741 | | | | |
| Heart | F | 1.01 ± 0.044 | 1.06 ± 0.048 | 1.18 ± 0.048 | 1.09 ± 0.042 | 1.05 ± 0.052 | 1.16 ± 0.069 | 1.09 ± 0.074 | 1.09 ± 0.030 | 1.15 ± 0.051 | 0.5940 | NA | 0.6610 | <0.0001 |
| | M | 1.70 ± 0.060 | 1.75 ± 0.045 | 1.75 ± 0.093 | 1.65 ± 0.066 | 1.64 ± 0.046 | 1.55 ± 0.039 | 1.67 ± 0.033 | 1.70 ± 0.029 | 1.60 ± 0.045 | | | | |
| Brain | F | 1.95 ± 0.026 | 1.94 ± 0.047 | 1.94 ± 0.032 | 1.92 ± 0.061 | 1.91 ± 0.019 | 1.84 ± 0.025 | 1.90 ± 0.065 | 1.96 ± 0.018 | 1.85 ± 0.051 | 0.0382 | 0.0083 | 0.1380 | <0.0001 |
| | M | 2.09 ± 0.030 | 2.08 ± 0.082 | 2.16 ± 0.041 | 2.11 ± 0.045 | 2.10 ± 0.051 | 2.11 ± 0.028 | 2.05 ± 0.093 | 2.08 ± 0.063 | 2.07 ± 0.058 | | | | |

Data reported as mean ± SE (n=6; N=108). The SW column shows the Shapiro-Wilk test p value results and the TSW column shows Shapiro-Wilk p values for log transformed data. Data that were normal were analyzed using multiple linear regression for diet dosage and sex effects. If data were not normal even following log-transformation, multiple linear regression was applied to data that were closest to normality.

**Supplemental Table 3-4.** Animal body weight at termination, growth during study period, and tissue weight (liver, heart, brain) for rats in the DHA diets.

| | | *Body and Tissue Weights (g)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| DHA Dose | | **0.074** | **0.165** | **0.392** | **0.646** | **1.35** | SW | DHA Dose | Sex |
| Body Weight | F | 301.5 ± 11.7 | 320.4 ± 13.8 | 308.2 ± 15.5 | 301.3 ± 7.77 | 303.8 ± 8.53 | 0.0812 | 0.9620 | <0.0001 |
| | M | 520.2 ± 16.7 | 526.6 ± 20.7 | 507.9 ± 9.39 | 522.6 ± 20.7 | 525.7 ± 16.2 | | | |
| Growth | F | 212.2 ± 9.59 | 229.7 ± 11.6 | 220.7 ± 11.8 | 210.1 ± 6.36 | 215.4 ± 9.87 | 0.6346 | 0.9250 | <0.0001 |
| | M | 420.9 ± 15.3 | 426.6 ± 17.2 | 409.0 ± 7.74 | 422.0 ± 17.4 | 424.9 ± 16.1 | | | |
| Liver | F | 12.1 ± 0.563 | 12.3 ± 0.755 | 11.8 ± 0.757 | 11.7 ± 0.423 | 11.3 ± 0.800 | 0.3607 | 0.2380 | <0.0001 |
| | M | 20.9 ± 1.25 | 21.1 ± 0.969 | 20 ± 0.567 | 20.2 ± 0.984 | 20.2 ± 1.02 | | | |
| Heart | F | 1.10 ± 0.054 | 1.12 ± 0.045 | 1.11 ± 0.059 | 1.1 ± 0.041 | 1.12 ± 0.044 | 0.6125 | 0.7870 | <0.0001 |
| | M | 1.69 ± 0.073 | 1.77 ± 0.094 | 1.66 ± 0.064 | 1.66 ± 0.054 | 1.69 ± 0.075 | | | |
| Brain | F | 1.93 ± 0.054 | 1.95 ± 0.028 | 1.92 ± 0.031 | 1.92 ± 0.037 | 2.03 ± 0.030 | 0.6944 | 0.2070 | <0.0001 |
| | M | 2.17 ± 0.039 | 2.07 ± 0.041 | 2.17 ± 0.038 | 2.15 ± 0.052 | 2.13 ± 0.038 | | | |

Data reported as mean ± SE (n=6; N=60). The SW column shows the Shapiro-Wilk test p value results and the TSW column shows Shapiro-Wilk p values for log transformed data. Data that were normal were analyzed using multiple linear regression for diet dosage and sex effects. If data were not normal even following log-transformation, multiple linear regression was applied to data that were closest to normality.

**Supplemental Table 3-5**. Equations used for DHA bioequivalence calculation

| | Female | | | | Male | | | |
|---|---|---|---|---|---|---|---|---|
| | ALA Equation | $R^2$ | DHA Equation | $R^2$ | ALA Equation | $R^2$ | DHA Equation | $R^2$ |
| | | | | *Serum* | | | | |
| $DHA_{OH}/ARA_{OH}$ | y=2.1269x -4.8427 | 0.61 | y=1.3240ln(x) -0.6715 | 0.88 | y=3.0262x -5.1523 | 0.45 | y=1.2482ln(x) -0.8446 | 0.81 |
| DHA/ARA | y=1.6825x -1.2925 | 0.42 | y=0.8447ln(x) +1.5890 | 0.87 | y=3.1785x -1.7953 | 0.48 | y=0.7135ln(x) +1.4049 | 0.91 |
| | | | | *Liver* | | | | |
| $DHA_{OH}/ARA_{OH}$ | y=2.2966x -1.5572 | 0.71 | y=1.0347ln(x) +1.9322 | 0.94 | y=4.0836x -2.2439 | 0.62 | y=1.0397ln(x) +1.7213 | 0.94 |
| DHA/ARA | y=2.5907x -0.8025 | 0.74 | y=0.699ln(x) +1.8870 | 0.89 | y=2.5023x -1.0712 | 0.53 | y=0.7466n(x) +1.7679 | 0.94 |
| | | | | *Heart* | | | | |
| $DHA_{OH}/ARA_{OH}$ | y=4.9895x -2.6313 | 0.60 | y=1.2450ln(x) +2.0807 | 0.94 | y=3.9687x -2.5930 | 0.39 | y=1.0429ln(x) +1.7103 | 0.93 |
| DHA/ARA | y=3.8284x -1.9600 | 0.31 | y=1.0484ln(x) +2.1030 | 0.90 | y=4.8591x -2.5242 | 0.43 | y=0.8940ln(x) +1.4981 | 0.82 |

Equation parameters used for determination of DHA bioequivalence: Y equals the ratio of interest, and x equals level of dietary ALA or DHA as applicable.

**Supplemental Table 3-6**. Complete multiple linear regression statistics for total non-esterified oxylipins by FA precursor and for non-esterified FA for rats that received ALA diets in rat serum (A), liver (B), heart (C), and Brain (D).

**A. Serum**

| ALA Dose | | **0.097** | **0.212** | **0.305** | **0.537** | **0.764** | **0.946** | **1.32** | **1.78** | **1.98** | SW (P) | Log-T SW (P) | ALA Dose | Sex | Dose*Sex |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | *Oxylipins (nmols/g)* | | | | | | | | |
| DHA | F | 0.046 ± 0.007 | 0.058 ± 0.005 | 0.072 ± 0.010 | 0.102 ± 0.014 | 0.078 ± 0.014 | 0.144 ± 0.025 | 0.081 ± 0.009 | 0.099 ± 0.015 | 0.072 ± 0.015 | <0.0001 | 0.8455 | 0.0001 | 0.0676 | 0.1966 |
| | M | 0.034 ± 0.004 | 0.046 ± 0.009 | 0.055 ± 0.007 | 0.091 ± 0.023 | 0.083 ± 0.010 | 0.084 ± 0.015 | 0.076 ± 0.007 | 0.079 ± 0.013 | 0.093 ± 0.018 | | | | | |
| | | d | cd | bc | a | ab | a | ab | ab | ab | | | | | |
| EPA | F | 0.017 ± 0.004 | 0.023 ± 0.004 | 0.066 ± 0.007 | 0.100 ± 0.007 | 0.099 ± 0.012 | 0.173 ± 0.03 | 0.200 ± 0.037 | 0.295 ± 0.038 | 0.213 ± 0.019 | <0.0001 | 0.1178 | <0.0001 | 0.6660 | 0.0876 |
| | M | 0.010 ± 0.002 | 0.023 ± 0.005 | 0.046 ± 0.004 | 0.085 ± 0.015 | 0.141 ± 0.021 | 0.176 ± 0.042 | 0.207 ± 0.038 | 0.239 ± 0.031 | 0.428 ± 0.073 | | | | | |
| | | g | f | e | de | cd | bc | abc | ab | a | | | | | |
| ALA | F | 0.027 ± 0.007 | 0.032 ± 0.003 | 0.050 ± 0.011 | 0.069 ± 0.005 | 0.082 ± 0.015 | 0.158 ± 0.025 | 0.156 ± 0.011 | 0.139 ± 0.024 | 0.159 ± 0.022 | 0.0001 | 0.8665 | <0.0001 | 0.7160 | 0.6060 |
| | M | 0.020 ± 0.003 | 0.035 ± 0.009 | 0.039 ± 0.006 | 0.104 ± 0.022 | 0.109 ± 0.020 | 0.108 ± 0.011 | 0.109 ± 0.009 | 0.131 ± 0.024 | 0.214 ± 0.032 | | | | | |
| | | e | de | d | c | bc | ab | ab | ab | a | | | | | |
| ARA | F | 2.63 ± 0.174 | 2.07 ± 0.240 | 2.97 ± 0.532 | 2.37 ± 0.267 | 1.40 ± 0.216 | 1.89 ± 0.386 | 1.52 ± 0.326 | 2.00 ± 0.178 | 1.20 ± 0.102 | 0.0326 | 0.0051 | 0.0003 | 0.0880 | 0.0278 |
| | | ab | bcdef | a | abcd | ef | bcdef | def | bcdef | f | | | | | |
| | M | 2.15 ± 0.244 | 2.05 ± 0.306 | 2.28 ± 0.241 | 1.93 ± 0.229 | 2.16 ± 0.271 | 1.95 ± 0.499 | 1.75 ± 0.232 | 1.51 ± 0.255 | 2.48 ± 0.387 | | | | | |
| | | abcde | bcdef | abcde | bcdef | abcde | bcdef | cdef | def | abc | | | | | |
| LA | F | 0.769 ± 0.145 | 0.475 ± 0.03 | 0.542 ± 0.056 | 0.580 ± 0.022 | 0.428 ± 0.066 | 0.495 ± 0.054 | 0.472 ± 0.020 | 0.441 ± 0.044 | 0.398 ± 0.044 | <0.0001 | 0.0929 | 0.0039 | 0.0022 | 0.2381 |
| | M | 0.397 ± 0.049 | 0.439 ± 0.092 | 0.392 ± 0.032 | 0.656 ± 0.144 | 0.444 ± 0.073 | 0.431 ± 0.041 | 0.328 ± 0.018 | 0.323 ± 0.028 | 0.432 ± 0.045 | | | | | |
| | | ab | bc | abc | a | bc | abc | c | c | bc | | | | | |
| | | | | | | | *Fatty acids (nmols/g)* | | | | | | | | |
| DHA | F | 3.51 ± 0.242 | 5.46 ± 0.418 | 6.63 ± 0.721 | 7.67 ± 1.15 | 7.73 ± 0.691 | 9.73 ± 1.07 | 8.56 ± 1.02 | 9.02 ± 0.299 | 8.72 ± 0.659 | 0.0008 | 0.5866 | <0.0001 | 0.0262 | 0.4240 |
| | M | 3.16 ± 0.348 | 4.50 ± 0.427 | 5.69 ± 0.220 | 6.54 ± 0.221 | 6.87 ± 0.623 | 6.82 ± 0.614 | 8.40 ± 1.02 | 7.77 ± 0.595 | 9.90 ± 1.58 | | | | | |
| | | e | d | cd | bc | abc | ab | ab | ab | a | | | | | |

**A. Serum (cont)**

| ALA Dose | | **0.097** | **0.212** | **0.305** | **0.537** | **0.764** | **0.946** | **1.32** | **1.78** | **1.98** | SW (P) | Log-T SW (P) | ALA Dose | Sex | Dose*Sex |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EPA | F | 0.042 ± 0.006 | 0.061 ± 0.005 | 0.120 ± 0.014 | 0.207 ± 0.018 | 0.261 ± 0.034 | 0.330 ± 0.028 | 0.495 ± 0.045 | 0.503 ± 0.031 | 0.571 ± 0.062 | 0.0098 | 0.0080 | <0.0001 | 0.2370 | 0.2680 |
| | M | 0.029 ± 0.008 | 0.068 ± 0.006 | 0.103 ± 0.014 | 0.166 ± 0.022 | 0.216 ± 0.024 | 0.316 ± 0.037 | 0.438 ± 0.028 | 0.488 ± 0.029 | 0.675 ± 0.075 | | | | | |
| | | g | f | e | d | cd | bc | ab | a | a | | | | | |
| ALA | F | 1.01 ± 0.165 | 1.61 ± 0.098 | 2.88 ± 0.224 | 4.01 ± 0.383 | 5.41 ± 1.31 | 5.95 ± 0.457 | 9.69 ± 0.928 | 8.98 ± 0.832 | 10.6 ± 0.712 | <0.0001 | 0.3899 | <0.0001 | 0.9940 | 0.9580 |
| | M | 0.901 ± 0.196 | 1.90 ± 0.251 | 2.52 ± 0.382 | 5.28 ± 0.838 | 5.60 ± 0.797 | 6.24 ± 0.570 | 7.12 ± 0.278 | 8.51 ± 0.643 | 12.4 ± 1.19 | | | | | |
| | | f | e | d | c | c | bc | ab | ab | a | | | | | |
| ARA | F | 7.91 ± 0.448 | 10.3 ± 0.395 | 10.5 ± 1.03 | 10.1 ± 0.912 | 8.69 ± 0.819 | 10.1 ± 1.01 | 9.60 ± 1.15 | 9.25 ± 0.445 | 8.86 ± 0.359 | 0.0903 | NA | 0.5230 | 0.5260 | 0.1690 |
| | M | 8.92 ± 0.841 | 9.42 ± 0.660 | 10.2 ± 0.523 | 9.30 ± 0.434 | 10.1 ± 0.59 | 9.13 ± 0.543 | 9.75 ± 0.963 | 8.47 ± 0.767 | 12.2 ± 1.38 | | | | | |
| ADA | F | 162.6 ± 18.8 | 190.5 ± 30.7 | 138.0 ± 24.7 | 118.3 ± 18.1 | 76.4 ± 14.6 | 82.7 ± 13.3 | 72.4 ± 6.15 | 67.7 ± 6.98 | 53.1 ± 2.76 | 0.0444 | 0.0108 | <0.0001 | 0.1620 | 0.5940 |
| | M | 166.9 ± 35.3 | 167.4 ± 34.4 | 178.2 ± 19.1 | 130.7 ± 19.5 | 101.6 ± 15.4 | 94.5 ± 12.5 | 83.4 ± 15.7 | 68.5 ± 10.8 | 90.9 ± 18.4 | | | | | |
| | | a | a | ab | bc | cd | cd | d | d | d | | | | | |
| GLA | F | 7.18 ± 1.30 | 4.00 ± 0.903 | 7.98 ± 1.08 | 5.96 ± 0.404 | 5.64 ± 1.77 | 7.00 ± 1.27 | 8.26 ± 0.703 | 6.43 ± 0.614 | 6.78 ± 0.452 | 0.2773 | NA | 0.4170 | 0.1080 | 0.4720 |
| | M | 4.52 ± 1.57 | 4.79 ± 0.182 | 5.38 ± 0.896 | 7.28 ± 1.26 | 5.03 ± 0.752 | 5.32 ± 0.927 | 5.28 ± 0.580 | 5.75 ± 0.829 | 8.07 ± 1.26 | | | | | |

**B. Liver**

| ALA Dose | | **0.097** | **0.212** | **0.305** | **0.537** | **0.764** | **0.946** | **1.32** | **1.78** | **1.98** | SW (P) | Log-T SW (P) | ALA Dose | Sex | Dose*Sex |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | *Oxylipins (nmols/g)* | | | | | | | | |
| DHA | F | 4.25 ± 0.809 | 4.32 ± 0.575 | 6.68 ± 1.07 | 6.55 ± 0.858 | 7.49 ± 1.17 | 7.04 ± 1.33 | 5.78 ± 0.725 | 6.71 ± 1.91 | 5.52 ± 0.557 | 0.0003 | 0.0130 | 0.0123 | 0.0009 | 0.1926 |
| | M | 1.99 ± 0.491 | 4.56 ± 0.363 | 3.86 ± 1.23 | 4.34 ± 0.578 | 5.73 ± 0.813 | 4.77 ± 0.623 | 5.62 ± 1.09 | 3.93 ± 0.507 | 5.62 ± 0.738 | | | | | |
| | | b | a | a | a | a | a | a | a | a | | | | | |
| EPA | F | 0.101 ± 0.065 | 0.239 ± 0.098 | 1.09 ± 0.177 | 1.51 ± 0.168 | 2.44 ± 0.418 | 2.41 ± 0.303 | 4.47 ± 0.155 | 4.36 ± 0.599 | 5.59 ± 0.756 | 0.0001 | <0.0001 | <0.0001 | 0.8870 | 0.6630 |
| | M | 0.279 ± 0.211 | 0.328 ± 0.126 | 0.581 ± 0.162 | 1.47 ± 0.365 | 1.99 ± 0.160 | 3.78 ± 0.627 | 4.09 ± 0.369 | 4.35 ± 0.432 | 5.10 ± 0.494 | | | | | |
| | | f | f | ef | de | d | c | b | b | a | | | | | |

**B. Liver (cont)**

| ALA Dose | | **0.097** | **0.212** | **0.305** | **0.537** | **0.764** | **0.946** | **1.32** | **1.78** | **1.98** | SW (P) | Log-T SW (P) | ALA Dose | Sex | Dose*Sex |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ALA | F | 0.262 ± 0.044 | 0.507 ± 0.065 | 0.653 ± 0.069 | 0.843 ± 0.153 | 1.19 ± 0.218 | 1.29 ± 0.161 | 1.94 ± 0.249 | 1.81 ± 0.375 | 2.57 ± 0.356 | 0.0008 | 0.2705 | <0.0001 | 0.0046 | 0.4076 |
| | M | 0.143 ± 0.026 | 0.372 ± 0.028 | 0.465 ± 0.052 | 0.722 ± 0.088 | 1.02 ± 0.253 | 1.10 ± 0.175 | 1.47 ± 0.204 | 1.49 ± 0.266 | 2.12 ± 0.221 | | | | | |
| | | g | f | ef | de | cd | bc | ab | b | a | | | | | |
| ARA | F | 10.5 ± 0.931 | 8.70 ± 0.884 | 10.5 ± 0.905 | 7.71 ± 0.576 | 6.19 ± 0.611 | 5.48 ± 0.706 | 5.80 ± 0.505 | 4.85 ± 0.64 | 5.15 ± 0.417 | 0.0076 | 0.6220 | <0.0001 | 0.4020 | 0.9940 |
| | M | 8.70 ± 1.08 | 10.0 ± 0.743 | 8.06 ± 0.789 | 7.56 ± 0.166 | 6.37 ± 0.382 | 6.15 ± 0.748 | 5.97 ± 0.282 | 4.28 ± 0.622 | 4.64 ± 0.355 | | | | | |
| | | a | ab | ab | b | c | cd | cd | e | de | | | | | |
| LA | F | 10.9 ± 1.79 | 10.9 ± 1.19 | 10.7 ± 0.785 | 8.96 ± 1.56 | 8.56 ± 1.01 | 7.38 ± 0.715 | 8.82 ± 0.656 | 7.50 ± 1.18 | 8.17 ± 0.676 | 0.0003 | 0.4500 | 0.0037 | <0.0001 | 0.3426 |
| | M | 5.89 ± 0.403 | 9.29 ± 0.696 | 7.51 ± 1.40 | 7.33 ± 0.520 | 7.38 ± 1.07 | 6.89 ± 1.06 | 7.55 ± 0.594 | 5.31 ± 0.656 | 7.09 ± 0.800 | | | | | |
| | | b | a | ab | ab | b | bc | ab | c | bc | | | | | |
| | | | | | | | *Fatty acids (nmols/g)* | | | | | | | | |
| DHA | F | 207.2 ± 13.5 | 235.8 ± 15.4 | 306.4 ± 33.9 | 346.4 ± 38.2 | 322.5 ± 21.1 | 366.3 ± 20.7 | 361.9 ± 20.8 | 384 ± 32.5 | 408.2 ± 31.1 | 0.0094 | 0.5353 | <0.0001 | 0.0022 | 0.5105 |
| | M | 146.2 ± 7.59 | 222.4 ± 14.6 | 277.4 ± 20.4 | 280.2 ± 13.1 | 289.8 ± 20.8 | 320.6 ± 24.9 | 350.1 ± 39.3 | 338.6 ± 23.2 | 358.5 ± 14.8 | | | | | |
| | | e | d | c | bc | bc | abc | ab | ab | a | | | | | |
| EPA | F | 1.52 ± 0.173 | 2.02 ± 0.183 | 4.47 ± 0.696 | 7.25 ± 0.833 | 9.99 ± 0.872 | 11.4 ± 0.79 | 19.6 ± 0.911 | 20.6 ± 1.38 | 25.0 ± 2.18 | 0.0002 | 0.0327 | <0.0001 | 0.4180 | 0.5940 |
| | M | 0.949 ± 0.128 | 2.30 ± 0.144 | 4.17 ± 0.312 | 6.26 ± 0.974 | 8.16 ± 0.550 | 14.0 ± 0.774 | 18.5 ± 1.27 | 20.9 ± 0.771 | 22.5 ± 1.61 | | | | | |
| | | f | e | d | c | bc | b | a | a | a | | | | | |
| ALA | F | 54.1 ± 6.72 | 103.6 ± 5.67 | 164.8 ± 31.9 | 219.6 ± 19.4 | 293.3 ± 29.4 | 358.6 ± 35.5 | 551.6 ± 47.1 | 584.4 ± 58.2 | 721.2 ± 33.6 | 0.0247 | 0.1594 | <0.0001 | 0.0011 | 0.3950 |
| | M | 31.8 ± 2.67 | 74.5 ± 7.43 | 127.7 ± 13.6 | 189.9 ± 27.7 | 226.0 ± 32.6 | 279.5 ± 39.5 | 382.1 ± 46.4 | 515.6 ± 22.8 | 618.1 ± 24.9 | | | | | |
| | | g | f | e | d | cd | c | b | ab | a | | | | | |
| ARA | F | 310.0 ± 20.5 | 265.7 ± 14.3 | 311.3 ± 24.5 | 298.6 ± 32.4 | 241.0 ± 22.3 | 239.2 ± 20.5 | 284.1 ± 19.0 | 263.8 ± 22.4 | 255.7 ± 14.5 | 0.0093 | 0.7277 | 0.0035 | 0.4332 | 0.6532 |
| | M | 260.1 ± 12.1 | 319.9 ± 25.2 | 343.7 ± 26.6 | 273.2 ± 11.3 | 264.8 ± 18.2 | 278.4 ± 17.1 | 292.1 ± 19.7 | 260.6 ± 23.2 | 240.4 ± 14.7 | | | | | |
| | | abc | ab | a | abc | bc | bc | abc | bc | c | | | | | |

**B. Liver (cont)**

| ALA Dose | | **0.097** | **0.212** | **0.305** | **0.537** | **0.764** | **0.946** | **1.32** | **1.78** | **1.98** | SW (P) | Log-T SW (P) | ALA Dose | Sex | Dose*Sex |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ADA | F | 1222 ± 639.2 | 1608 ± 567.1 | 2114 ± 1092 | 1031.4 ± 489.3 | 783.7 ± 358.8 | 306.7 ± 194.7 | 458.5 ± 290.5 | 400.0 ± 253.1 | 317.3 ± 317.3 | <0.0001 | <0.0001 | 0.0001 | 0.9470 | 0.7523 |
| | M | 1245 ± 389.1 | 2312 ± 1024 | 1942 ± 986.7 | 882.1 ± 609.1 | 447.4 ± 283.0 | 394.0 ± 249.6 | 222.8 ± 222.8 | 346.5 ± 221.8 | 299.2 ± 189.5 | | | | | |
| | | abc | ab | a | bc | c | c | c | c | c | | | | | |
| GLA | F | 573.4 ± 58.2 | 371.3 ± 77.2 | 599.0 ± 146.7 | 548.9 ± 81.6 | 288.9 ± 94.7 | 357.4 ± 74.2 | 546.9 ± 45.9 | 361.8 ± 135.7 | 113.4 ± 113.3 | 0.0014 | 0.0001 | 0.0025 | <0.0001 | 0.0433 |
| | | a | abc | a | a | bcd | abcd | ab | abcd | cd | | | | | |
| | M | 119.9 ± 53.6 | 117.0 ± 79.9 | 204.9 ± 96.8 | 201.8 ± 97.2 | 126.2 ± 79.9 | 146.0 ± 95.3 | 103.3 ± 65.9 | 134.4 ± 94.5 | 147.4 ± 94.0 | | | | | |
| | | cd | cd | cd | cd | cd | cd | d | cd | cd | | | | | |

**C. Heart**

| ALA Dose | | **0.097** | **0.212** | **0.305** | **0.537** | **0.764** | **0.946** | **1.32** | **1.78** | **1.98** | SW (P) | Log-T SW (P) | ALA Dose | Sex | Dose*Sex |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | *Oxylipins (nmols/g)* | | | | | | | | |
| DHA | F | 1.24 ± 0.115 | 2.03 ± 0.326 | 3.19 ± 0.601 | 2.43 ± 0.386 | 2.78 ± 0.311 | 2.65 ± 0.292 | 2.73 ± 0.320 | 2.90 ± 0.192 | 2.74 ± 0.454 | 0.0060 | <0.0001 | 0.0019 | 0.0026 | 0.8984 |
| | M | 1.11 ± 0.287 | 1.79 ± 0.290 | 1.54 ± 0.307 | 2.62 ± 0.215 | 2.17 ± 0.307 | 1.79 ± 0.376 | 2.46 ± 0.264 | 2.47 ± 0.466 | 2.03 ± 0.281 | | | | | |
| | | c | b | ab | ab | ab | ab | ab | a | ab | | | | | |
| EPA | F | 0.002 ± 0.002 | 0.021 ± 0.003 | 0.027 ± 0.004 | 0.07 ± 0.018 | 0.113 ± 0.016 | 0.162 ± 0.018 | 0.238 ± 0.019 | 0.258 ± 0.032 | 0.257 ± 0.042 | <0.0001 | <0.0001 | <0.0001 | 0.3418 | 0.0144 |
| | | h | gh | fgh | efg | de | cd | a | a | a | | | | | |
| | M | 0.001 ± 0.001 | 0.010 ± 0.006 | 0.010 ± 0.003 | 0.047 ± 0.013 | 0.044 ± 0.008 | 0.076 ± 0.015 | 0.143 ± 0.008 | 0.226 ± 0.038 | 0.175 ± 0.014 | | | | | |
| | | h | h | h | fgh | fgh | ef | cd | ab | bc | | | | | |
| ALA | F | 0.129 ± 0.018 | 0.298 ± 0.049 | 0.422 ± 0.053 | 0.434 ± 0.066 | 0.724 ± 0.044 | 0.933 ± 0.110 | 1.34 ± 0.142 | 1.30 ± 0.247 | 1.31 ± 0.213 | <0.0001 | 0.1598 | <0.0001 | <0.0001 | 0.1567 |
| | M | 0.104 ± 0.010 | 0.154 ± 0.026 | 0.184 ± 0.027 | 0.427 ± 0.044 | 0.480 ± 0.057 | 0.581 ± 0.107 | 0.740 ± 0.018 | 1.16 ± 0.153 | 1.16 ± 0.111 | | | | | |
| | | f | e | e | d | cd | bc | ab | a | a | | | | | |
| ARA | F | 3.23 ± 0.227 | 3.67 ± 0.489 | 3.66 ± 0.366 | 2.72 ± 0.279 | 2.71 ± 0.213 | 2.48 ± 0.168 | 2.36 ± 0.196 | 2.32 ± 0.145 | 1.90 ± 0.174 | 0.0025 | 0.1708 | <0.0001 | 0.0064 | 0.4540 |
| | M | 3.73 ± 0.287 | 3.07 ± 0.272 | 2.70 ± 0.146 | 2.85 ± 0.182 | 2.35 ± 0.284 | 2.01 ± 0.159 | 2.02 ± 0.140 | 1.94 ± 0.258 | 1.77 ± 0.119 | | | | | |
| | | a | ab | ab | bc | cd | d | de | de | e | | | | | |

**C. Heart (cont)**

| ALA Dose | | **0.097** | **0.212** | **0.305** | **0.537** | **0.764** | **0.946** | **1.32** | **1.78** | **1.98** | SW (P) | Log-T SW (P) | ALA Dose | Sex | Dose*Sex |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LA | F | 7.64 ± 1.13 | 8.72 ± 1.07 | 9.19 ± 1.11 | 6.36 ± 1.06 | 8.11 ± 0.772 | 7.23 ± 0.460 | 8.49 ± 0.763 | 6.79 ± 1.14 | 5.47 ± 0.504 | 0.0244 | 0.5768 | 0.0822 | <0.0001 | 0.0851 |
| | M | 5.51 ± 0.480 | 4.39 ± 0.454 | 4.73 ± 0.627 | 6.04 ± 0.643 | 5.41 ± 0.581 | 5.06 ± 0.806 | 5.03 ± 0.109 | 4.64 ± 0.323 | 5.18 ± 0.405 | | | | | |
| | | | | | | | *Fatty acids (nmols/g)* | | | | | | | | |
| DHA | F | 9.49 ± 2.52 | 16.7 ± 3.80 | 29.8 ± 6.54 | 25.0 ± 6.81 | 27.8 ± 7.09 | 26.8 ± 4.53 | 32.9 ± 7.57 | 38.2 ± 5.42 | 25.7 ± 5.82 | 0.0001 | 0.3817 | <0.0001 | 0.0339 | 0.6368 |
| | M | 12.5 ± 3.06 | 13.7 ± 2.83 | 14.5 ± 4.20 | 24.5 ± 7.28 | 24.7 ± 4.38 | 18.3 ± 4.00 | 21.6 ± 4.95 | 20.5 ± 2.98 | 25.3 ± 4.52 | | | | | |
| | | c | bc | ab | ab | a | ab | a | a | a | | | | | |
| EPA | F | 0.148 ± 0.016 | 0.236 ± 0.020 | 0.377 ± 0.040 | 0.568 ± 0.058 | 0.940 ± 0.098 | 1.10 ± 0.141 | 1.54 ± 0.046 | 1.70 ± 0.085 | 1.72 ± 0.221 | 0.0001 | 0.3168 | <0.0001 | 0.0010 | 0.4193 |
| | M | 0.103 ± 0.020 | 0.193 ± 0.011 | 0.258 ± 0.045 | 0.458 ± 0.065 | 0.611 ± 0.052 | 0.856 ± 0.089 | 1.20 ± 0.054 | 1.40 ± 0.073 | 1.47 ± 0.076 | | | | | |
| | | f | e | d | c | b | b | a | a | a | | | | | |
| ALA | F | 22.5 ± 3.07 | 49.3 ± 6.80 | 66.0 ± 5.60 | 76.5 ± 11.1 | 121.8 ± 9.18 | 143.9 ± 18.9 | 188.7 ± 7.92 | 193.7 ± 24.0 | 193.2 ± 32.5 | <0.0001 | 0.1504 | <0.0001 | <0.0001 | 0.2678 |
| | M | 16.8 ± 2.28 | 30.3 ± 2.34 | 31.6 ± 4.35 | 62.5 ± 6.37 | 84.1 ± 9.84 | 95.1 ± 11.9 | 119.2 ± 6.16 | 145.9 ± 11.2 | 166.6 ± 14.0 | | | | | |
| | | f | e | e | d | c | bc | ab | a | a | | | | | |
| ARA | F | 26.2 ± 3.64 | 34.7 ± 5.93 | 46.4 ± 5.52 | 35.4 ± 5.27 | 34.7 ± 5.47 | 30.9 ± 4.95 | 36.1 ± 1.97 | 34.8 ± 3.13 | 22.0 ± 4.50 | 0.3062 | NA | 0.0075 | 0.6012 | 0.4730 |
| | M | 51.1 ± 6.84 | 31.0 ± 5.69 | 30.4 ± 5.74 | 30.8 ± 6.22 | 35.9 ± 4.92 | 27.5 ± 3.28 | 28.3 ± 3.15 | 25.8 ± 3.23 | 29.4 ± 1.28 | | | | | |
| | | a | ab | a | ab | ab | ab | ab | ab | b | | | | | |

**D. Brain**

| ALA Dose | | **0.097** | **0.212** | **0.305** | **0.537** | **0.764** | **0.946** | **1.32** | **1.78** | **1.98** | SW (P) | Log-T SW (P) | ALA Dose | Sex | Dose*Sex |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | *Oxylipins (nmols/g)* | | | | | | | | |
| DHA | F | 0.674 ± 0.194 | 0.485 ± 0.094 | 0.752 ± 0.138 | 0.661 ± 0.146 | 0.477 ± 0.137 | 0.610 ± 0.079 | 0.547 ± 0.142 | 0.759 ± 0.15 | 0.513 ± 0.126 | 0.1754 | NA | 0.9739 | 0.0388 | 0.8260 |
| | M | 0.594 ± 0.171 | 0.689 ± 0.171 | 0.869 ± 0.149 | 0.939 ± 0.080 | 0.755 ± 0.173 | 0.633 ± 0.124 | 0.684 ± 0.186 | 0.675 ± 0.08 | 0.863 ± 0.167 | | | | | |
| ALA | F | 0.011 ± 0.002 | 0.016 ± 0.003 | 0.020 ± 0.006 | 0.012 ± 0.002 | 0.012 ± 0.003 | 0.009 ± 0.003 | 0.011 ± 0.005 | 0.015 ± 0.006 | 0.004 ± 0.001 | <0.0001 | <0.0001 | 0.0037 | 0.5498 | 0.4000 |
| | M | 0.026 ± 0.007 | 0.012 ± 0.004 | 0.010 ± 0.004 | 0.011 ± 0.003 | 0.011 ± 0.004 | 0.007 ± 0.003 | 0.006 ± 0.003 | 0.005 ± 0.001 | 0.010 ± 0.004 | | | | | |
| | | a | ab | ab | ab | ab | b | b | b | b | | | | | |

**D. Brain (cont)**

| ALA Dose | | **0.097** | **0.212** | **0.305** | **0.537** | **0.764** | **0.946** | **1.32** | **1.78** | **1.98** | SW (P) | Log-T SW (P) | ALA Dose | Sex | Dose*Sex |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ARA | F | 3.23 ± 0.442 | 3.00 ± 0.244 | 3.50 ± 0.476 | 3.04 ± 0.325 | 2.55 ± 0.194 | 3.19 ± 0.341 | 2.86 ± 0.165 | 3.78 ± 0.403 | 2.62 ± 0.371 | 0.6172 | NA | 0.9371 | 0.0055 | 0.7260 |
| | M | 3.26 ± 0.356 | 3.46 ± 0.425 | 4.21 ± 0.588 | 3.99 ± 0.184 | 3.29 ± 0.497 | 3.26 ± 0.354 | 3.40 ± 0.491 | 3.56 ± 0.415 | 4.02 ± 0.526 | | | | | |
| LA | F | 1.48 ± 0.627 | 1.34 ± 0.562 | 1.89 ± 0.732 | 1.13 ± 0.475 | 1.18 ± 0.556 | 1.08 ± 0.606 | 0.800 ± 0.568 | 1.32 ± 0.617 | 0.660 ± 0.369 | <0.0001 | <0.0001 | 0.1440 | 0.9050 | 0.8720 |
| | M | 2.20 ± 1.14 | 1.41 ± 0.742 | 1.27 ± 0.473 | 1.26 ± 0.596 | 1.16 ± 0.588 | 0.666 ± 0.333 | 0.534 ± 0.345 | 0.899 ± 0.506 | 1.69 ± 0.703 | | | | | |
| | | | | | | | *Fatty acids (nmols/g)* | | | | | | | | |
| DHA | F | 72.4 ± 7.87 | 82.4 ± 4.73 | 101.4 ± 4.79 | 84.6 ± 7.95 | 74.0 ± 12.7 | 88.3 ± 8.15 | 77.4 ± 3.05 | 91.1 ± 6.74 | 95.9 ± 20.9 | 0.0009 | <0.0001 | 0.0447 | 0.5565 | 0.5820 |
| | M | 68.3 ± 3.03 | 77.4 ± 6.40 | 92.6 ± 6.53 | 103.4 ± 12.6 | 90.4 ± 11.6 | 77.6 ± 7.03 | 78.1 ± 5.98 | 98.3 ± 7.14 | 102.8 ± 17.0 | | | | | |
| | | c | abc | ab | ab | abc | abc | bc | ab | a | | | | | |
| EPA | F | 0.053 ± 0.013 | 0.071 ± 0.009 | 0.098 ± 0.022 | 0.114 ± 0.017 | 0.134 ± 0.022 | 0.211 ± 0.039 | 0.199 ± 0.016 | 0.247 ± 0.017 | 0.283 ± 0.057 | <0.0001 | 0.0002 | <0.0001 | 0.4880 | 0.3870 |
| | M | 0.045 ± 0.012 | 0.062 ± 0.014 | 0.090 ± 0.016 | 0.113 ± 0.026 | 0.138 ± 0.026 | 0.157 ± 0.022 | 0.198 ± 0.022 | 0.241 ± 0.009 | 0.286 ± 0.038 | | | | | |
| | | f | e | de | d | cd | bc | ab | ab | a | | | | | |
| ALA | F | 12.6 ± 2.26 | 15.4 ± 2.06 | 14.7 ± 4.13 | 13.5 ± 3.35 | 11.3 ± 3.54 | 16.2 ± 6.70 | 12.7 ± 3.64 | 15.7 ± 4.00 | 13.2 ± 4.81 | 0.0001 | <0.0001 | 0.8220 | 0.4910 | 0.8890 |
| | M | 15.7 ± 4.42 | 15.8 ± 4.49 | 17.6 ± 2.22 | 16.3 ± 6.37 | 14.9 ± 5.92 | 9.99 ± 3.64 | 9.50 ± 3.79 | 17.5 ± 2.52 | 20.1 ± 6.31 | | | | | |
| ARA | F | 180.3 ± 18.5 | 184.3 ± 9.17 | 199.3 ± 11.5 | 199.0 ± 16.1 | 153.9 ± 27.0 | 200.9 ± 16.1 | 193.3 ± 14.6 | 202.2 ± 16.8 | 197.5 ± 37.6 | 0.0537 | NA | 0.0988 | 0.3822 | 0.6030 |
| | M | 161.4 ± 7.79 | 190.7 ± 16.7 | 223.9 ± 10.5 | 217.2 ± 16.1 | 179.7 ± 8.80 | 169.2 ± 18.3 | 192.6 ± 11.8 | 230.6 ± 14.7 | 214.8 ± 37.6 | | | | | |

Data reported as mean ± SE (n=6; N=108). The SW column shows the Shapiro-Wilk test p value results and the TSW column shows Shapiro-Wilk p values for log transformed data. Data that were normal were analyzed using multiple linear regression for diet dosage and sex effects, and dose*sex interactions. If data were not normal even following log-transformation, multiple linear regression was applied to data that were closest to normality. Diet group means within each row with differing letters below the means indicate significant differences between diet groups as determined by Fisher's Protected LSD test ($p<0.05$). NA: not applicable ND: not detected.

**Supplemental Table 3-7**. Complete multiple linear regression statistics for total non-esterified oxylipins by FA precursor and for non-esterified FA for rats that received DHA diets in rat serum (A), liver (B), heart (C), and Brain (D).

**A. Serum**

| DHA Dose | | **0.074** | **0.165** | **0.392** | **0.646** | **1.35** | SW (P) | Log-T SW (P) | DHA Dose | Sex | Dose* Sex | Diet ALA vs DHA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *Oxylipins (nmols/g)* | | | | | | | |
| DHA | F | 0.084 ± 0.015 | 0.144 ± 0.042 | 0.286 ± 0.034 | 0.307 ± 0.057 | 0.492 ± 0.079 | 0.0078 | 0.3644 | <0.0001 | 0.4590 | 0.9490 | <0.0001 |
| | M | 0.076 ± 0.012 | 0.148 ± 0.017 | 0.209 ± 0.027 | 0.253 ± 0.048 | 0.491 ± 0.056 | | | | | | |
| | | d | c | b | b | a | | | | | | |
| EPA | F | 0.007 ± 0.002 | 0.016 ± 0.003 | 0.084 ± 0.018 | 0.107 ± 0.031 | 0.269 ± 0.033 | <0.0001 | 0.5204 | <0.0001 | 0.6180 | 0.7740 | 0.8540 |
| | M | 0.008 ± 0.001 | 0.019 ± 0.004 | 0.061 ± 0.007 | 0.126 ± 0.019 | 0.320 ± 0.100 | | | | | | |
| | | d | c | b | b | a | | | | | | |
| ALA | F | 0.005 ± 0.001 | 0.006 ± 0.001 | 0.006 ± 0.001 | 0.005 ± 0.001 | 0.005 ± 0.001 | 0.3763 | NA | 0.1142 | 0.0337 | 0.4630 | <0.0001 |
| | M | 0.007 ± 0.001 | 0.008 ± 0.001 | 0.007 ± 0.001 | 0.006 ± 0.001 | 0.006 ± 0.001 | | | | | | |
| ARA | F | 2.99 ± 0.511 | 2.36 ± 0.481 | 1.91 ± 0.389 | 1.25 ± 0.297 | 1.23 ± 0.228 | 0.0006 | 0.7703 | <0.0001 | 0.6370 | 0.8472 | 0.0183 |
| | M | 2.88 ± 0.390 | 2.32 ± 0.477 | 1.98 ± 0.251 | 1.36 ± 0.174 | 1.59 ± 0.528 | | | | | | |
| | | a | ab | b | c | c | | | | | | |
| LA | F | 0.481 ± 0.065 | 0.483 ± 0.066 | 0.551 ± 0.035 | 0.451 ± 0.046 | 0.518 ± 0.075 | 0.2913 | NA | 0.7228 | 0.0084 | 0.9297 | 0.2944 |
| | M | 0.454 ± 0.036 | 0.370 ± 0.010 | 0.395 ± 0.055 | 0.363 ± 0.046 | 0.438 ± 0.051 | | | | | | |
| | | | | | *Fatty acids (nmols/g)* | | | | | | | |
| DHA | F | 6.08 ± 0.438 | 7.81 ± 0.999 | 14.3 ± 1.55 | 17.1 ± 1.18 | 25.0 ± 2.25 | 0.0068 | 0.7783 | <0.0001 | 0.1690 | 0.2510 | <0.0001 |
| | M | 5.48 ± 0.401 | 9.25 ± 0.553 | 11.7 ± 0.611 | 14.4 ± 0.744 | 19.5 ± 1.11 | | | | | | |
| | | d | c | b | b | a | | | | | | |
| EPA | F | 0.014 ± 0.001 | 0.036 ± 0.008 | 0.140 ± 0.028 | 0.242 ± 0.032 | 0.525 ± 0.091 | <0.0001 | 0.3356 | <0.0001 | 0.9660 | 0.3470 | 0.0799 |
| | M | 0.019 ± 0.002 | 0.041 ± 0.003 | 0.102 ± 0.006 | 0.239 ± 0.038 | 0.414 ± 0.068 | | | | | | |
| | | e | d | c | b | a | | | | | | |

**A. Serum (cont)**

| DHA Dose | | **0.074** | **0.165** | **0.392** | **0.646** | **1.35** | SW (P) | Log-T SW (P) | DHA Dose | Sex | Dose* Sex | Diet ALA vs DHA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ALA | F | 0.087 ± 0.041 | 0.097 ± 0.058 | 0.150 ± 0.037 | 0.095 ± 0.024 | 0.190 ± 0.058 | 0.0205 | 0.2810 | 0.8800 | 0.6400 | 0.2310 | <0.0001 |
| | M | 0.130 ± 0.035 | 0.067 ± 0.03 | 0.107 ± 0.019 | 0.094 ± 0.032 | 0.090 ± 0.018 | | | | | | |
| ARA | F | 8.86 ± 0.764 | 8.31 ± 1.06 | 8.01 ± 0.913 | 7.03 ± 0.995 | 7.30 ± 0.745 | 0.1794 | NA | 0.0497 | 0.1517 | 0.8140 | <0.0001 |
| | M | 8.10 ± 0.845 | 7.88 ± 0.492 | 6.79 ± 0.474 | 6.59 ± 0.302 | 6.82 ± 0.590 | | | | | | |
| | | a | ab | ab | b | ab | | | | | | |
| ADA | F | 151.0 ± 22.5 | 107.3 ± 24.6 | 70.9 ± 11.0 | 43.8 ± 6.23 | 27.9 ± 5.02 | <0.0001 | 0.9664 | <0.0001 | 0.4610 | 0.8570 | <0.0001 |
| | M | 183.0 ± 39.8 | 107.2 ± 14.7 | 72.2 ± 6.22 | 45.3 ± 6.44 | 27.3 ± 2.24 | | | | | | |
| | | a | b | b | c | d | | | | | | |
| GLA | F | 4.16 ± 0.487 | 3.08 ± 1.33 | 3.73 ± 0.857 | 2.59 ± 0.642 | 2.41 ± 0.808 | 0.5343 | NA | 0.1090 | 0.7660 | 0.6050 | <0.0001 |
| | M | 4.12 ± 0.332 | 2.99 ± 0.273 | 2.75 ± 0.299 | 1.89 ± 0.674 | 1.69 ± 0.753 | | | | | | |

**B. Liver**

| DHA Dose | | **0.074** | **0.165** | **0.392** | **0.646** | **1.35** | SW (P) | Log-T SW (P) | DHA Dose | Sex | Dose* Sex | Diet ALA vs DHA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *Oxylipins (nmols/g)* | | | | | | | |
| DHA | F | 6.68 ± 1.29 | 11.5 ± 1.39 | 12.6 ± 1.82 | 14.7 ± 1.40 | 17.9 ± 3.19 | 0.0016 | 0.1556 | <0.0001 | 0.0350 | 0.4429 | <0.0001 |
| | M | 5.05 ± 1.11 | 6.59 ± 0.574 | 12.1 ± 2.80 | 12.7 ± 3.42 | 15.6 ± 2.34 | | | | | | |
| | | c | b | ab | a | a | | | | | | |
| EPA | F | 0.016 ± 0.003 | 0.126 ± 0.047 | 0.872 ± 0.187 | 2.53 ± 0.231 | 4.33 ± 0.521 | 0.0013 | <0.0001 | <0.0001 | 0.0312 | 0.0696 | 0.0076 |
| | M | 0.027 ± 0.012 | 0.273 ± 0.134 | 1.21 ± 0.224 | 3.27 ± 0.449 | 5.40 ± 0.576 | | | | | | |
| | | d | d | c | b | a | | | | | | |
| ALA | F | 0.066 ± 0.016 | 0.098 ± 0.030 | 0.078 ± 0.019 | 0.063 ± 0.02 | 0.064 ± 0.014 | 0.0159 | <0.0001 | 0.3084 | 0.0467 | 0.8930 | <0.0001 |
| | M | 0.071 ± 0.023 | 0.028 ± 0.015 | 0.058 ± 0.010 | 0.059 ± 0.017 | 0.038 ± 0.010 | | | | | | |

**B. Liver (cont)**

| DHA Dose | | **0.074** | **0.165** | **0.392** | **0.646** | **1.35** | SW (P) | Log-T SW (P) | DHA Dose | Sex | Dose* Sex | Diet ALA vs DHA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ARA | F | 7.65 ± 0.814 | 8.85 ± 1.22 | 5.52 ± 0.598 | 4.30 ± 0.446 | 2.42 ± 0.292 | 0.0001 | 0.9193 | <0.0001 | 0.6420 | 0.4800 | <0.0001 |
| | M | 9.73 ± 1.45 | 7.05 ± 0.470 | 5.62 ± 0.493 | 3.92 ± 0.395 | 2.86 ± 0.193 | | | | | | |
| | | a | a | b | c | d | | | | | | |
| LA | F | 8.91 ± 1.56 | 12.8 ± 1.80 | 8.83 ± 1.14 | 7.74 ± 0.486 | 6.41 ± 0.425 | 0.0010 | 0.8025 | 0.0033 | 0.0190 | 0.6528 | 0.0936 |
| | M | 10.0 ± 2.36 | 7.05 ± 1.31 | 7.66 ± 1.04 | 5.72 ± 0.576 | 5.88 ± 0.497 | | | | | | |
| | | ab | a | abc | bc | c | | | | | | |
| | | | | | *Fatty acids (nmols/g)* | | | | | | | |
| DHA | F | 286.0 ± 18.2 | 432.7 ± 34.5 | 466.0 ± 30.3 | 596.9 ± 36.4 | 704.5 ± 38.3 | 0.1279 | NA | <0.0001 | 0.0219 | 0.4230 | <0.0001 |
| | M | 257.6 ± 31.4 | 328.5 ± 40.7 | 484.6 ± 57.7 | 518.3 ± 14.7 | 601.7 ± 22.8 | | | | | | |
| | | e | d | c | b | a | | | | | | |
| EPA | F | 0.386 ± 0.021 | 2.16 ± 0.524 | 5.00 ± 0.745 | 10.9 ± 1.14 | 21.6 ± 2.89 | <0.0001 | 0.0581 | <0.0001 | 0.3180 | 0.5280 | 0.9290 |
| | M | 0.719 ± 0.050 | 1.91 ± 0.243 | 5.95 ± 0.61 | 13.3 ± 2.32 | 20.9 ± 1.62 | | | | | | |
| | | e | d | c | b | a | | | | | | |
| ALA | F | 5.62 ± 2.53 | 14.8 ± 3.80 | 11.5 ± 2.80 | 13.6 ± 3.34 | 14.6 ± 3.35 | 0.1133 | NA | 0.9820 | 0.1310 | 0.0580 | <0.0001 |
| | M | 12.5 ± 1.79 | 9.68 ± 3.16 | 10.1 ± 3.66 | 6.16 ± 2.92 | 6.52 ± 3.06 | | | | | | |
| ARA | F | 268.5 ± 14.6 | 285.5 ± 29.7 | 206.9 ± 13.4 | 193.6 ± 11.7 | 171.4 ± 27.3 | 0.0006 | 0.0784 | <0.0001 | 0.7140 | 0.7250 | <0.0001 |
| | M | 288.9 ± 33.9 | 241.5 ± 28.0 | 237.0 ± 18.1 | 186.2 ± 14.0 | 151.9 ± 4.40 | | | | | | |
| | | a | ab | bc | cd | d | | | | | | |
| ADA | F | 1526 ± 442.7 | 2202 ± 681.1 | 706 ± 479.3 | 0.001 ± 0.000 | 0.001 ± 0.000 | <0.0001 | 0.0001 | 0.0003 | 0.5732 | 0.7164 | 0.1040 |
| | M | 2997 ± 1405 | 1265 ± 838.7 | 1020 ± 476.7 | 279.2 ± 176.7 | 0.001 ± 0.000 | | | | | | |
| | | a | ab | bc | c | c | | | | | | |

**B. Liver (cont)**

| DHA Dose | | **0.074** | **0.165** | **0.392** | **0.646** | **1.35** | SW (P) | Log-T SW (P) | DHA Dose | Sex | Dose* Sex | Diet ALA vs DHA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GLA | F | 403.7 ± 92.8 a | 294.6 ± 136.1 ab | 254.1 ± 85.7 abc | 73.5 ± 73.4 cd | 0.015 ± 0.000 d | 0.0464 | NA | 0.0001 | 0.0002 | 0.0305 | <0.0001 |
| | M | 112.4 ± 71.1 bcd | 80.8 ± 80.8 cd | 40.0 ± 39.9 d | 0.015 ± 0.000 d | 0.015 ± 0.000 d | | | | | | |

**C. Heart**

| DHA Dose | | **0.074** | **0.165** | **0.392** | **0.646** | **1.35** | SW (P) | Log-T SW (P) | DHA Dose | Sex | Dose* Sex | Diet ALA vs DHA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *Oxylipins (nmols/g)* | | | | | | | |
| DHA | F | 2.37 ± 0.238 f | 3.91 ± 0.476 def | 6.66 ± 0.630 c | 9.29 ± 1.06 b | 13.2 ± 1.26 a | 0.4306 | NA | <0.0001 | 0.7090 | 0.0020 | <0.0001 |
| | M | 2.92 ± 0.498 f | 3.17 ± 0.293 ef | 4.99 ± 0.299 cde | 5.24 ± 0.525 cd | 9.49 ± 0.878 b | | | | | | |
| EPA | F | ND d | ND d | 0.032 ± 0.008 cd | 0.128 ± 0.032 b | 0.225 ± 0.032 a | <0.0001 | <0.0001 | <0.0001 | 0.4766 | 0.0002 | 0.8530 |
| | M | ND d | 0.004 ± 0.004 d | 0.017 ± 0.002 cd | 0.060 ± 0.024 c | 0.114 ± 0.018 b | | | | | | |
| ALA | F | 0.019 ± 0.006 | 0.017 ± 0.003 | 0.027 ± 0.008 | 0.032 ± 0.006 | 0.028 ± 0.005 | 0.0018 | <0.0001 | 0.1440 | 0.2930 | 0.6400 | <0.0001 |
| | M | 0.017 ± 0.006 | 0.012 ± 0.006 | 0.029 ± 0.008 | 0.023 ± 0.008 | 0.021 ± 0.005 | | | | | | |
| ARA | F | 2.7 ± 0.118 | 2.36 ± 0.154 | 2.11 ± 0.142 | 1.86 ± 0.179 | 1.18 ± 0.076 | 0.6971 | NA | <0.0001 | 0.7500 | 0.2910 | <0.0001 |
| | M | 3.14 ± 0.290 a | 2.33 ± 0.210 b | 2.11 ± 0.229 b | 1.33 ± 0.140 c | 1.11 ± 0.126 d | | | | | | |
| LA | F | 5.08 ± 0.450 | 6.45 ± 0.268 | 6.29 ± 0.701 | 7.68 ± 0.464 | 6.41 ± 0.608 | 0.0097 | 0.1655 | 0.2440 | 0.0001 | 0.6791 | 0.0115 |
| | M | 5.47 ± 0.605 | 3.81 ± 0.157 | 5.03 ± 0.637 | 4.70 ± 0.573 | 5.20 ± 0.751 | | | | | | |

**C. Heart (cont)**

| DHA Dose | | **0.074** | **0.165** | **0.392** | **0.646** | **1.35** | SW (P) | Log-T SW (P) | DHA Dose | Sex | Dose* Sex | Diet ALA vs DHA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *Fatty acids (nmols/g)* | | | | | | | |
| DHA | F | 28.9 ± 9.62 | 24.9 ± 4.32 | 55.9 ± 8.56 | 94.9 ± 22.4 | 108.3 ± 18.2 | 0.0005 | 0.7912 | <0.0001 | 0.0197 | 0.1900 | <0.0001 |
| | M | 23.3 ± 4.93 | 23.0 ± 3.49 | 43.5 ± 6.67 | 46.3 ± 9.24 | 65.1 ± 11.8 | | | | | | |
| | | c | c | b | ab | a | | | | | | |
| EPA | F | 0.057 ± 0.005 | 0.136 ± 0.019 | 0.426 ± 0.076 | 0.918 ± 0.125 | 1.47 ± 0.192 | <0.0001 | 0.2063 | <0.0001 | 0.0870 | 0.3880 | 0.0238 |
| | M | 0.066 ± 0.007 | 0.107 ± 0.007 | 0.274 ± 0.006 | 0.569 ± 0.102 | 1.01 ± 0.065 | | | | | | |
| | | e | d | c | b | a | | | | | | |
| ALA | F | 5.47 ± 0.698 | 5.46 ± 0.627 | 6.03 ± 0.716 | 7.94 ± 0.752 | 6.95 ± 0.863 | 0.0008 | 0.1711 | 0.0255 | <0.0001 | 0.7521 | <0.0001 |
| | M | 3.89 ± 0.545 | 4.11 ± 0.479 | 5.69 ± 1.01 | 4.11 ± 0.427 | 5.13 ± 0.592 | | | | | | |
| | | b | ab | ab | ab | a | | | | | | |
| ARA | F | 35.5 ± 5.65 | 22.5 ± 3.72 | 28.2 ± 3.69 | 30.1 ± 4.67 | 18.1 ± 2.85 | 0.5887 | NA | 0.0003 | 0.7877 | 0.5146 | <0.0001 |
| | M | 37.0 ± 4.81 | 25.5 ± 4.18 | 30.4 ± 2.14 | 20.7 ± 3.28 | 17.2 ± 2.55 | | | | | | |
| | | a | bc | ab | bc | c | | | | | | |

**D. Brain**

| DHA Dose | | **0.074** | **0.165** | **0.392** | **0.646** | **1.35** | SW (P) | Log-T SW (P) | DHA Dose | Sex | Dose* Sex | Diet ALA vs DHA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *Oxylipins (nmols/g)* | | | | | | | |
| DHA | F | 0.421 ± 0.118 | 0.350 ± 0.051 | 0.564 ± 0.157 | 0.465 ± 0.076 | 1.16 ± 0.251 | 0.0001 | 0.4165 | 0.0192 | 0.0547 | 0.0780 | 0.8466 |
| | M | 0.744 ± 0.229 | 1.25 ± 0.415 | 0.518 ± 0.176 | 0.975 ± 0.177 | 0.929 ± 0.310 | | | | | | |
| | | b | ab | b | ab | a | | | | | | |
| DHA | F | 0.421 ± 0.118 | 0.350 ± 0.051 | 0.564 ± 0.157 | 0.465 ± 0.076 | 1.16 ± 0.251 | | | 0.2164 | 0.0245 | 0.0608 | 0.3219 |
| | M | 0.744 ± 0.229 | 1.25 ± 0.415 | 0.518 ± 0.176 | 0.975 ± 0.177 | 0.929 ± 0.310 | | | adjusted by Run | | | |

**D. Brain (cont)**

| DHA | Dose | **0.074** | **0.165** | **0.392** | **0.646** | **1.35** | SW (P) | Log-T SW (P) | DHA Dose | Sex | Dose* Sex | Diet ALA vs DHA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ALA | F | 0.008 ± 0.002 | 0.003 ± 0.001 | 0.011 ± 0.003 | 0.013 ± 0.003 | 0.010 ± 0.004 | 0.0037 | <0.0001 | 0.4730 | 0.3390 | 0.3753 | 0.0999 |
| | M | 0.013 ± 0.003 | 0.013 ± 0.006 | 0.007 ± 0.003 | 0.010 ± 0.003 | 0.012 ± 0.002 | | | | | | |
| ARA | F | 2.81 ± 0.336 | 2.59 ± 0.187 | 2.87 ± 0.434 | 2.70 ± 0.299 | 3.81 ± 0.607 | 0.0237 | 0.5675 | 0.7145 | 0.0626 | 0.0628 | 0.4761 |
| | M | 3.67 ± 0.504 | 4.43 ± 0.631 | 2.83 ± 0.506 | 3.79 ± 0.429 | 3.35 ± 0.802 | | | | | | |
| LA | F | 0.700 ± 0.465 | 0.254 ± 0.066 | 0.795 ± 0.366 | 0.991 ± 0.566 | 2.64 ± 1.45 | <0.0001 | <0.0001 | 0.0133 | 0.5113 | 0.5834 | 0.4459 |
| | M | 1.23 ± 0.533 | 1.01 ± 0.618 | 0.842 ± 0.575 | 1.54 ± 0.473 | 2.41 ± 1.73 | | | | | | |
| | | ab | b | b | ab | a | | | | | | |
| LA | F | 0.700 ± 0.465 | 0.254 ± 0.066 | 0.795 ± 0.366 | 0.991 ± 0.566 | 2.64 ± 1.45 | | | 0.2036 | 0.5636 | 0.5611 | 0.2889 |
| | M | 1.23 ± 0.533 | 1.01 ± 0.618 | 0.842 ± 0.575 | 1.54 ± 0.473 | 2.41 ± 1.73 | | | adjusted by Run | | | |
| | | | | | *Fatty acids (nmols/g)* | | | | | | | |
| DHA | F | 82.3 ± 6.17 | 80.1 ± 7.78 | 85.6 ± 8.19 | 81.1 ± 6.25 | 116.6 ± 12.0 | 0.3564 | NA | 0.0033 | 0.9943 | 0.1472 | 0.1035 |
| | M | 79.4 ± 7.32 | 92.5 ± 8.64 | 78.8 ± 7.27 | 100.9 ± 12.5 | 94.2 ± 7.06 | | | | | | |
| | | b | b | b | ab | a | | | | | | |
| DHA | F | 82.3 ± 6.17 | 80.1 ± 7.78 | 85.6 ± 8.19 | 81.1 ± 6.25 | 116.6 ± 12.0 | | | 0.0665 | 0.9834 | 0.1495 | 0.0468 |
| | M | 79.4 ± 7.32 | 92.5 ± 8.64 | 78.8 ± 7.27 | 100.9 ± 12.5 | 94.2 ± 7.06 | | | adjusted by Run | | | |
| EPA | F | 0.043 ± 0.005 | 0.066 ± 0.007 | 0.124 ± 0.017 | 0.202 ± 0.019 | 0.412 ± 0.035 | 0.0035 | 0.4076 | <0.0001 | 1.0000 | 0.2420 | <0.0001 |
| | M | 0.045 ± 0.007 | 0.082 ± 0.013 | 0.111 ± 0.007 | 0.222 ± 0.026 | 0.328 ± 0.034 | | | | | | |
| | | e | d | c | b | a | | | | | | |
| ALA | F | 9.12 ± 3.01 | 6.99 ± 2.73 | 11.4 ± 3.14 | 13.4 ± 3.81 | 19.4 ± 5.87 | 0.0062 | <0.0001 | 0.0112 | 0.9265 | 0.3774 | 0.2330 |
| | M | 13.4 ± 3.10 | 11.9 ± 4.19 | 7.32 ± 2.96 | 8.46 ± 2.57 | 18.1 ± 5.06 | | | | | | |
| | | b | b | b | b | a | | | | | | |

**D. Brain (cont)**

| DHA | Dose | **0.074** | **0.165** | **0.392** | **0.646** | **1.35** | SW (P) | Log-T SW (P) | DHA Dose | Sex | Dose* Sex | Diet ALA vs DHA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ALA | F | 9.12 ± 3.01 | 6.99 ± 2.73 | 11.4 ± 3.14 | 13.4 ± 3.81 | 19.4 ± 5.87 | | | 0.3990 | 0.7560 | 0.3010 | 0.0900 |
| | M | 13.4 ± 3.10 | 11.9 ± 4.19 | 7.32 ± 2.96 | 8.46 ± 2.57 | 18.1 ± 5.06 | | adjusted by Run | | | | |
| ARA | F | 200.3 ± 17.1 | 180.8 ± 18.1 | 174.2 ± 14.7 | 171.0 ± 15.4 | 208.3 ± 14.9 | 0.3596 | NA | 0.5630 | 0.7740 | 0.0734 | 0.4610 |
| | M | 188.7 ± 9.28 | 209.9 ± 17.6 | 168.1 ± 13.0 | 191.8 ± 16.6 | 161.9 ± 10.6 | | | | | | |

Data reported as mean ± SE (n=6; N=60). The SW column shows the Shapiro-Wilk test p value results and the TSW column shows Shapiro-Wilk p values for log transformed data. Data that were normal were analyzed using multiple linear regression for diet dosage and sex effects, and dose*sex interactions. If data were not normal even following log-transformation, multiple linear regression was applied to data that were closest to normality. Diet group means within each row with differing letters below the means indicate significant differences between diet groups as determined by Fisher's Protected LSD test ($p<0.05$). ALA vs. DHA indicates results of multiple linear regression applied for comparison. NA: not applicable ND: not detected.

## Appendix 3: Supplemental figures and tables for Chapter 4

*Abbreviations*: ARA, arachidonic acid; ALA, α-linolenic acid; BEL, bromoenol lactone; COX, cyclooxygenase; CYP, cytochrome P450; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DiHDoPE, dihydroxy-docosapentaenoic acid; DiHDoHE, dihydroxy-docosahexaenoic acid; DiHETE, dihydroxy-eicosatetraenoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; DiHODE, dihydroxy-octadecadienoic acid; diHOME, dihydroxy-octadecenoic acid; EPA, eicosapentaenoic acid; EpDoPE, epoxy-docosapentaenoic acid; EpETrE, epoxy-eicosatrienoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; GLA, γ-linolenic 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; HX, hepoxilin; k, keto; LA, linoleic acid; LOX, lipoxygenase; LT, leukotriene; LX, lipoxin; MAFP, methyl arachidonyl fluorophosphonate; PAF-AH, platelet-activating factor acetylhydrolase; PG, prostaglandin; $PLA_2$, phospholipase $A_2$; PYR, pyrrophenone; triHOME, trihydroxy-octadecenoic acid; TX, thromboxane; VAR, varespladib.

**Supplemental Table 4-1.** Internal standard composition.

| Internal Standard | (ng/100µl) |
|---|---|
| 6-k-$PGF_{1\alpha}$-d4 | 1.125 |
| $TXB_2$-d4 | 0.300 |
| $PGF_2\alpha$-d4 | 1.50 |
| $PGE_2$-d4 | 0.375 |
| $PGD_2$-d4 | 0.600 |
| 13,14-dihydro-15-keto-PGF2α-d4 | 1.50 |
| 8-iso $PGF_2\alpha$-d4 | 3.375 |
| $LTB_4$-d4 | 1.50 |
| 20-HETE-d6 | 3.00 |
| 15-HETE-d8 | 1.125 |
| 12-HETE-d8 | 1.50 |
| 5-HETE-d8 | 2.25 |
| 13-HODE-d4 | 0.750 |
| 9-HODE-d4 | 1.50 |
| 12,13-DiHOME-d4 | 0.1875 |
| 9,10-DiHOME-d4 | 0.09375 |
| 14,15-DHET-d11 | 0.300 |
| 11,12-DHET-d11 | 0.375 |
| 8,9-DHET-d11 | 1.50 |
| 15d-$PGJ_2$-d4 | 3.00 |
| EPA-d5 | 3.00 |
| 5-OxoETE-d7 | 10.125 |
| $RvD_1$-d5 | 1.125 |

Concentration of deuterated internal standards diluted in 50% ethanol, of which 100uL was added to each sample. Rv: Resolvin

**Supplemental Table 4-2.** $PLA_2$ isoform primers used for RT-qPCR

| $PLA_2$ isoform | Gene name | Forward primer | Reverse primer | Fasta sequecence | Amplicon length |
|---|---|---|---|---|---|
| $sPLA_2$ IB | PLA2G1B | GCGTTACTGCACACAGCATCA | GAGTCTGGCAGCACCTGTCTA | NM_031585.1 | 171 |
| $sPLA_2$ IIA | PLA2G2A | AAGCCATACCACCATCCCATC | CTGGACCTGAATTGAGCCAAAG | NM_031598.3 | 97 |
| $sPLA_2$ IIC | PLA2G2C | TATGCGTTCAGGGGAAAGGGA | CTGCCAGTTCAGCTCCAACAGA | NM_019202.2 | 183 |
| $sPLA_2$ IID | PLA2G2D | GGCTGCTGCTGGCTGGTATAA | TCCTTGGGTTGTCCTTTGCC | NM_001013428.1 | 148 |
| $sPLA_2$ IIE | PLA2G2E | GTGGACCAGACGGATTGGTG | AGTCGTTCTACCAGCACAGAA | NM_001106696.1 | 138 |
| $sPLA_2$ IIF | PLA2G2F | TGAAGTCCATGGTGGAAGCC | CATCTCATCCATGGGGTGGC | NM_001109587.1 | 107 |
| $sPLA_2$ V | PLA2G5 | ATCAGCATCACGCACGGAAT | GCGCTTCATTTCTTGGGTTCTTT | NM_017174.1 | 174 |
| $sPLA_2$ X | PLA2G10 (1) | TGTCTCAGCGAAGCAACCA | GCGATCGAGGACCAACACAA | NM_017176.2 | 91 |
| $sPLA_2$ X | PLA2G10 (2) | AAGCAACCAGGAGGTCACAT | GGTAGTAGCAGCACCAGTCAAT | NM_017176.2 | 168 |
| $sPLA_2$ XIIA | PLA2G12A | CCAGGAACAGGACCAGACCAC | ATATCCATAGCGTGGGGCAGG | NM_001108565.1 | 172 |
| $sPLA_2$ XIIB | PLA2G12B | AGCTTCGAGTCGGTCAACAG | GCCCAAGTCCATACTTCCCG | XM_006256432.2 | 189 |
| $cPLA_2$ α | PLA2G4A | AGGGGACCTTTGGCGATATG | GCATCCATCAATGTGATCTCCAA | NM_133551.2 | 187 |
| $cPLA_2$ β | PLA2G4B | CCGTTCCTGTGGATTTGGTCT | CAGTCAGAGGATGTCACTAGGTC | NM_001107764.2 | 144 |
| $cPLA_2$ γ | PLA2G4C | ACTGGTTAGACCTGAGCCTG | TAGCCCCTGTTAAGAACTTCAG | NM_001145982.1 | 146 |
| $cPLA_2$ δ | PLA2G4D | TTCCCGAGTCCCAGATTTGC | ATGAGGGCTCCTTCTCTGAGT | XM_001080051.5 | 147 |
| $cPLA_2$ ε | PLA2G4E | GAGGTGGCACGAGGTCTATG | AGGTAGCCATTGTCCAGGTTG | XM_017592204.1 | 123 |
| $cPLA_2$ ζ | PLA2G4F | ACTCTAAGGACCCCCGTGAA | TGTTTGTCGTTCCACTCCTGG | XM_017592206.1 | 131 |
| $iPLA_2$ β | PNPLA9 | CTCCCTGGACAGAACTCAGCC | CAGGTGGTCATGGCTTCGCT | NM_001005560.1 | 139 |
| $iPLA_2$ γ | PNPLA8 | CCTCGCGATTGATGGTGGAG | GCTAATATGGCCCCTGTGCTT | NM_001108020.2 | 135 |
| $iPLA_2$ δ | PNPLA6 | TAGTTGGGGAGTATGGCCGA | TAAGGCGAGTCACGACCTGT | XM_008769012.2 | 168 |
| $iPLA_2$ ε | PNPLA3 | GCGGCTTCCTAGGCTTCTAC | TCCATGATGTGATCGAGAGGG | NM_001282324.1 | 157 |
| $iPLA_2$ ζ | PNPLA2 | CGGATTCTAGAGCACCTGCC | GCCACTCCAACAAACGGATG | NM_001108509.2 | 184 |

**Supplemental Table 4-2. Cont**

| PLA$_2$ isoform | Gene name | Forward primer | Reverse primer | Fasta sequecence | Amplicon length |
|---|---|---|---|---|---|
| iPLA$_2$ η | PNPLA4 | CATTTTCGTCCGCACGTCAG | TCATGGTAGTGGCTGCAAGT | XM_006256967.3 | 97 |
| Lp-PLA$_2$ G7A | PLA2G7 | CTAGGAGTTGGCTCACGCTG | GCTTCCGTTTGTTGTTCTGTTCA | NM_001009353.1 | 90 |
| Lp-PLA$_2$ G7B | PAFAH2 | CGGATGTGATGGAGGGTCAC | GCAAGCGACAGGATCCGATG | NM_177932.2 | 195 |
| LPLA$_2$ | PLA2G15 | CTATGGTGGAGAGTCTTGTGGG | AAGGCCCGTTTTCATTTGGGG | NM_001004277.2 | 99 |

**Supplemental Table 4-3**. Effects of 3 doses of VAR and MAFP on PUFA release

| | | MAFP | | | VAR | | | |
|---|---|---|---|---|---|---|---|---|
| | CTR | 0.1μM | 1μM | 10μM | 0.01 μM | 0.1μM | 1μM | p |
| ARA | 5053 ± 865 | 3936 ± 819.6 | 2688 ± 1627 | 2377 ± 463.8 | 3286 ± 698.6 | 2257 ± 481.4 | 2919 ± 905.4 | 0.0217 |
| DHA | 5218 ± 472.2 | 5584 ± 1088 | 5103 ± 1927 | 2956 ± 146.8 | 2800 ± 535.9 | 1117 ± 151.4 | 1437 ± 274.4 | 0.0001 |
| ALA | 60.4 ± 15.2 | 64.9 ± 20.8 | 25.9 ± 5.17 | 4.68 ± 10.3 | 63.4 ± 5.96 | 55.7 ± 10.6 | 60.6 ± 4.51 | <.0001 |
| EPA | 14.9 ± 1.88 | 17.6 ± 1.18 | 8.01 ± 1.02 | 4.99 ± 1.19 | 15 ± 1.55 | 14.7 ± 1.82 | 15.3 ± 0.472 | <.0001 |

Results are shown as pmol/g of tissue/min of incubation (mean ± SE). n=6 for CTR, MAFP 10μM, and VAR 0.1μM (these data are shown in Figure 2), n=3 for remaining doses.

Indicates a significant difference when compared to control ($p<0.05$). Statistical significance between inhibitors is not shown.

**Supplemental Table 4-4.** Effects of 3 doses of VAR and MAFP on oxylipin formation

| | | MAFP | | | VAR | | | |
|---|---|---|---|---|---|---|---|---|
| | CTR | 0.1μM | 1μM | 10μM | 0.01 μM | 0.1μM | 1μM | p |
| **ARA COX Oxylipins** | | | | | | | | |
| $PGD_2$ | 2.5 ± 0.225 | 2.04 ± 0.024 | 2.08 ± 0.044 | 2.36 ± 0.229 | 2.31 ± 0.072 | 2.54 ± 0.106 | 2.24 ± 0.057 | 0.591 |
| $PGE_2$ | 2.14 ± 0.129 | 1.97 ± 0.134 | 1.91 ± 0.171 | 1.94 ± 0.132 | 2.19 ± 0.146 | 2.22 ± 0.095 | 2.11 ± 0.223 | 0.0038 |
| $11\beta PGE_2$ | 1.35 ± 0.12 | 1.18 ± 0.029 | 1.18 ± 0.044 | 1.21 ± 0.075 | 1.34 ± 0.03 | 1.43 ± 0.071 | 1.3 ± 0.05 | 0.0796 |
| 15k $PGE_2$ | 2.07 ± 0.429 | 2.25 ± 0.409 | 1.26 ± 0.216 | 1.21 ± 0.197 | 2.08 ± 0.14 | 2.04 ± 0.294 | 1.82 ± 0.464 | 0.101 |
| $TXB_2$ | 0.926 ± 0.065 | 0.977 ± 0.059 | 1.13 ± 0.097 | 1.1 ± 0.096 | 1.08 ± 0.086 | 1 ± 0.077 | 1.07 ± 0.087 | 0.0001 |
| $PGF_2\alpha$ | 9.9 ± 0.727 | 9.86 ± 1.75 | 10.2 ± 1.73 | 9.24 ± 0.85 | 11.1 ± 1.58 | 11.2 ± 0.885 | 11 ± 1.69 | <.0001 |
| 15k $PGF_2\alpha$ | 5.59 ± 0.39 | 5.79 ± 0.478 | 4.92 ± 0.642 | 4.77 ± 0.416 | 5.86 ± 0.557 | 6.22 ± 0.337 | 6.1 ± 0.713 | <.0001 |
| 12-HHTrE | 64.5 ± 10.9 | 47.2 ± 5.24 | 39.8 ± 4.51 | 49.4 ± 4.14 | 45 ± 6.29 | 60.9 ± 9.53 | 42.9 ± 3.98 | 0.3933 |
| 6k $PGF_1\alpha$ | 63 ± 4.9 | 69.4 ± 5.48 | 68 ± 7.69 | 61.4 ± 5.38 | 71 ± 9.35 | 67.2 ± 4.85 | 73.6 ± 8.41 | 0.0217 |
| **ARA LOX Oxylipins** | | | | | | | | |
| 5-HETE | 14 ± 1.32 | 11.1 ± 1.23 | 6.42 ± 1.39 | 7.29 ± 1.3 | 12.9 ± 1.97 | 11.8 ± 0.602 | 11.9 ± 0.245 | 0.0021 |
| 8-HETE | 10.3 ± 1.32 | 9.63 ± 2.05 | 4.69 ± 0.658 | 3.57 ± 0.407 | 10.6 ± 0.645 | 9.99 ± 0.529 | 10.5 ± 0.266 | <.0001 |
| 9-HETE | 10.1 ± 1.51 | 13.9 ± 4.28 | 5.42 ± 1.34 | 5.12 ± 1.35 | 12 ± 2.34 | 9.33 ± 0.984 | 8.92 ± 2.21 | 0.0197 |
| 11-HETE | 12.6 ± 1.2 | 10.8 ± 0.716 | 6.96 ± 1.07 | 7.65 ± 0.806 | 10.7 ± 0.536 | 9.8 ± 0.546 | 10.2 ± 1.03 | 0.0006 |
| 12-HETE | 51.5 ± 4.59 | 49.9 ± 8.4 | 47.4 ± 7.75 | 45.3 ± 5.11 | 57.2 ± 8.85 | 55.3 ± 4.51 | 54.6 ± 9.35 | 0.084 |
| 15-HETE | 21 ± 2.78 | 16.5 ± 1.21 | 11.4 ± 1.59 | 10.6 ± 1.92 | 16.4 ± 0.815 | 16.3 ± 0.946 | 16.1 ± 0.785 | 0.001 |
| $LTB_4$ | 0.245 ± 0.069 | 0.187 ± 0.05 | 0.127 ± 0.025 | 0.108 ± 0.035 | 0.218 ± 0.038 | 0.195 ± 0.027 | 0.248 ± 0.051 | 0.1491 |
| 12epi $LTB_4$ | 0.272 ± 0.066 | 0.192 ± 0.038 | 0.167 ± 0.025 | 0.137 ± 0.026 | 0.232 ± 0.024 | 0.194 ± 0.031 | 0.242 ± 0.041 | 0.1803 |
| 6S-$LXA_4$ | 2.57 ± 0.97 | 1.07 ± 0.419 | 0.744 ± 0.2 | 1.58 ± 0.682 | 0.976 ± 0.234 | 1.78 ± 0.283 | 1.06 ± 0.096 | 0.1984 |
| **ARA CYP Oxylipins** | | | | | | | | |
| 16-HETE | 3.12 ± 0.613 | 2.22 ± 0.279 | 1.07 ± 0.567 | 1.21 ± 0.442 | 2.41 ± 0.577 | 2.68 ± 0.267 | 2.34 ± 0.357 | 0.0002 |
| 5,6 EpETrE | 5.31 ± 1.53 | 2.97 ± 0.132 | 2.38 ± 0.451 | 2.62 ± 1.709 | 4.26 ± 0.312 | 3.26 ± 1.22 | 3.78 ± 0.777 | 0.2315 |
| 8,9 EpETrE | 1.2 ± 0.3 | 0.812 ± 0.08 | 0.662 ± 0.055 | 0.5 ± 0.349 | 1.1 ± 0.135 | 0.842 ± 0.265 | 0.986 ± 0.087 | 0.1764 |
| 8,9 DiHETrE | 0.293 ± 0.048 | 0.215 ± 0.06 | 0.113 ± 0.04 | 0.131 ± 0.044 | 0.217 ± 0.031 | 0.268 ± 0.056 | 0.249 ± 0.03 | 0.0012 |

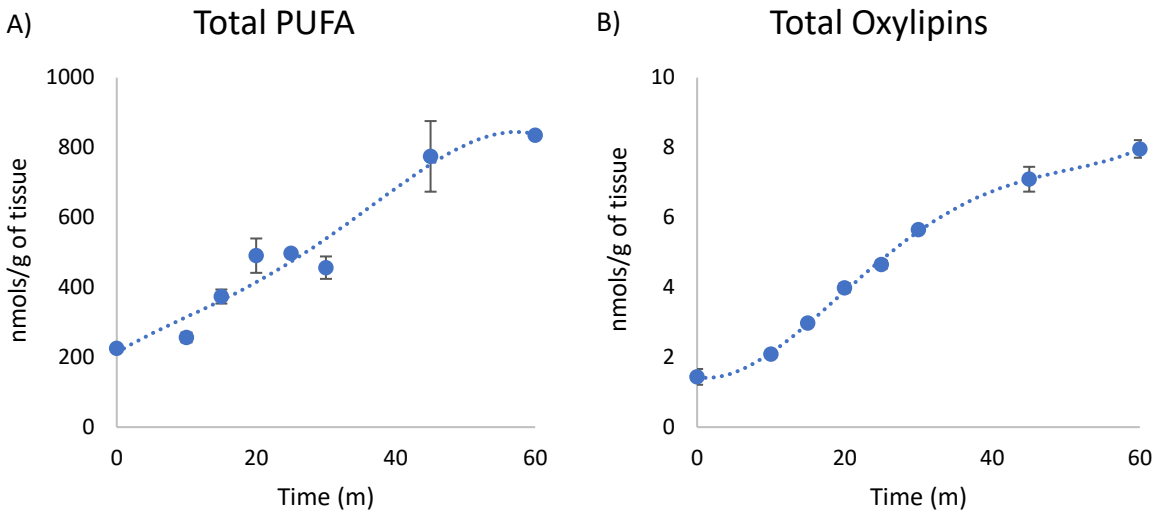
| | | MAFP | | | VAR | | | |
|---|---|---|---|---|---|---|---|---|
| | CTR | 0.1μM | 1μM | 10μM | 0.01 μM | 0.1μM | 1μM | p |
| 11,12 EpETrE | 2.13 ± 0.413 | 1.35 ± 0.179 | 1.55 ± 0.079 | 0.406 ± 0.386 | 2.17 ± 0.315 | 1.69 ± 0.522 | 1.9 ± 0.074 | 0.0194 |
| 11,12 DiHETrE | 0.217 ± 0.034 | 0.166 ± 0.025 | 0.141 ± 0.005 | 0.124 ± 0.022 | 0.158 ± 0.013 | 0.167 ± 0.021 | 0.14 ± 0.01 | 0.0003 |
| 14,15 EpETrE | 0.891 ± 0.07 | 0.74 ± 0.133 | 0.616 ± 0.05 | 0.067 ± 0.145 | 1 ± 0.257 | 0.829 ± 0.281 | 0.944 ± 0.132 | 0.0239 |
| 14,15 DiHETrE | 0.205 ± 0.029 | 0.157 ± 0.017 | 0.107 ± 0.009 | 0.091 ± 0.018 | 0.137 ± 0.011 | 0.155 ± 0.018 | 0.131 ± 0.011 | <.0001 |
| **LA LOX Oxylipins** | | | | | | | | |
| 9-HODE | 135 ± 19.3 | 96.8 ± 8.02 | 55.1 ± 5.06 | 42.5 ± 10.3 | 134 ± 12.4 | 144.5 ± 8.35 | 137.7 ± 10.6 | <.0001 |
| 13-HODE | 48.7 ± 7.43 | 37.8 ± 3.14 | 20.1 ± 2.23 | 17.8 ± 4.4 | 49.8 ± 2.96 | 54.7 ± 4.64 | 50.7 ± 5.26 | <.0001 |
| 9,10,13 triHOME | 17 ± 3.68 | 13.6 ± 1.75 | 7.9 ± 1.98 | 7.9 ± 3.95 | 17.5 ± 1.9 | 17.1 ± 2.34 | 18.8 ± 5.83 | 0.0156 |
| 9,12,13 triHOME | 6.15 ± 1.48 | 5.18 ± 0.816 | 2.84 ± 0.815 | 3.39 ± 1.54 | 6.7 ± 0.984 | 6 ± 1.08 | 6.83 ± 1.87 | 0.0337 |
| **LA CYP Oxylipins** | | | | | | | | |
| 9,10 EpOME | 3.01 ± 0.45 | 1.8 ± 0.561 | 0.013 ± 0.432 | -0.961 ± 0.371 | 3.37 ± 0.172 | 3.1 ± 0.676 | 3.17 ± 0.197 | <.0001 |
| 9,10 diHOME | 0.423 ± 0.054 | 0.412 ± 0.049 | 0.232 ± 0.004 | 0.116 ± 0.016 | 0.415 ± 0.041 | 0.451 ± 0.068 | 0.411 ± 0.038 | <.0001 |
| 12,13 EpOME | 2.22 ± 0.246 | 1.67 ± 0.709 | -0.1 ± 0.191 | -0.872 ± 0.375 | 2.94 ± 0.367 | 2.31 ± 0.607 | 2.63 ± 0.318 | <.0001 |
| 12,13 diHOME | 0.445 ± 0.086 | 0.518 ± 0.07 | 0.285 ± 0.03 | 0.117 ± 0.038 | 0.514 ± 0.088 | 0.482 ± 0.08 | 0.478 ± 0.114 | <.0001 |
| **GLA Oxylipins** | | | | | | | | |
| 13-HOTrE-y | 0.206 ± 0.032 | 0.164 ± 0.045 | 0.094 ± 0.04 | 0.089 ± 0.03 | 0.215 ± 0.072 | 0.312 ± 0.058 | 0.239 ± 0.065 | 0.0014 |
| **DGLA COX Oxylipins** | | | | | | | | |
| 15k $PGE_1$ | 0.073 ± 0.025 | 0.082 ± 0.01 | 0.045 ± 0.014 | 0.042 ± 0.008 | 0.071 ± 0.004 | 0.072 ± 0.012 | 0.07 ± 0.025 | 0.6186 |
| $PGF_1\alpha$ | 0.219 ± 0.021 | 0.235 ± 0.052 | 0.26 ± 0.04 | 0.251 ± 0.029 | 0.253 ± 0.031 | 0.269 ± 0.026 | 0.263 ± 0.033 | 0.0735 |
| **DGLA LOX Oxylipins** | | | | | | | | |
| 8-HETrE | 0.457 ± 0.059 | 0.413 ± 0.124 | 0.153 ± 0.116 | 0.123 ± 0.081 | 0.564 ± 0.164 | 0.452 ± 0.064 | 0.424 ± 0.095 | 0.0092 |
| 15-HETrE | 1.82 ± 0.287 | 1.53 ± 0.167 | 1.35 ± 0.328 | 1.25 ± 0.288 | 1.95 ± 0.298 | 1.96 ± 0.247 | 1.85 ± 0.287 | 0.0619 |
| **DHA LOX Oxylipins** | | | | | | | | |
| 4-HDoHE | 9.62 ± 1.41 | 8.38 ± 0.633 | 6.95 ± 1.95 | 6.72 ± 0.793 | 4.01 ± 0.804 | 2.61 ± 0.246 | 3.1 ± 0.3 | <.0001 |
| 7-HDoHE | 1.92 ± 0.256 | 2 ± 0.399 | 1.4 ± 0.344 | 1.3 ± 0.223 | 1.08 ± 0.052 | 0.769 ± 0.115 | 0.933 ± 0.264 | 0.0064 |
| 8-HDoHE | 5.47 ± 0.74 | 4.55 ± 0.561 | 2.54 ± 0.789 | 3.27 ± 0.583 | 2.23 ± 0.082 | 1.39 ± 0.337 | 1.01 ± 0.356 | <.0001 |

| | | MAFP | | | VAR | | | |
|---|---|---|---|---|---|---|---|---|
| | CTR | 0.1μM | 1μM | 10μM | 0.01μM | 0.1μM | 1μM | p |
| 10-HDoHE | 2 ± 0.321 | 1.77 ± 0.212 | 1.15 ± 0.483 | 1.28 ± 0.236 | 0.82 ± 0.057 | 0.65 ± 0.065 | 0.727 ± 0.15 | 0.0006 |
| 11-HDoHE | 2.51 ± 0.176 | 2.56 ± 0.794 | 1.75 ± 0.635 | 1.67 ± 0.258 | 1.43 ± 0.18 | 0.765 ± 0.211 | 0.918 ± 0.361 | 0.0002 |
| 13-HDoHE | 3.3 ± 0.567 | 3.33 ± 0.605 | 2.39 ± 0.919 | 2.54 ± 0.486 | 1.39 ± 0.191 | 1.08 ± 0.114 | 1.46 ± 0.385 | <.0001 |
| 14-HDoHE | 11.6 ± 2 | 11.2 ± 4.18 | 9.3 ± 3.57 | 9.73 ± 1.98 | 7.53 ± 2.07 | 7.19 ± 1.27 | 6.85 ± 2.01 | 0.0534 |
| 16-HDoHE | 2.99 ± 0.368 | 2.51 ± 0.365 | 2.08 ± 0.55 | 1.75 ± 0.256 | 1.25 ± 0.278 | 0.919 ± 0.128 | 0.916 ± 0.175 | <.0001 |
| 17-HDoHE | 38.6 ± 6.31 | 37.5 ± 9.93 | 31.7 ± 9.31 | 28 ± 4.64 | 24 ± 4.49 | 19.3 ± 2.12 | 21.3 ± 4.77 | <.0001 |
| **DHA CYP Oxylipins** | | | | | | | | |
| 20-HDoHE | 5.4 ± 1.25 | 4.3 ± 0.753 | 3.43 ± 1.05 | 2.97 ± 0.527 | 2.53 ± 0.772 | 1.27 ± 0.488 | 1.49 ± 0.666 | 0.0011 |
| 16,17 EpDoPE | 5.37 ± 1.17 | 4.9 ± 1.45 | 4.05 ± 1.22 | 2.64 ± 0.592 | 2.31 ± 0.244 | 0.925 ± 0.428 | 0.976 ± 0.157 | 0.0022 |
| 19,20 EpDoPE | 1.3 ± 0.193 | 1.43 ± 0.556 | 1.17 ± 0.341 | 0.88 ± 0.282 | 0.533 ± 0.244 | 0.348 ± 0.095 | 0.16 ± 0.339 | 0.0142 |
| 16,17 DiHDoPE | 0.038 ± 0.004 | 0.036 ± 0.009 | 0.018 ± 0.004 | 0.02 ± 0.006 | 0.012 ± 0.006 | 0.022 ± 0.005 | 0.012 ± 0.005 | 0.0054 |
| **ALA LOX Oxylipins** | | | | | | | | |
| 9-HOTrE | 1.29 ± 0.331 | 0.785 ± 0.096 | 0.292 ± 0.083 | 0.267 ± 0.102 | 1.14 ± 0.168 | 1.76 ± 0.382 | 1.05 ± 0.104 | <.0001 |
| 13-HOTrE | 1.59 ± 0.432 | 0.923 ± 0.346 | 0.812 ± 0.296 | 0.995 ± 0.239 | 1.16 ± 0.437 | 2.24 ± 0.602 | 1.24 ± 0.389 | 0.007 |
| **EPA LOX Oxylipins** | | | | | | | | |
| 12-HEPE | 0.687 ± 0.122 | 0.89 ± 0.203 | 0.58 ± 0.095 | 0.757 ± 0.215 | 0.867 ± 0.136 | 1.01 ± 0.216 | 0.709 ± 0.128 | 0.3192 |
| **EPA CYP Oxylipins** | | | | | | | | |
| 18-HEPE | 0.263 ± 0.072 | 0.189 ± 0.079 | 0.007 ± 0.033 | -0.059 ± 0.044 | 0.148 ± 0.021 | 0.248 ± 0.038 | 0.192 ± 0.088 | 0.0012 |
| 14,15 diHETE | 0.322 ± 0.108 | 0.218 ± 0.169 | 0.231 ± 0.082 | 0.095 ± 0.192 | 0.306 ± 0.097 | 0.363 ± 0.057 | 0.638 ± 0.123 | 0.3256 |
| **Non-quantifiable oxylipins** | | | | | | | | |
| $HXB_3$ | 5.21 ± 1.03 | 5.45 ± 0.969 | 4.24 ± 0.866 | 3.24 ± 0.5 | 5.53 ± 1.13 | 5.19 ± 0.812 | 6.15 ± 2.07 | 0.0609 |
| 9,10 EpODE | 0.715 ± 0.101 | 0.481 ± 0.14 | 0.073 ± 0.069 | 0.059 ± 0.16 | 0.942 ± 0.082 | 0.967 ± 0.181 | 0.902 ± 0.062 | 0.0001 |
| 15,16 diHODE | 5.1 ± 0.842 | 5.15 ± 1.39 | 4.36 ± 1.17 | 4.73 ± 1.09 | 5.65 ± 1.83 | 4.76 ± 0.95 | 5.16 ± 1.5 | 0.5503 |
| 5-iso $PGF_{2\alpha}$-VI | 3.3 ± 0.579 | 3.16 ± 0.718 | 1.95 ± 0.652 | 2.58 ± 0.523 | 1.75 ± 0.401 | 3.61 ± 0.857 | 1.97 ± 0.203 | 0.33 |
| 8-iso $PGF_{2\alpha}$-III | 6.5 ± 0.736 | 9.32 ± 0.754 | 6.45 ± 1.47 | 7.58 ± 0.869 | 6.83 ± 1.25 | 6.62 ± 0.626 | 5.59 ± 0.366 | 0.2136 |

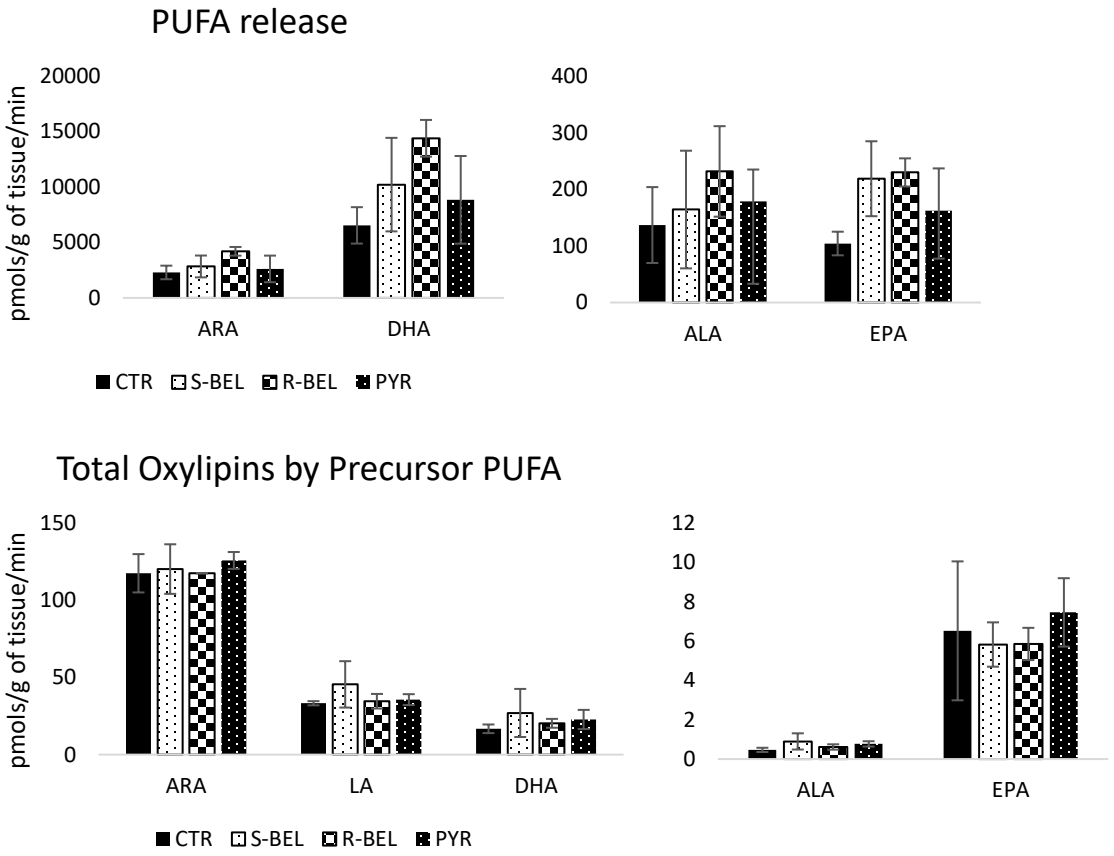
Results are shown as pmol/g of tissue/min of incubation (mean ± SE). n=6 for CTR, MAFP 10Mm, and VAR 0.1Mm (these data are shown in Figures 4-6), n=3 for remaining doses].

Indicates a significant difference when compared to control ($p<0.05$). Statistical significance between inhibitors is not shown.

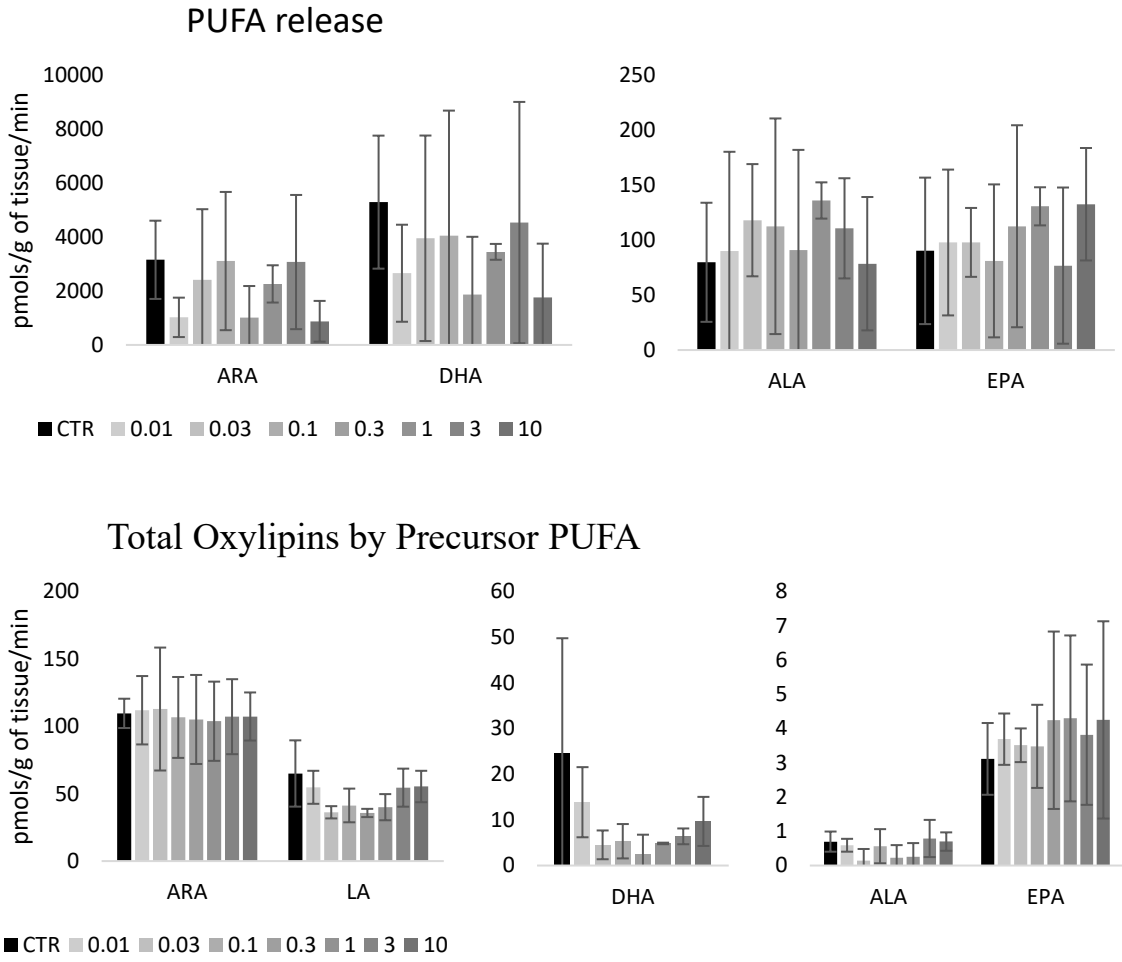
**Supplemental Figure 4-1.** Representative time course for A) total PUFA release and B) total oxylipins formed in duplicates of the same rat heart. Error bars are SD.



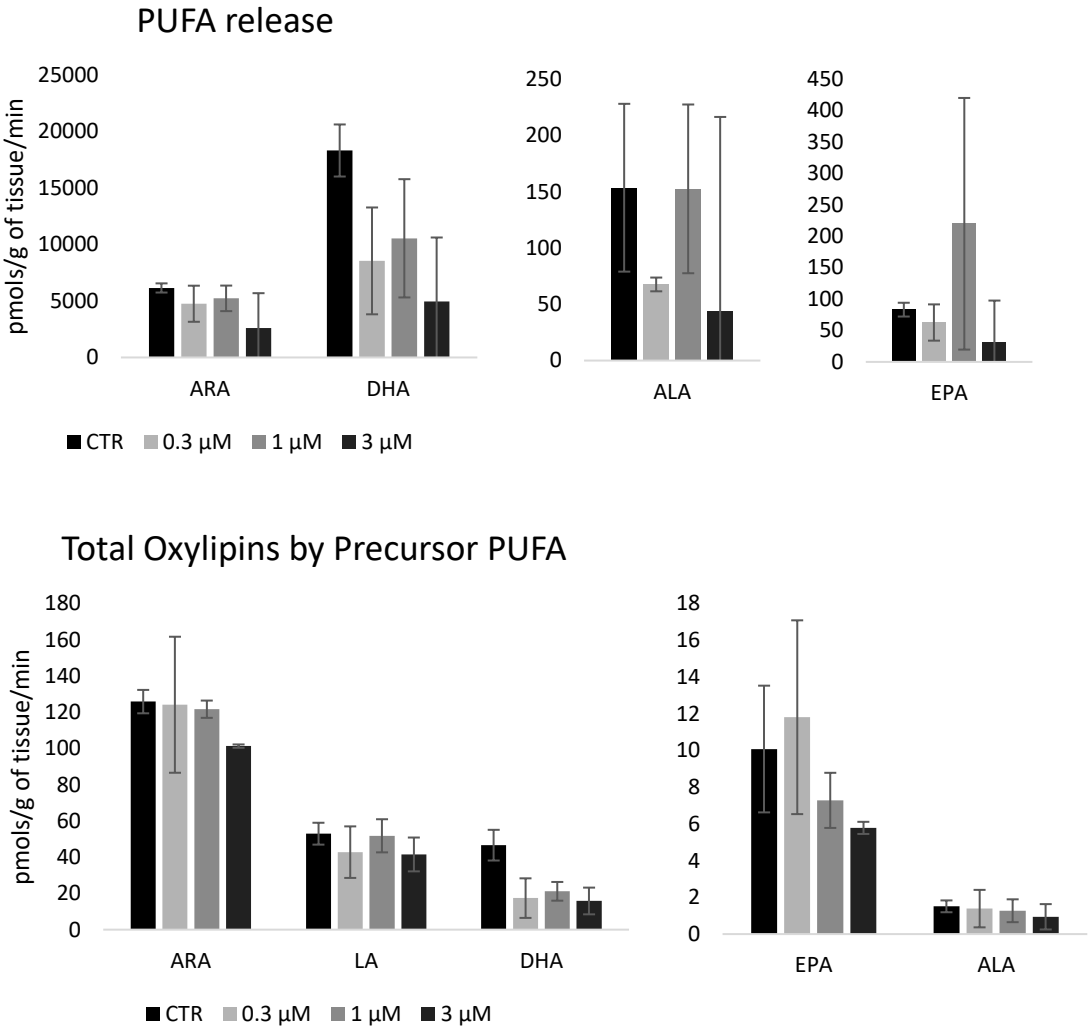
**Supplemental Figure 4-2.** Effects of specific inhibitors for iPLA$_2$β, iPLA$_2$γ, and cPLA$_2$α on PUFA release and oxylipin formation. Data show one rat heart homogenate analyzed in duplicate (mean ± SD), which was representative of multiple experiments conducted under different conditions (i.e. other incubation times or inhibitor doses). CTR – control samples without inhibitor, S-BEL – 10μM of S-BEL (iPLA$_2$β inhibitor), R-BEL – 10μM of R-BEL (iPLA$_2$γ inhibitor), PYR –10μM Pyrrophenone (cPLA$_2$α inhibitor).



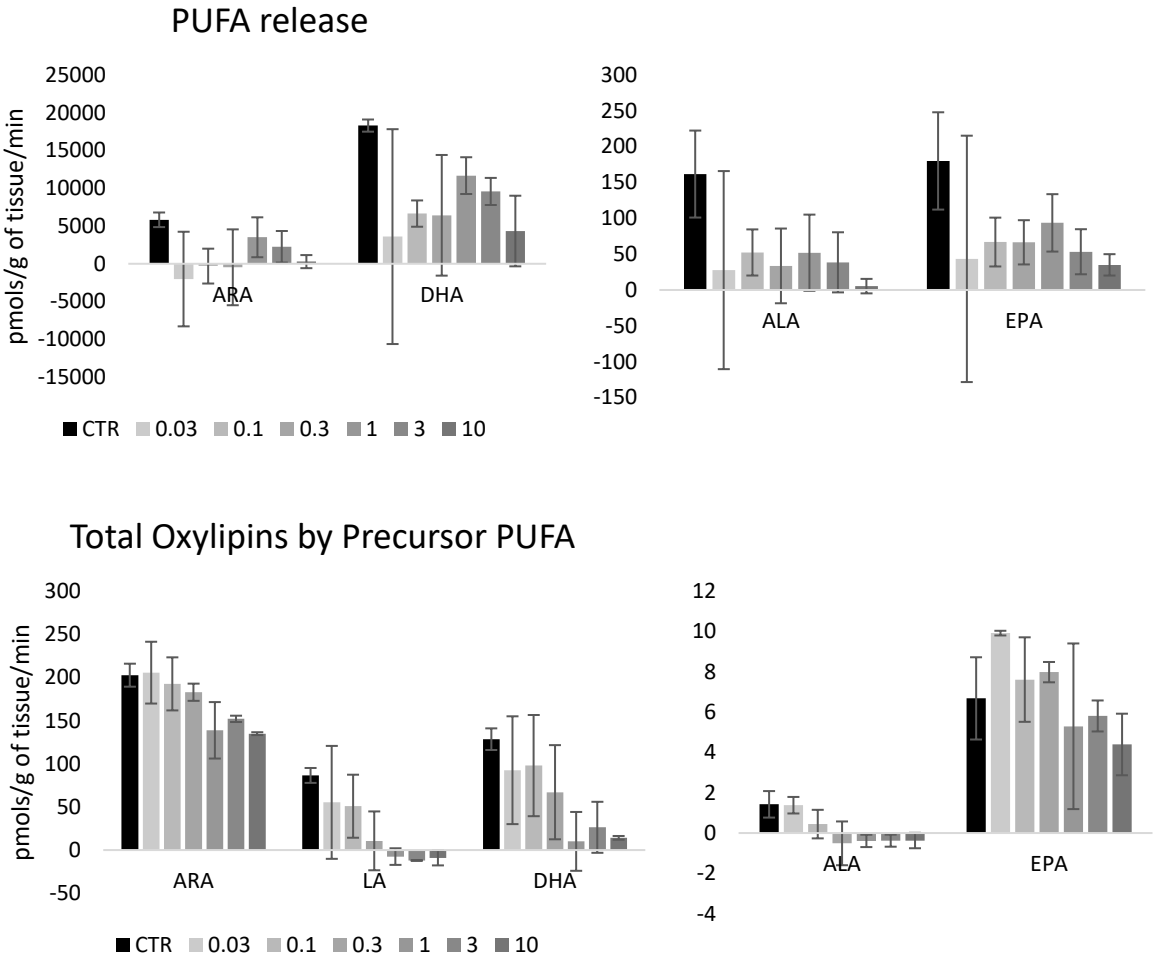
**Supplemental Figure 4-3.** Effect of dose of VAR, an inhibitor of $sPLA_2$ IIA, V, and X, on PUFA release and oxylipin formation in rat heart. The results are shown for n=2 with mean±SD Numbers in the legend indicate dose in μM.



**Supplemental Figure 4-4.** Effects of dose of LY311727, another inhibitor for $sPLA_2$ IIA, V, and X, on PUFA release and oxylipin formation. Data show one rat heart homogenate analyzed in duplicate (mean ± SD).

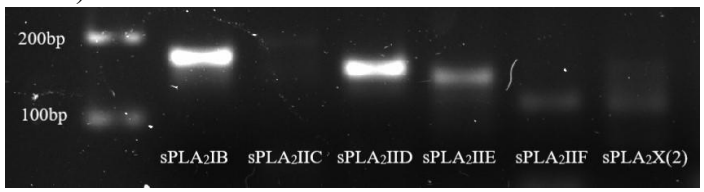


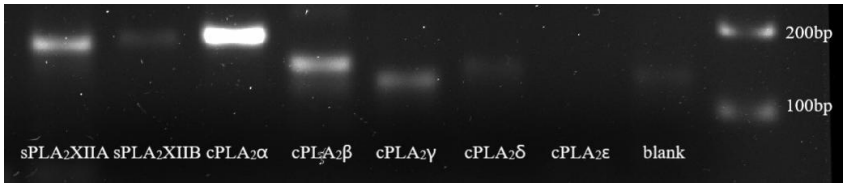
**Supplemental Figure 4-5.** Effects of dose of MAFP, and inhibitor of $cPLA_2$ and $iPLA_2$, on PUFA release and oxylipin formation. Data are from one rat heart homogenate analyzed in duplicate (mean ± SD). Numbers in the legend indicate dose in μM.

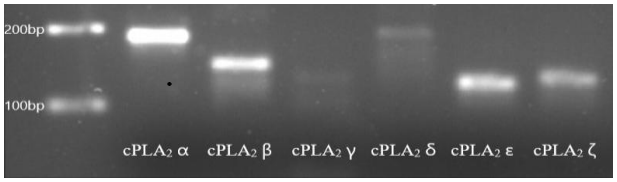


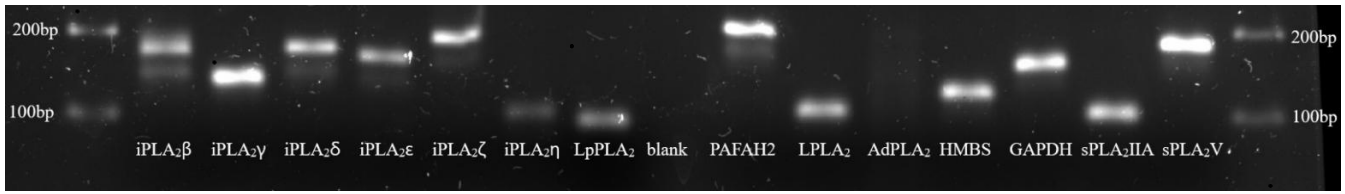
**Supplemental Figure 4-6**. RT-qPCR products were run in a agarose gel to verify product amplicon length. Amplicon legnth is repported in Supplemental Table 2 and were generally between 100-200bp. A) Images of gels for results from heart samples. B) Images of gels for results from testis (T) and brain (B) samples to confirm primers of isoforms not found in heart samples.

A) Heart results for each $PLA_2$ isoform:

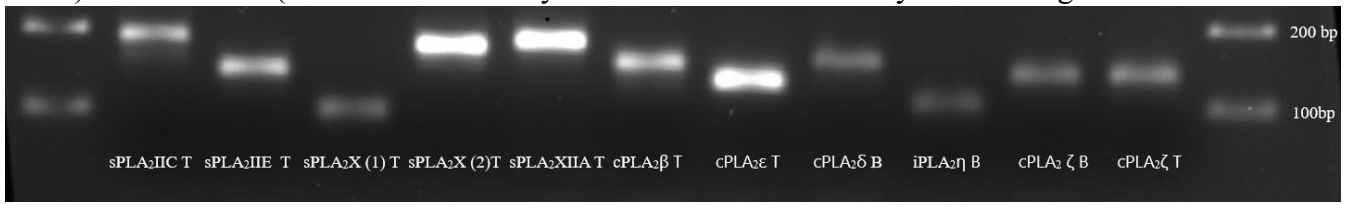


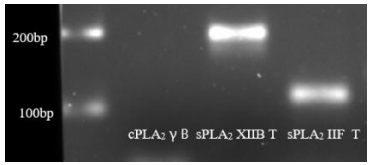



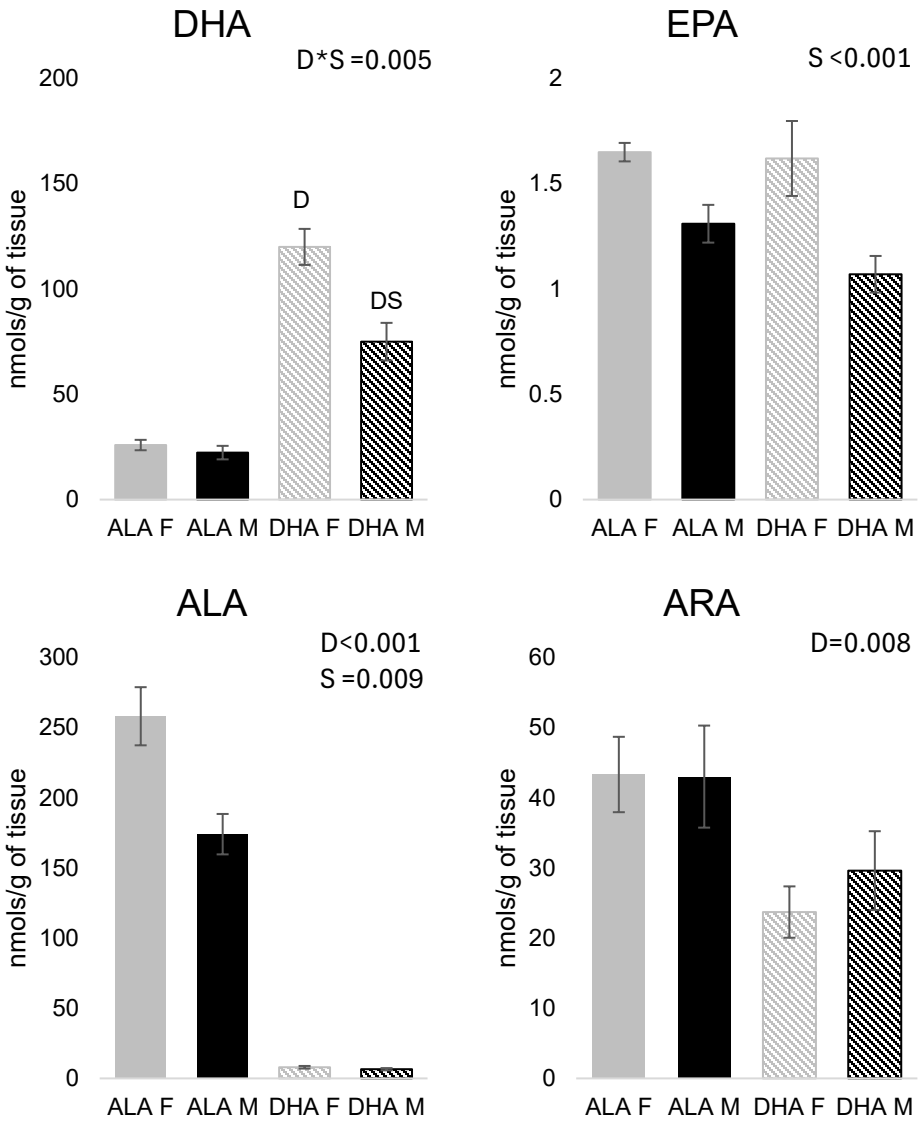




B) Other tissues (Testis is indicated by T and Brain is indicated by B following the $PLA_2$ isoform abreviation):

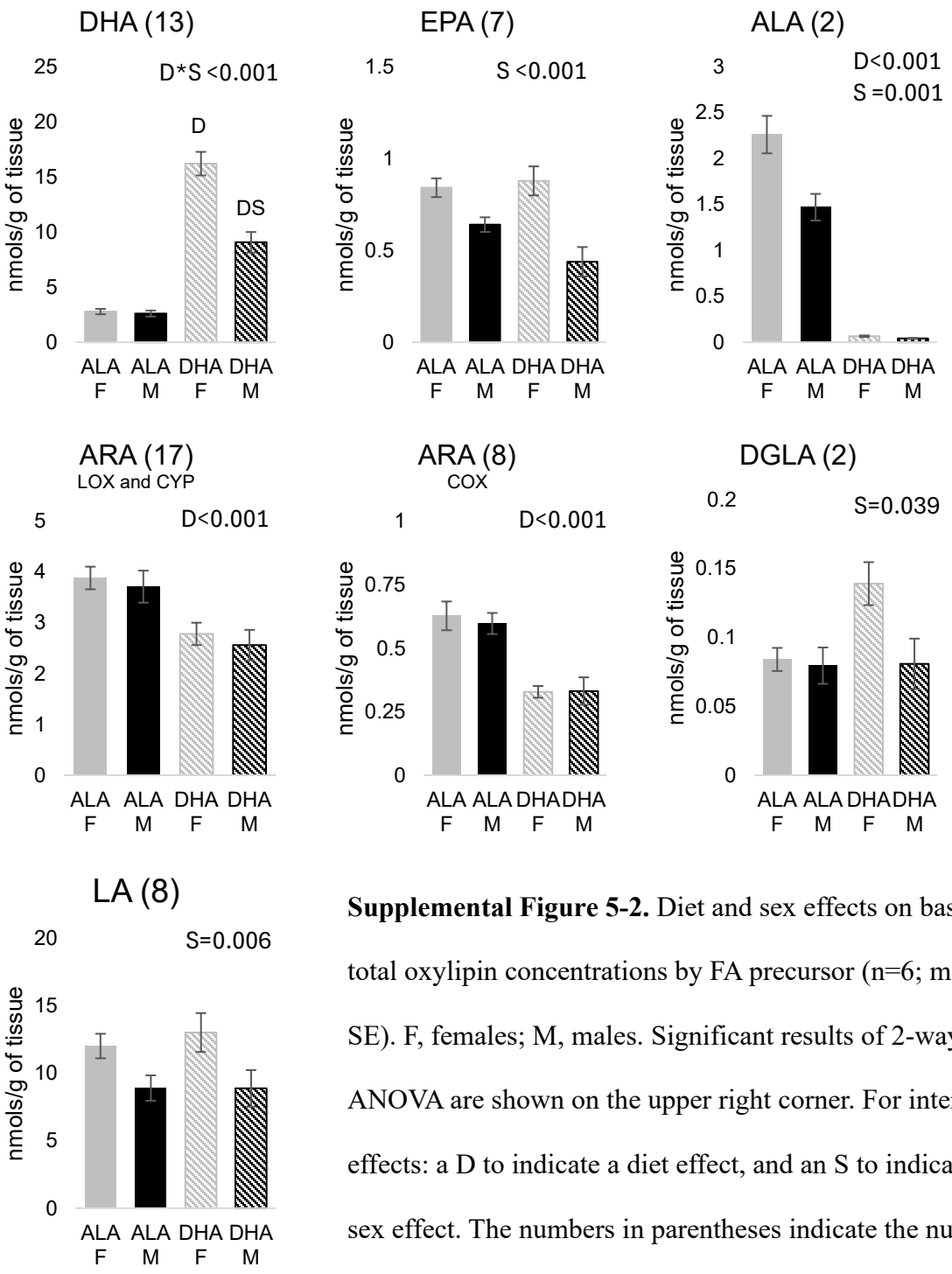


# Appendix 4: Supplemental figures and tables for Chapter 5




**Supplemental Figure 5-1**. Diet and sex effects on baseline FA levels (n=6; mean ± SE). F, females; M, males. Significant results of 2-way ANOVA are shown on the upper right corner. For interaction effects: a D to indicate a diet effect, and an S to indicate a sex effect.




**Supplemental Figure 5-2.** Diet and sex effects on baseline total oxylipin concentrations by FA precursor (n=6; mean ± SE). F, females; M, males. Significant results of 2-way ANOVA are shown on the upper right corner. For interaction effects: a D to indicate a diet effect, and an S to indicate a sex effect. The numbers in parentheses indicate the number

**Supplemental Table 5-1.** Oil amounts and fatty acid composition of diets used.

| | **ALA diet** | **DHA diet** |
|---|---|---|
| *Dietary oils (g/100g)* | | |
| DHA oil | 0.000 | 1.15 |
| Flax oil | 2.29 | 0.000 |
| Coconut oil | 1.98 | 2.19 |
| Safflower oil | 2.42 | 2.82 |
| Olive oil | 0.310 | 0.840 |
| *Analyzed Fatty acids (g/100g)* | | |
| SFA | 2.35 | 2.16 |
| MUFA | 1.15 | 1.08 |
| PUFA | 3.56 | 3.54 |
| LA | 2.24 | 2.18 |
| ALA | 1.32 | 0.009 |
| EPA | 0.001 | 0.002 |
| DHA (g/100g diet or %diet wt) | 0.000 | 1.35 |
| ALA and DHA (energy %) | 3.16 | 3.22 |

AIN93G-based diets were formulated as follows: cornstarch (39.8g/100g), casein (20g/100g), dytrose (13.2g/100g), sucrose (10g/100g), fibre (5g/100g), AIN93G-MX (3.5g/100g), AIN93G-VX (1g/100g), L-cystine (0.3g/100g), choline bitartrate, 41.1% choline (0.25g/100g), TBHQ (0.0014g/100g), oil (7g/100g). Diet fatty acids were analyzed in duplicate.

**Supplemental Table 5-2**. Effects of sex and dietary ALA and DHA on $PLA_2$ activity towards non-esterified PUFA in rat hearts (n=6; mean ± SE).

| | Group | ALA F | ALA M | DHA F | DHA M | Post hoc | SW | Diet | Sex | Treatment (Treat) | Diet* Sex | Diet* Treat | Sex* Treat | Diet*Sex *Treat |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Fatty acids | | | | | | | | |
| DHA | Control | 133 ± 11.0 | 97.1 ± 11.4 | 205 ± 23.0 | 155 ± 18.7 | S (A:D) | 0.6961 | 0.0001 | 0.0172 | <0.0001 | 0.6667 | 0.0862 | 0.0303 | 0.9368 |
| | MAFP | 89.8 ± 7.43 | 65.4 ± 7.80 | 132 ± 14.2 | 103 ± 15.6 | *S (A:D) | | | | | | | | |
| | VAR | 32.1 ± 5.39 | 26.7 ± 4.18 | 78.0 ± 8.48 | 65.5 ± 2.95 | *$ (A:D) | | | | | | | | |
| EPA | Control | 1.83 ± 0.086 | 1.40 ± 0.114 s | 1.49 ± 0.200 | 1.04 ± 0.043 ds | | 0.2813 | 0.0539 | 0.0023 | <0.0001 | 0.7047 | 0.0226 | 0.0265 | 0.5842 |
| | MAFP | 0.739 ± 0.064 * | 0.510 ± 0.067 * | 0.582 ± 0.080 * | 0.422 ± 0.025 * | | | | | | | | | |
| | VAR | 1.45 ± 0.085 *$ | 0.988 ± 0.055 *$s | 1.24 ± 0.227 *$ | 0.951 ± 0.073 $ | | | | | | | | | |
| ALA | Control | 153 ± 20.1 | 116 ± 10.4 | 5.52 ± 0.869 | 4.69 ± 0.830 | | 0.0025 (T) | <0.0001 | 0.0300 | <0.0001 | 0.1383 | 0.0513 | 0.6950 | 0.1586 |
| | MAFP | 39.6 ± 15.8 | 26.5 ± 6.51 | 2.59 ± 0.428 | 1.65 ± 1.07 | * | | | | | | | | |
| | VAR | 193 ± 29.9 | 132 ± 10.8 | 7.22 ± 1.19 | 6.24 ± 0.997 | $ | | | | | | | | |
| ARA | Control | 80.3 ± 8.4 | 54.3 ± 8.92 | 43.0 ± 8.02 | 37.2 ± 4.79 | | 0.5670 | 0.0008 | 0.0732 | <0.0001 | 0.1549 | 0.1361 | 0.2604 | 0.1298 |
| | MAFP | 37.6 ± 5.98 | 33.3 ± 6.74 | 24.9 ± 5.20 | 17.8 ± 3.05 | * | | | | | | | | |
| | VAR | 49.5 ± 7.75 | 31.6 ± 4.06 | 22.0 ± 5.57 | 28.9 ± 3.35 | * | | | | | | | | |

Treatments: Control – incubation without inhibitor, VAR – 0.1 μM of VAR (sPLA2 inhibitor – remaining activity is primarily iPLA2 derived), MAFP – 10 μM of MAFP (iPLA2 inhibitor – remaining activity is sPLA2 derived). Complete results of 3-way repeated measures ANOVA are shown. For interaction effects, relevant differences as determined by BH post hoc tests were denoted with a * to indicate differences from Control, a $ to indicate differences between inhibitors, a D to indicate a diet effect, and an S to indicate a sex effect. T indicates a treatment effect. Effects applicable to the treatment group are shown on the post hoc column, with (A:D) indicating results were averaged over diet, (A:S) averaged over sex, or (A:T) averaged over treatment. When a 3-way interaction analysis was conducted, results are shown in superscript, beside mean ± SE with the difference being from the same sex or diet group within that treatment. SW column showns the Shapiro-Wilk results and whether log transformation was applied (T), or log + 1 (T+1). Oxylipin totals are included for complete display of statistical analysis results. Significant ANOVA p values to be interpreted are highlighted in yellow. Abbreviations: ARA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; F, female; M, male; PUFA, polyunsaturated fatty acids.

**Supplemental Table 5-3**. Effects of sex and dietary ALA and DHA on $PLA_2$ activity towards non-esterified oxylipins in rat hearts (n=6; mean ± SE).

| | Group | ALA F | ALA M | DHA F | DHA M | Post hoc | SW | Diet | Sex | Treatment (Treat) | Diet* Sex | Diet* Treat | Sex* Treat | Diet*Sex *Treat |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | DHA Oxylipins | | | | | | | | | |
| 4-HDoHE | Control | 1.56 ± 0.158 | 1.34 ± 0.095 | 3.09 ± 0.479 | 2.52 ± 0.676 | | <0.0001 (T) | 0.0103 | 0.8726 | <0.0001 | 0.9470 | 0.9270 | 0.1838 | 0.9631 |
| | MAFP | 1.05 ± 0.108 | 0.843 ± 0.130 | 1.52 ± 0.195 | 1.49 ± 0.369 | * | | | | | | | | |
| | VAR | 0.269 ± 0.046 | 0.432 ± 0.058 | 0.797 ± 0.201 | 0.913 ± 0.199 | *$ | | | | | | | | |
| 7-HDoHE | Control | 0.291 ± 0.051 | 0.255 ± 0.022 | 0.507 ± 0.079 | 0.450 ± 0.129 | | 0.0081 (T+1) | 0.0311 | 0.8717 | <0.0001 | 0.8863 | 0.0708 | 0.5236 | 0.6646 |
| | MAFP | 0.212 ± 0.033 | 0.189 ± 0.033 | 0.233 ± 0.074 | 0.258 ± 0.056 | * | | | | | | | | |
| | VAR | 0.039 ± 0.015 | 0.084 ± 0.019 | 0.108 ± 0.042 | 0.103 ± 0.035 | *$ | | | | | | | | |
| 8-HDoHE | Control | 0.315 ± 0.041 | 0.239 ± 0.010 | 0.494 ± 0.096 | 0.464 ± 0.142 | | 0.0004 (T+1) | 0.0589 | 0.4109 | <0.0001 | 0.7490 | 0.1095 | 0.7299 | 0.9803 |
| | MAFP | 0.241 ± 0.029 | 0.185 ± 0.021 | 0.254 ± 0.073 | 0.223 ± 0.062 | * | | | | | | | | |
| | VAR | 0.088 ± 0.017 | 0.087 ± 0.012 | 0.125 ± 0.056 | 0.132 ± 0.063 | *$ | | | | | | | | |
| 10-HDoHE | Control | 0.417 ± 0.111 | 0.279 ± 0.025 | 0.601 ± 0.048 | 0.395 ± 0.030 | | 0.0001 (T+1) | 0.0078 | 0.0341 | <0.0001 | 0.1797 | 0.5711 | 0.1194 | 0.7448 |
| | MAFP | 0.21 ± 0.043 | 0.189 ± 0.014 | 0.348 ± 0.051 | 0.238 ± 0.089 | * | | | | | | | | |
| | VAR | 0.040 ± 0.014 | 0.072 ± 0.016 | 0.186 ± 0.024 | 0.097 ± 0.045 | *$ | | | | | | | | |
| 11-HDoHE | Control | 0.330 ± 0.070 | 0.199 ± 0.018 | 0.521 ± 0.032 | 0.371 ± 0.040 | S | 0.0294 | 0.0001 | 0.0547 | <0.0001 | 0.4117 | 0.0636 | 0.0048 | 0.7230 |
| | MAFP | 0.163 ± 0.027 | 0.157 ± 0.017 | 0.276 ± 0.032 | 0.246 ± 0.067 | * (A:D) | | | | | | | | |
| | VAR | 0.027 ± 0.010 | 0.072 ± 0.014 | 0.149 ± 0.027 | 0.109 ± 0.018 | *$ (A:D) | | | | | | | | |
| 13-HDoHE | Control | 0.419 ± 0.107 | 0.244 ± 0.034 | 0.64 ± 0.059 | 0.475 ± 0.035 | | 0.1001 (T+1) | 0.0013 | 0.0694 | <0.0001 | 0.3838 | 0.1825 | 0.1705 | 0.3193 |
| | MAFP | 0.215 ± 0.041 | 0.214 ± 0.016 | 0.382 ± 0.038 | 0.351 ± 0.110 | * | | | | | | | | |
| | VAR | 0.039 ± 0.011 | 0.067 ± 0.016 | 0.193 ± 0.038 | 0.072 ± 0.058 | *$ | | | | | | | | |
| 14-HDoHE | Control | 0.319 ± 0.037 | 0.273 ± 0.035 | 0.665 ± 0.143 d | 0.493 ± 0.095 d | | 0.0230 (T+1) | 0.0018 | 0.3368 | <0.0001 | 0.4453 | 0.0400 | 0.0060 | 0.6783 |
| | MAFP | 0.241 ± 0.041 | 0.184 ± 0.027 | 0.467 ± 0.071 *d | 0.282 ± 0.046 * | | | | | | | | | |
| | VAR | 0.045 ± 0.013 *$ | 0.112 ± 0.032 * | 0.124 ± 0.054 *$ | 0.178 ± 0.022 * | | | | | | | | | |
| 16-HDoHE | Control | 0.375 ± 0.063 | 0.288 ± 0.025 | 0.569 ± 0.135 | 0.473 ± 0.093 | | 0.0015 (T+1) | 0.0327 | 0.4004 | <0.0001 | 0.9222 | 0.4208 | 0.0220 | 0.9000 |
| | MAFP | 0.304 ± 0.052 | 0.187 ± 0.020 * | 0.420 ± 0.036 | 0.271 ± 0.033 * | (A:D) | | | | | | | | |
| | VAR | 0.047 ± 0.011 *$ | 0.102 ± 0.024 * | 0.089 ± 0.083 *$ | 0.180 ± 0.077 * | (A:D) | | | | | | | | |
| 17-HDoHE | Control | 0.909 ± 0.131 | 0.688 ± 0.084 | 1.24 ± 0.213 | 1.08 ± 0.297 | | 0.1717 (T+2) | 0.0435 | 0.6232 | <0.0001 | 0.3918 | 0.4827 | 0.0279 | 0.8024 |
| | MAFP | 0.641 ± 0.087 * | 0.403 ± 0.046* | 0.755 ± 0.131* | 0.606 ± 0.132* | (A:D) | | | | | | | | |
| | VAR | 0.093 ± 0.029 *$ | 0.199 ± 0.052* | 0.051 ± 0.123*$ | 0.390 ± 0.136* | (A:D) | | | | | | | | |

**Supplemental Table 5-3 (cont)**. Effects of sex and dietary ALA and DHA on $PLA_2$ activity towards non-esterified oxylipins in rat hearts (n=6; mean ± SE).

| | Group | ALA F | ALA M | DHA F | DHA M | Post hoc | SW | Diet | Sex | Treatment (Treat) | Diet* Sex | Diet* Treat | Sex* Treat | Diet*Sex *Treat |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 16,17-EpDoPE | Control | 4.44 ± 0.430 | 3.56 ± 0.264 | 6.7 ± 0.568 | 6.03 ± 0.628 | D | 0.3803 | 0.0014 | 0.2727 | <0.0001 | 0.9151 | 0.0201 | 0.5701 | 0.5512 |
| | MAFP | 2.75 ± 0.356 | 2.15 ± 0.381 | 3.49 ± 0.949 | 3.49 ± 0.545 | * (A:S) | | | | | | | | |
| | VAR | 0.444 ± 0.088 | 0.469 ± 0.120 | 1.69 ± 0.707 | 1.16 ± 0.221 | *$ (A:S) | | | | | | | | |
| 16,17-DiHDoPE | Control | 0.0047 ± 0.0021 | 0.0024 ± 0.0015 | 0.0134 ± 0.0007 d | 0.0063 ± 0.0020 s | | 0.4302 | <0.0001 | 0.0001 | <0.0001 | 0.0069 | 0.0194 | 0.0380 | 0.4842 |
| | MAFP | 0.0023 ± 0.0014 | ND | 0.0112 ± 0.0011 d | 0.0016 ± 0.0016 *s | | | | | | | | | |
| | VAR | ND * | ND | 0.0034 ± 0.0015 *$ | ND * | | | | | | | | | |
| 19,20-EpDoPE | Control | 0.388 ± 0.056 | 0.290 ± 0.021 | 0.537 ± 0.055 | 0.542 ± 0.067 | | 0.6821 | 0.0006 | 0.6697 | <0.0001 | 0.4465 | 0.1403 | 0.0852 | 0.7212 |
| | MAFP | 0.237 ± 0.038 | 0.163 ± 0.026 | 0.345 ± 0.053 | 0.308 ± 0.079 | * | | | | | | | | |
| | VAR | -0.004 ± 0.017 | 0.044 ± 0.024 | 0.0775 ± 0.029 | 0.144 ± 0.044 | *$ | | | | | | | | |
| 20-HDoHE | Control | 1.13 ± 0.191 | 0.758 ± 0.089 | 1.47 ± 0.444 | 1.45 ± 0.175 | | 0.1492 (T+1) | 0.1272 | 0.6773 | <0.0001 | 0.5023 | 0.7115 | 0.3953 | 0.4469 |
| | MAFP | 0.759 ± 0.116 | 0.523 ± 0.120 | 0.954 ± 0.249 | 0.794 ± 0.191 | * | | | | | | | | |
| | VAR | 0.176 ± 0.051 | 0.201 ± 0.080 | 0.409 ± 0.174 | 0.418 ± 0.089 | *$ | | | | | | | | |
| Total DHA Oxylipins | Control | 10.9 ± 1.3 | 8.41 ± 0.542 | 17.0 ± 1.79 | 14.7 ± 1.54 | D | 0.3876 | 0.0001 | 0.1772 | <0.0001 | 0.9723 | 0.0073 | 0.1269 | 0.8433 |
| | MAFP | 7.03 ± 0.809 | 5.39 ± 0.770 | 9.46 ± 1.22 | 8.56 ± 1.43 | *D (A:S) | | | | | | | | |
| | VAR | 1.30 ± 0.13 | 1.94 ± 0.386 | 4.00 ± 1.21 | 3.89 ± 0.68 | *$D (A:S) | | | | | | | | |
| EPA Oxylipins | | | | | | | | | | | | | | |
| 5-HEPE | Control | 0.0163 ± 0.0097 | 0.0312 ± 0.0097 | 0.0128 ± 0.00998 | 0.0226 ± 0.0195 | | 0.0007 (T+1) | 0.4256 | 0.1079 | 0.0001 | 0.4476 | 0.1903 | 0.2893 | 0.7402 |
| | MAFP | -0.0152 ± 0.0079 | 0.0045 ± 0.0088 | -0.0025 ± 0.0098 | -0.0043 ± 0.012 | * | | | | | | | | |
| | VAR | 0.0078 ± 0.0055 | 0.0405 ± 0.0071 | -0.0024 ± 0.0105 | 0.016 ± 0.015 | $ | | | | | | | | |
| 8-HEPE | Control | 0.0191 ± 0.0104 | 0.0438 ± 0.0455 | 0.0326 ± 0.0284 | 0.0392 ± 0.026 | | 0.4963 | 0.7283 | 0.3034 | 0.1127 | 0.4092 | 0.8248 | 0.7520 | 0.7596 |
| | MAFP | -0.0208 ± 0.0103 | 0.0341 ± 0.0188 | 0.018 ± 0.0222 | 0.0247 ± 0.0159 | | | | | | | | | |
| | VAR | 0.012 ± 0.0203 | 0.0509 ± 0.0247 | 0.0342 ± 0.0211 | 0.0343 ± 0.0238 | | | | | | | | | |
| 11-HEPE | Control | 0.242 ± 0.071 | 0.226 ± 0.0356 | 0.120 ± 0.024 | 0.212 ± 0.065 | | 0.8305 | 0.7803 | 0.1393 | <0.0001 | 0.9716 | 0.1465 | 0.3772 | 0.2162 |
| | MAFP | -0.014 ± 0.038 | 0.098 ± 0.040 | 0.036 ± 0.030 | 0.109 ± 0.060 | * | | | | | | | | |
| | VAR | 0.127 ± 0.043 | 0.181 ± 0.034 | 0.168 ± 0.035 | 0.160 ± 0.034 | $ | | | | | | | | |
| 12-HEPE | Control | 0.0384 ± 0.0059 | 0.0656 ± 0.0187 | 0.0737 ± 0.0158 | 0.039 ± 0.0065 | | 0.4307 | 0.7420 | 0.9013 | 0.0256 | 0.0270 | 0.7636 | 0.4348 | 0.7123 |
| | MAFP | 0.0146 ± 0.0267 | 0.0602 ± 0.0167 | 0.0604 ± 0.0205 | 0.0347 ± 0.0078 | | | | | | | | | |
| | VAR | 0.0459 ± 0.020 | 0.0777 ± 0.0152 | 0.0883 ± 0.0125 | 0.0456 ± 0.0101 | $ | | | | | | | | |

**Supplemental Table 5-3 (cont)**. Effects of sex and dietary ALA and DHA on $PLA_2$ activity towards non-esterified oxylipins in rat hearts (n=6; mean ± SE).

| | Group | ALA F | ALA M | DHA F | DHA M | Post hoc | SW | Diet | Sex | Treatment (Treat) | Diet* Sex | Diet* Treat | Sex* Treat | Diet*Sex *Treat |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 14,15-EpETE | Control | 0.0057 ± 0.0025 | 0.0057 ± 0.0022 | 0.0019 ± 0.0025 | 0.0020 ± 0.0019 | | 0.6987 | 0.3497 | 0.7035 | <0.0001 | 0.6631 | 0.2294 | 0.8819 | 0.5860 |
| | MAFP | -0.0018 ± 0.0020 | -0.0029 ± 0.0018 | -0.0039 ± 0.0017 | -0.0010 ± 0.0019 | * | | | | | | | | |
| | VAR | 0.0005 ± 0.0022 | 0.0014 ± 0.0013 | -0.0006 ± 0.0025 | 0.0006 ± 0.0023 | *$ | | | | | | | | |
| 17,18-EpETE | Control | 0.0057 ± 0.0033 | 0.0033 ± 0.0022 | -0.0014 ± 0.0025 | -0.0020 ± 0.0026 | | 0.3201 | 0.3212 | 0.9484 | 0.0070 | 0.3837 | 0.0006 | 0.3644 | 0.5214 |
| | MAFP | -0.0012 ± 0.0029 * | -0.0033 ± 0.0016 * | -0.0021 ± 0.0035 | 0.0016 ± 0.0023 | (A:S) | | | | | | | | |
| | VAR | 0.0009 ± 0.0035 * | -0.0008 ± 0.0010 * | -0.0053 ± 0.0029 | -0.0013 ± 0.0034 | (A:S) | | | | | | | | |
| 18-HEPE | Control | 0.116 ± 0.022 | 0.077 ± 0.006 | 0.079 ± 0.032 | 0.042 ± 0.011 | | 0.0818 | 0.1072 | 0.6570 | <0.0001 | 0.7063 | 0.1143 | 0.0939 | 0.8769 |
| | MAFP | 0.032 ± 0.028 | 0.002 ± 0.016 | 0.020 ± 0.021 | 0.021 ± 0.021 | * | | | | | | | | |
| | VAR | 0.114 ± 0.021 | 0.137 ± 0.044 | 0.051 ± 0.018 | 0.082 ± 0.035 | $ | | | | | | | | |
| Total EPA Oxylipins | Control | 0.484 ± 0.121 | 0.453 ± 0.094 | 0.318 ± 0.049 | 0.354 ± 0.082 | | 0.1600 | 0.4075 | 0.1685 | <0.0001 | 0.4389 | 0.0890 | 0.3309 | 0.3275 |
| | MAFP | -0.006 ± 0.062 | 0.193 ± 0.053 | 0.126 ± 0.066 | 0.186 ± 0.068 | * | | | | | | | | |
| | VAR | 0.308 ± 0.041 | 0.488 ± 0.0673 | 0.333 ± 0.042 | 0.337 ± 0.084 | $ | | | | | | | | |
| ALA Oxylipins | | | | | | | | | | | | | | |
| 9-HOTrE | Control | 0.779 ± 0.162 | 0.54 ± 0.060 | 0.019 ± 0.004 | 0.021 ± 0.002 | D (A:S) | <0.0001 (T+1) | 0.0000 | 0.3252 | <0.0001 | 0.3444 | <0.0001 | 0.6910 | 0.5906 |
| | MAFP | 0.111 ± 0.079 * | 0.022 ± 0.036 * | 0.004 ± 0.003 | 0.005 ± 0.002 | (A:S) | | | | | | | | |
| | VAR | 0.871 ± 0.108 *$ | 0.826 ± 0.196 *$ | 0.030 ± 0.007 | 0.022 ± 0.004 | D (A:S) | | | | | | | | |
| 13-HOTrE | Control | 0.560 ± 0.168 | 0.341 ± 0.061 | 0.016 ± 0.004 | 0.017 ± 0.007 | D (A:S) | <0.0001 (T+10) | 0.0000 | 0.6598 | <0.0001 | 0.6150 | <0.0001 | 0.2611 | 0.2879 |
| | MAFP | -0.043 ± 0.057 * | 0.061 ± 0.034 * | 0.002 ± 0.005 | 0.010 ± 0.005 | (A:S) | | | | | | | | |
| | VAR | 0.559 ± 0.132 $ | 0.544 ± 0.082 $ | 0.020 ± 0.003 | 0.018 ± 0.003 | D (A:S) | | | | | | | | |
| TALA | Control | 1.34 ± 0.302 | 0.881 ± 0.104 | 0.036 ± 0.002 d | 0.038 ± 0.009 d | | 0.9965 (T) | 0.0000 | 0.2504 | <0.0001 | 0.0692 | 0.0002 | 0.8548 | 0.0164 |
| | MAFP | 0.068 ± 0.104 * | 0.083 ± 0.021 *s | 0.005 ± 0.006 *d | 0.015 ± 0.005*d | | | | | | | | | |
| | VAR | 1.43 ± 0.124 $ | 1.37 ± 0.249 $ | 0.050 ± 0.008 $d | 0.040 ± 0.005 $d | | | | | | | | | |
| ARA COX Oxylipins | | | | | | | | | | | | | | |
| 6-keto-$PGF_{1\alpha}$ | Control | 0.599 ± 0.077 | 2.11 ± 0.436 | 0.66 ± 0.162 | 1.23 ± 0.231 | | 0.5788 | 0.0923 | 0.0010 | 0.8148 | 0.0816 | <0.0001 | 0.5089 | 0.2642 |
| | MAFP | 0.707 ± 0.095 | 2.22 ± 0.411 | 0.593 ± 0.162 * | 1.02 ± 0.260 * | (A:S) | | | | | | | | |
| | VAR | 0.586 ± 0.077 $ | 2.06 ± 0.406 $ | 0.692 ± 0.195 $ | 1.29 ± 0.307 $ | (A:S) | | | | | | | | |
| $PGD_2$ | Control | 0.046 ± 0.006 | 0.070 ± 0.010 s | 0.051 ± 0.005 | 0.046 ± 0.005 d | (A:T) | 0.7178 | 0.1724 | 0.0239 | 0.0463 | 0.0039 | 0.2931 | 0.1584 | 0.2126 |
| | MAFP | 0.034 ± 0.007 | 0.073 ± 0.009 s | 0.046 ± 0.005 | 0.037 ± 0.008 d | (A:T) | | | | | | | | |
| | VAR | 0.036 ± 0.004 | 0.075 ± 0.008 s | 0.052 ± 0.006 | 0.052 ± 0.005 d | (A:T) | | | | | | | | |

**Supplemental Table 5-3 (cont)**. Effects of sex and dietary ALA and DHA on $PLA_2$ activity towards non-esterified oxylipins in rat hearts (n=6; mean ± SE).

| | Group | ALA F | ALA M | DHA F | DHA M | Post hoc | SW | Diet | Sex | Treatment (Treat) | Diet* Sex | Diet* Treat | Sex* Treat | Diet*Sex *Treat |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $PGE_2$ | Control | 0.028 ± 0.003 | 0.062 ± 0.011 | 0.022 ± 0.006 | 0.034 ± 0.005 | | 0.0320 | 0.0211 | 0.0025 | 0.0004 | 0.0844 | 0.3389 | 0.1961 | 0.7705 |
| | MAFP | 0.023 ± 0.005 | 0.057 ± 0.010 | 0.017 ± 0.004 | 0.025 ± 0.006 | * | | | | | | | | |
| | VAR | 0.023 ± 0.002 | 0.062 ± 0.012 | 0.021 ± 0.006 | 0.034 ± 0.006 | $ | | | | | | | | |
| 11β-$PGE_2$ | Control | 0.029 ± 0.006 | 0.059 ± 0.010 | 0.029 ± 0.004 | 0.040 ± 0.006 | S (A:D) | 0.2998 | 0.1155 | 0.0053 | 0.0007 | 0.1042 | 0.4637 | 0.0109 | 0.6317 |
| | MAFP | 0.028 ± 0.005 | 0.053 ± 0.008 * | 0.025 ± 0.003 | 0.030 ± 0.008 * | (A:D) | | | | | | | | |
| | VAR | 0.025 ± 0.003 | 0.062 ± 0.010 $ | 0.029 ± 0.005 | 0.040 ± 0.007 $ | S (A:D) | | | | | | | | |
| 15-keto-$PGE_2$ | Control | 0.0102 ± 0.0025 | 0.0172 ± 0.0038 s | 0.0072 ± 0.0019 | 0.0081 ± 0.0016 d | (A:T) | 0.0003 (T+1) | 0.0068 | 0.0014 | 0.0005 | 0.0062 | 0.5441 | 0.4842 | 0.5607 |
| | MAFP | 0.0002 ± 0.0018 | 0.0119 ± 0.0022 s | 0.0038 ± 0.0015 | 0.0029 ± 0.0018 d | (A:T)* | | | | | | | | |
| | VAR | 0.0068 ± 0.0040 | 0.0191 ± 0.0021 s | 0.0062 ± 0.0014 | 0.0093 ± 0.0020 d | (A:T) $ | | | | | | | | |
| $PGF_{2\alpha}$ | Control | 0.022 ± 0.002 | 0.070 ± 0.014 | 0.022 ± 0.006 | 0.040 ± 0.009 | | 0.0944 T | 0.1293 | 0.0078 | 0.4377 | 0.3660 | 0.1980 | 0.2479 | 0.9935 |
| | MAFP | 0.027 ± 0.004 | 0.073 ± 0.014 | 0.023 ± 0.006 | 0.039 ± 0.010 | | | | | | | | | |
| | VAR | 0.022 ± 0.002 | 0.074 ± 0.018 | 0.024 ± 0.006 | 0.046 ± 0.012 | | | | | | | | | |
| $TXB_2$ | Control | 0.0115 ± 0.0009 | 0.022 ± 0.0043 | 0.0063 ± 0.0016 | 0.0109 ± 0.0010 | D (A:S) | 0.2106 | 0.0034 | 0.0020 | 0.0003 | 0.1720 | 0.0045 | 0.1056 | 0.3553 |
| | MAFP | 0.0129 ± 0.0012 * | 0.0269 ± 0.0044 * | 0.007 ± 0.0019 | 0.0122 ± 0.0024 | D (A:S) | | | | | | | | |
| | VAR | 0.0096 ± 0.0010 $ | 0.0224 ± 0.0042 $ | 0.0063 ± 0.0023 | 0.0125 ± 0.0016 | D (A:S) | | | | | | | | |
| 12-HHTrE | Control | 0.853 ± 0.076 | 1.27 ± 0.128 | 0.475 ± 0.075 | 0.665 ± 0.088 | | 0.0127 | 0.0003 | 0.0017 | 0.0097 | 0.1753 | 0.3406 | 0.2320 | 0.5705 |
| | MAFP | 0.593 ± 0.124 | 1.20 ± 0.179 | 0.343 ± 0.048 | 0.596 ± 0.148 | * | | | | | | | | |
| | VAR | 0.643 ± 0.070 | 1.20 ± 0.137 | 0.425 ± 0.088 | 0.656 ± 0.115 | | | | | | | | | |
| Total ARA COX Oxylipins | Control | 1.54 ± 0.146 | 3.68 ± 0.599 | 1.27 ± 0.252 | 2.07 ± 0.332 | | 0.1809 | 0.0213 | 0.0009 | 0.0613 | 0.0845 | 0.0212 | 0.7045 | 0.5153 |
| | MAFP | 1.42 ± 0.215 | 3.71 ± 0.628 | 1.06 ± 0.226 | 1.77 ± 0.416 | D (A:S) | | | | | | | | |
| | VAR | 1.35 ± 0.137 | 3.57 ± 0.580 | 1.25 ± 0.298 $ | 2.14 ± 0.438 $ | (A:S) | | | | | | | | |
| ARA LOX and CYP Oxylipins | | | | | | | | | | | | | | |
| 5-HETE | Control | 0.785 ± 0.123 | 0.885 ± 0.131 | 0.387 ± 0.074 | 0.429 ± 0.164 | | 0.1138 (T+1) | 0.0001 | 0.3065 | <0.0001 | 0.7207 | 0.5898 | 0.6435 | 0.9108 |
| | MAFP | 0.345 ± 0.075 | 0.388 ± 0.115 | 0.113 ± 0.0475 | 0.155 ± 0.098 | * | | | | | | | | |
| | VAR | 0.459 ± 0.091 | 0.662 ± 0.108 | 0.187 ± 0.036 | 0.278 ± 0.090 | *$ | | | | | | | | |
| 8-HETE | Control | 0.095 ± 0.026 | 0.149 ± 0.019 | 0.043 ± 0.013 | 0.049 ± 0.028 | | 0.0103 (T+1) | <0.0001 | 0.0677 | 0.0001 | 0.1615 | 0.4777 | 0.2023 | 0.3194 |
| | MAFP | 0.055 ± 0.015 | 0.051 ± 0.016 | 0.005 ± 0.008 | 0.007 ± 0.016 | * | | | | | | | | |
| | VAR | 0.056 ± 0.011 | 0.120 ± 0.016 | 0.023 ± 0.011 | 0.031 ± 0.011 | *$ | | | | | | | | |
| 9-HETE | Control | 0.250 ± 0.048 | 0.343 ± 0.068 | 0.094 ± 0.035 | 0.135 ± 0.0541 | | 0.0568 | 0.0001 | 0.0844 | <0.0001 | 0.2952 | 0.4749 | 0.4634 | 0.4137 |
| | MAFP | 0.119 ± 0.047 | 0.152 ± 0.043 | -0.001 ± 0.017 | 0.017 ± 0.030 | * | | | | | | | | |
| | VAR | 0.135 ± 0.043 | 0.297 ± 0.059 | 0.027 ± 0.034 | 0.042 ± 0.028 | *$ | | | | | | | | |

**Supplemental Table 5-3 (cont)**. Effects of sex and dietary ALA and DHA on $PLA_2$ activity towards non-esterified oxylipins in rat hearts (n=6; mean ± SE).

| | Group | ALA F | ALA M | DHA F | DHA M | Post hoc | SW | Diet | Sex | Treatment (Treat) | Diet* Sex | Diet* Treat | Sex* Treat | Diet*Sex *Treat |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 11-HETE | Control | 0.396 ± 0.105 | 0.348 ± 0.043 | 0.146 ± 0.019 d | 0.144 ± 0.011 d | | 0.0175 (T+1) | 0.0002 | 0.0746 | <0.0001 | 0.3066 | 0.0415 | 0.0120 | 0.1367 |
| | MAFP | 0.135 ± 0.059 * | 0.279 ± 0.032 s | 0.058 ± 0.016 | 0.109 ± 0.043 d | | | | | | | | | |
| | VAR | 0.129 ± 0.023 * | 0.301 ± 0.044 s | 0.099 ± 0.0226 | 0.127 ± 0.035 d | | | | | | | | | |
| 12-HETE | Control | 0.412 ± 0.087 | 0.599 ± 0.0796 | 0.243 ± 0.085 | 0.285 ± 0.056 | | 0.4817 | 0.0195 | 0.1735 | 0.1116 | 0.3927 | 0.1767 | 0.4950 | 0.4602 |
| | MAFP | 0.33 ± 0.155 | 0.532 ± 0.0877 | 0.203 ± 0.078 | 0.259 ± 0.064 | | | | | | | | | |
| | VAR | 0.313 ± 0.088 | 0.583 ± 0.098 | 0.278 ± 0.093 | 0.314 ± 0.059 | | | | | | | | | |
| 15-HETE | Control | 0.859 ± 0.189 | 0.654 ± 0.097 | 0.356 ± 0.073 | 0.287 ± 0.081 | (A:D) | 0.0954 | 0.0002 | 0.8861 | 0.0001 | 0.8609 | 0.2981 | 0.0268 | 0.3841 |
| | MAFP | 0.419 ± 0.141 * | 0.266 ± 0.070 * | 0.121 ± 0.043 * | 0.092 ± 0.049 * | (A:D) | | | | | | | | |
| | VAR | 0.401 ± 0.085 * | 0.687 ± 0.154 $ | 0.127 ± 0.066 * | 0.233 ± 0.103 $ | (A:D) | | | | | | | | |
| 6-trans-$LTB_4$ | Control | 0.022 ± 0.003 | 0.021 ± 0.004 | 0.014 ± 0.006 | 0.006 ± 0.004 | D (A:S) | 0.0146 | 0.1218 | 0.6466 | 0.0260 | 0.4111 | 0.0126 | 0.1222 | 0.2516 |
| | MAFP | 0.014 ± 0.002 * | 0.011 ± 0.002 * | 0.011 ± 0.005 | 0.008 ± 0.004 | (A:S) | | | | | | | | |
| | VAR | 0.009 ± 0.004 * | 0.017 ± 0.003 * | 0.013 ± 0.005 | 0.010 ± 0.004 | (A:S) | | | | | | | | |
| 6-trans-12epi-$LTB_4$ | Control | 0.038 ± 0.008 | 0.050 ± 0.008 | 0.030 ± 0.012 | 0.027 ± 0.007 | | 0.1398 | 0.1959 | 0.3776 | <0.0001 | 0.4630 | 0.1904 | 0.3439 | 0.7465 |
| | MAFP | 0.018 ± 0.012 | 0.025 ± 0.005 | 0.008 ± 0.012 | 0.009 ± 0.009 | * | | | | | | | | |
| | VAR | 0.015 ± 0.005 | 0.036 ± 0.008 | 0.019 ± 0.011 | 0.025 ± 0.005 | *$ | | | | | | | | |
| $LTB_4$ | Control | 0.0092 ± 0.0027 | 0.012 ± 0.0038 | 0.0036 ± 0.0007 | 0.0065 ± 0.0027 | | 0.5420 | 0.1678 | 0.0541 | 0.0094 | 0.6650 | 0.1614 | 0.1686 | 0.9005 |
| | MAFP | 0.0015 ± 0.0022 | 0.0036 ± 0.0016 | 0.0032 ± 0.0023 | 0.0033 ± 0.0031 | * | | | | | | | | |
| | VAR | 0.0041 ± 0.0031 | 0.0126 ± 0.0025 | 0.0025 ± 0.0014 | 0.0082 ± 0.0034 | $ | | | | | | | | |
| 6S-$LXA_4$ | Control | 0.075 ± 0.019 | 0.064 ± 0.026 | 0.034 ± 0.012 | 0.017 ± 0.018 | | 0.0136 (T+1) | 0.0181 | 0.7763 | 0.0046 | 0.3047 | 0.1605 | 0.4022 | 0.6809 |
| | MAFP | -0.006 ± 0.012 | 0.021 ± 0.009 | 0.020 ± 0.020 | -0.002 ± 0.016 | * | | | | | | | | |
| | VAR | 0.057 ± 0.020 | 0.088 ± 0.030 | 0.019 ± 0.016 | 0.033 ± 0.023 | $ | | | | | | | | |
| 5,6-EpETrE | Control | 0.322 ± 0.036 | 0.283 ± 0.041 | 0.092 ± 0.020 | 0.180 ± 0.068 | D (A:S) | 0.0008 (T+1) | 0.0003 | 0.2146 | <0.0001 | 0.1170 | 0.0025 | 0.6352 | 0.4765 |
| | MAFP | 0.083 ± 0.035 * | 0.070 ± 0.011 * | 0.025 ± 0.015 * | 0.059 ± 0.016 * | (A:S) | | | | | | | | |
| | VAR | 0.136 ± 0.019 *$ | 0.159 ± 0.023 *$ | 0.039 ± 0.007 * | 0.100 ± 0.013 * | D (A:S) | | | | | | | | |
| 8,9-EpETrE | Control | 0.105 ± 0.010 | 0.084 ± 0.017 | 0.032 ± 0.006 | 0.054 ± 0.021 | D (A:S) | 0.0052 (T+1) | 0.0006 | 0.5200 | <0.0001 | 0.0760 | 0.0162 | 0.4112 | 0.7217 |
| | MAFP | 0.031 ± 0.016 * | 0.018 ± 0.006 * | 0.006 ± 0.002 * | 0.019 ± 0.007 * | (A:S) | | | | | | | | |
| | VAR | 0.056 ± 0.012 *$ | 0.060 ± 0.011 *$ | 0.010 ± 0.002 | 0.036 ± 0.009 | D (A:S) | | | | | | | | |
| 11,12-EpETrE | Control | 0.031 ± 0.006 | 0.027 ± 0.005 | 0.010 ± 0.002 | 0.011 ± 0.001 | D (A:S) | 0.0244 | 0.0002 | 0.7502 | <0.0001 | 0.2516 | 0.0002 | 0.2691 | 0.8589 |
| | MAFP | 0.015 ± 0.004 * | 0.008 ± 0.002 * | 0.004 ± 0.001 * | 0.004 ± 0.001 * | D(A:S) | | | | | | | | |
| | VAR | 0.011 ± 0.002 * | 0.011 ± 0.002 * | 0.003 ± 0.001 * | 0.007 ± 0.003 * | (A:S) | | | | | | | | |

**Supplemental Table 5-3 (cont)**. Effects of sex and dietary ALA and DHA on $PLA_2$ activity towards non-esterified oxylipins in rat hearts (n=6; mean ± SE).

| | Group | ALA F | ALA M | DHA F | DHA M | Post hoc | SW | Diet | Sex | Treatment (Treat) | Diet* Sex | Diet* Treat | Sex* Treat | Diet*Sex *Treat |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 14,15-EpETrE | Control | 0.213 ± 0.029 | 0.187 ± 0.012 | 0.061 ± 0.006 d | 0.088 ± 0.015 d | | 0.0514 | <0.0001 | 0.6346 | <0.0001 | 0.0293 | <0.0001 | 0.0559 | 0.7065 |
| | MAFP | 0.102 ± 0.017 * | 0.060 ± 0.012 * | 0.018 ± 0.009 *d | 0.038 ± 0.007 * | | | | | | | | | |
| | VAR | 0.070 ± 0.013 * | 0.079 ± 0.007 * | 0.020 ± 0.006 *d | 0.065 ± 0.018 | | | | | | | | | |
| 14,15-DiHETrE | Control | 0.0040 ± 0.0005 | 0.0032 ± 0.0002 | 0.0018 ± 0.0004 | 0.0020 ± 0.0002 | D (A:S) | 0.8664 | 0.0022 | 0.4839 | <0.0001 | 0.3798 | 0.0359 | 0.6709 | 0.4355 |
| | MAFP | 0.0020 ± 0.0007 * | 0.0015 ± 0.0003 * | 0.0007 ± 0.0005 * | 0.0005 ± 0.0003 * | D(A:S) | | | | | | | | |
| | VAR | 0.0025 ± 0.0003 *$ | 0.0021 ± 0.0003 *$ | 0.0013 ± 0.0005 $ | 0.0016 ± 0.0003 $ | (A:S) | | | | | | | | |
| 16-HETE | Control | 0.080 ± 0.013 | 0.066 ± 0.012 | 0.033 ± 0.017 | 0.035 ± 0.012 | (A:D) | 0.1616 | 0.0165 | 0.5055 | 0.0001 | 0.8497 | 0.0978 | 0.0221 | 0.4153 |
| | MAFP | 0.026 ± 0.012 * | 0.028 ± 0.0051 * | 0.021 ± 0.007 * | 0.013 ± 0.010 * | (A:D) | | | | | | | | |
| | VAR | 0.038 ± 0.014 * | 0.076 ± 0.020 $ | 0.018 ± 0.012 * | 0.038 ± 0.011 $ | (A:D) | | | | | | | | |
| 18-HETE | Control | 0.0061 ± 0.0031 | 0.0007 ± 0.0011 | 0.0015 ± 0.0016 | 0.0011 ± 0.0008 | | 0.0289 | 0.5591 | 0.0917 | 0.0008 | 0.7513 | 0.0132 | 0.3356 | 0.1648 |
| | MAFP | -0.0018 ± 0.0021 * | -0.0014 ± 0.0006 * | 0.0019 ± 0.0023 | 0.0002 ± 0.0010 | | | | | | | | | |
| | VAR | 0.0065 ± 0.0016 $ | 0.0035 ± 0.0020 $ | 0.0039 ± 0.0014 | 0.0003 ± 0.0011 | | | | | | | | | |
| Total ARA LOX + CYP Oxylipins | Control | 3.63 ± 0.516 | 3.78 ± 0.361 | 1.58 ± 0.277 | 1.76 ± 0.303 | | 0.4978 | <0.0001 | 0.1413 | <0.0001 | 0.6041 | 0.0423 | 0.0981 | 0.4253 |
| | MAFP | 1.69 ± 0.431 | 1.91 ± 0.270 | 0.617 ± 0.149 | 0.792 ± 0.201 | | | | | | | | | |
| | VAR | 1.90 ± 0.245 | 3.19 ± 0.425 | 0.889 ± 0.238 | 1.35 ± 0.265 | | | | | | | | | |
| DGLA Oxylipins | | | | | | | | | | | | | | |
| 8-HETrE | Control | 0.041 ± 0.006 | 0.052 ± 0.018 | 0.021 ± 0.005 | 0.042 ± 0.018 | | 0.0033 (T+10) | 0.2079 | 0.0482 | <0.0001 | 0.6087 | 0.8440 | 0.5658 | 0.9361 |
| | MAFP | 0.007 ± 0.003 | 0.029 ± 0.013 | -0.007 ± 0.004 | 0.021 ± 0.010 | * | | | | | | | | |
| | VAR | 0.031 ± 0.007 | 0.040 ± 0.012 | 0.015 ± 0.005 | 0.038 ± 0.011 | $ | | | | | | | | |
| 15-HETrE | Control | 0.037 ± 0.005 | 0.037 ± 0.004 | 0.034 ± 0.012 | 0.041 ± 0.006 | | 0.5828 | 0.8649 | 0.2174 | <0.0001 | 0.9748 | 0.8385 | 0.0985 | 0.4837 |
| | MAFP | 0.017 ± 0.006 | 0.011 ± 0.006 | 0.014 ± 0.007 | 0.016 ± 0.005 | * | | | | | | | | |
| | VAR | 0.029 ± 0.006 | 0.055 ± 0.013 | 0.031 ± 0.012 | 0.044 ± 0.008 | $ | | | | | | | | |
| Total DGLA Oxylipins | Control | 0.078 ± 0.010 | 0.089 ± 0.018 | 0.055 ± 0.016 | 0.083 ± 0.018 | | 0.4776 | 0.2546 | 0.0260 | <0.0001 | 0.6817 | 0.9621 | 0.5963 | 0.8367 |
| | MAFP | 0.023 ± 0.008 | 0.041 ± 0.011 | 0.007 ± 0.009 | 0.037 ± 0.013 | * | | | | | | | | |
| | VAR | 0.059 ± 0.008 | 0.095 ± 0.022 | 0.047 ± 0.014 | 0.081 ± 0.016 | $ | | | | | | | | |
| LA Oxylipins | | | | | | | | | | | | | | |
| 9-HODE | Control | 1.62 ± 0.539 | 1.43 ± 0.200 | 0.923 ± 0.348 | 1.53 ± 0.187 | | 0.5373 (T+1) | 0.9009 | 0.4299 | <0.0001 | 0.8012 | 0.1283 | 0.5480 | 0.1779 |
| | MAFP | 0.239 ± 0.214 | 0.285 ± 0.075 | 0.457 ± 0.212 | 0.465 ± 0.154 | * | | | | | | | | |
| | VAR | 1.47 ± 0.214 | 1.78 ± 0.398 | 1.56 ± 0.331 | 1.42 ± 0.230 | $ | | | | | | | | |

**Supplemental Table 5-3 (cont)**. Effects of sex and dietary ALA and DHA on $PLA_2$ activity towards non-esterified oxylipins in rat hearts (n=6; mean ± SE).

| | Group | ALA F | ALA M | DHA F | DHA M | Post hoc | SW | Diet | Sex | Treatment (Treat) | Diet* Sex | Diet* Treat | Sex* Treat | Diet*Sex *Treat |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 13-HODE | Control | 3.62 ± 0.555 | 2.18 ± 0.449 | 2.03 ± 0.343 | 2.68 ± 0.532 | | 0.3035 | 0.1632 | 0.8272 | <0.0001 | 0.0280 | 0.9638 | 0.1883 | 0.7836 |
| | MAFP | 0.412 ± 0.607 | 0.423 ± 0.416 | -0.675 ± 0.55 | 0.721 ± 0.458 | * | | | | | | | | |
| | VAR | 3.89 ± 0.799 | 3.14 ± 0.259 | 2.69 ± 0.27 | 3.27 ± 0.329 | *$ | | | | | | | | |
| 9,10,13-TriHOME | Control | 0.553 ± 0.166 | 0.706 ± 0.142 | 0.241 ± 0.1 | 0.628 ± 0.161 | (A:D) | 0.1325 (T+1) | 0.0900 | 0.1459 | <0.0001 | 0.5322 | 0.3551 | 0.0428 | 0.9116 |
| | MAFP | -0.121 ± 0.097 * | 0.035 ± 0.020 * | -0.234 ± 0.102 * | 0.0092 ± 0.087 * | (A:D) | | | | | | | | |
| | VAR | 1.34 ± 0.636 *$ | 0.986 ± 0.186 $ | 0.572 ± 0.139 *$ | 0.598 ± 0.139 $ | (A:D) | | | | | | | | |
| 9,12,13-TriHOME | Control | 0.641 ± 0.206 | 0.848 ± 0.117 | 0.278 ± 0.171 | 0.706 ± 0.126 | | 0.4809 | 0.1072 | 0.0571 | <0.0001 | 0.6836 | 0.5794 | 0.5809 | 0.2789 |
| | MAFP | -0.077 ± 0.134 | 0.073 ± 0.045 | -0.344 ± 0.151 | 0.109 ± 0.081 | * | | | | | | | | |
| | VAR | 0.869 ± 0.179 | 1.13 ± 0.171 | 0.691 ± 0.281 | 0.746 ± 0.141 | *$ | | | | | | | | |
| 9,10-EpOME | Control | 0.487 ± 0.065 | 0.425 ± 0.050 | 0.291 ± 0.046 | 0.419 ± 0.086 | | <0.0001 (T+1) | 0.2823 | 0.5844 | <0.0001 | 0.3061 | 0.5959 | 0.2424 | 0.8653 |
| | MAFP | -0.062 ± 0.047 | -0.002 ± 0.027 | -0.093 ± 0.043 | 0.020 ± 0.047 | * | | | | | | | | |
| | VAR | 0.772 ± 0.352 | 0.504 ± 0.082 | 0.445 ± 0.043 | 0.482 ± 0.086 | *$ | | | | | | | | |
| 9,10-DiHOME | Control | 0.037 ± 0.006 | 0.025 ± 0.003 | 0.029 ± 0.006 | 0.031 ± 0.003 | | <0.0001 (T+1) | 0.3559 | 0.1531 | <0.0001 | 0.0792 | 0.1600 | 0.0568 | 0.0454 |
| | MAFP | 0.009 ± 0.003 * | 0.008 ± 0.003 * | 0.007 ± 0.002 * | 0.009 ± 0.001 * | | | | | | | | | |
| | VAR | 0.060 ± 0.018 *$ | 0.025 ± 0.002 $s | 0.029 ± 0.004 $d | 0.031 ± 0.005 $ | | | | | | | | | |
| 12,13-EpOME | Control | 0.251 ± 0.048 | 0.294 ± 0.023 | 0.176 ± 0.064 | 0.302 ± 0.047 | | 0.3533 | 0.6647 | 0.0498 | <0.0001 | 0.1341 | 0.7422 | 0.9041 | 0.8915 |
| | MAFP | 0.015 ± 0.011 | 0.0108 ± 0.031 | -0.071 ± 0.040 | 0.059 ± 0.034 | * | | | | | | | | |
| | VAR | 0.242 ± 0.070 | 0.257 ± 0.023 | 0.201 ± 0.061 | 0.314 ± 0.058 | $ | | | | | | | | |
| 12,13-DiHOME | Control | 0.052 ± 0.016 | 0.029 ± 0.003 | 0.032 ± 0.007 | 0.035 ± 0.004 | | 0.0004 (T+1) | 0.7669 | 0.3200 | <0.0001 | 0.2230 | 0.3733 | 0.0646 | 0.1403 |
| | MAFP | 0.009 ± 0.004 | 0.009 ± 0.004 | 0.008 ± 0.004 | 0.011 ± 0.002 | * | | | | | | | | |
| | VAR | 0.046 ± 0.013 | 0.027 ± 0.004 | 0.038 ± 0.004 | 0.036 ± 0.005 | $ | | | | | | | | |
| Total LA Oxylipins | Control | 7.26 ± 1.21 | 5.93 ± 0.799 | 4.00 ± 0.836 | 6.34 ± 1.01 | | 0.5204 | 0.1037 | 0.3939 | <0.0001 | 0.1028 | 0.4444 | 0.3954 | 0.5710 |
| | MAFP | 0.425 ± 0.510 | 0.842 ± 0.346 | -0.945 ± 0.849 | 1.40 ± 0.643 | * | | | | | | | | |
| | VAR | 8.69 ± 1.54 | 7.85 ± 1.00 | 6.22 ± 0.855 | 6.90 ± 0.924 | *$ | | | | | | | | |

**Supplemental Table 5-3 (cont)**. Effects of sex and dietary ALA and DHA on $PLA_2$ activity towards non-esterified oxylipins in rat hearts (n=6; mean ± SE).

Treatments: Control – incubation without inhibitor, VAR – 0.1 μM of VAR ($sPLA_2$ inhibitor – remaining activity is primarily $iPLA_2$ derived), MAFP – 10 μM of MAFP ($iPLA_2$ inhibitor – remaining activity is primarily $sPLA_2$ derived). Complete results of 3-way repeated measures ANOVA are shown. For interaction effects, relevant differences as determined by BH post hoc tests were denoted with a * to indicate differences from Control, a $ to indicate differences between inhibitors, a D to indicate a diet effect, and an S to indicate a sex effect. T indicates a treatment effect. Effects applicable to the treatment group are shown on the post hoc column, with (A:D) indicating results were averaged over diet, (A:S) averaged over sex, or (A:T) averaged over treatment. When a 3-way interaction analysis was conducted, results are shown in superscript, beside mean ± SE with the difference being from the same sex or diet group within that treatment. SW column shows the Shapiro-Wilk results and whether log transformation was applied (T), or log + 1 (T+1). Oxylipin totals are included for complete display of statistical analysis results. Significant ANOVA p values to be interpreted are highlighted in yellow. Abbreviations: ARA, arachidonic acid; ALA, α-linolenic acid; COX, cyclooxygenase; CYP, cytochrome P450; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DiHDoPE, dihydroxy-docosapentaenoic acid; DiHDoHE, dihydroxy-docosahexaenoic acid; DiHETE, dihydroxy-eicosatetraenoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; DiHODE, dihydroxy-octadecadienoic acid; diHOME, dihydroxy-octadecenoic acid; EPA, eicosapentaenoic acid; EpDoPE, epoxy-docosapentaenoic acid; EpETrE, epoxy-eicosatrienoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; F, females; 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; HX, hepoxilin; LA, linoleic acid; LOX, lipoxygenase; LT, leukotriene; LX, lipoxin; M, males; MAFP, methyl arachidonyl fluorophosphonate; PG, prostaglandin; $PLA_2$, phospholipase $A_2$; triHOME, trihydroxy-octadecenoic acid; TX, thromboxane; VAR, varespladib.