Investigating the metabolic, transcriptomic and DNA methylation signatures of high dietary arachidonic acid in zebrafish

Epigenetic and transcriptional effects in the next generation

Anne-Catrin Adam

Thesis for the Degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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Contents

Scientific environment

This PhD thesis started in August 2013 and was accomplished at the National Institute of Nutrition and Seafood Research (NIFES) in Bergen, Norway. The work was administered through the Department of Biology at the University of Bergen (UiB), Norway.

The work was performed under supervision of Dr. Kai Kristoffer Lie (NIFES) and co-supervision of Dr. Kaja Helvik Skjærven (NIFES) and Professor Rune Waagbø (NIFES, UiB).

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NATIONAL INSTITUTE OF NUTRITION AND SEAFOOD RESEARCH

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Abstract

Background. Nutrition is one of the important environmental determinants of individual health. Alteration in dietary omega-3 and omega-6 polyunsaturated fatty acid (n-3 and n-6 PUFA) profiles is known to affect the health and welfare of farmed fish, but is also a consideration for human health, particularly considering current dietary habits (Western diet). In aquaculture fish feeds, marine ingredients are increasingly substituted with plant-based materials. The resulting lower dietary n-3/n-6 PUFA ratio due to higher plant oil inclusion in the diets, can affect both metabolism and physiology of the fish. Today's global nutrition and lifestyle habits of humans, i.e. increasing amounts of vegetable oils combined with a higher consumption of animal products, have been associated with the increased incidence of chronic diseases such as cardiovascular diseases, cancer, obesity, metabolic syndrome and other chronic inflammatory diseases. Diet can affect individual phenotypes and several animal studies supported the notion that metabolic influences and epigenetic regulation of gene expression during early development can be linked to health outcomes later in life and across generations. This project aimed to investigate the effects of high dietary n-6 PUFAs, particularly arachidonic acid (ARA), on parental metabolic profiles and on both hepatic gene expression and DNA methylation profiles in the first and following generation of zebrafish (*Danio rerio*).

Design. In a transgenerational feeding trial, zebrafish (F_0) were fed a plant-based diet either low (control) or high in ARA (high ARA) from 27 DPF onwards, whereas progeny (F_1) from both groups were fed only the control diet. Body weight was recorded at juvenile stage in F_0 and at adult stage in both F_0 and F_1. The effect on the metabolic fingerprint using metabolomics was analysed in juvenile fish after feeding the experimental diets for 17 days. Diet associated changes in the gene expression and DNA methylation profiles in adult male F_0 and F_1 livers were investigated using RNA-sequencing and reduced representation bisulfite sequencing, respectively.

Results. In the parental (F_0) generation, high dietary ARA-associated metabolic profiles were characterized by increased levels of dicarboxylic acids, pro-inflammatory eicosanoids, oxidized lipids and amino acids, a lower n-3/n-6 PUFA ratio and changed levels of n-3 and n-6 PUFAs, complex lipids and metabolites with known anti-oxidative properties. Differential expressed genes (DEGs) involved in β-oxidation, RXR and PPAR signalling were observed for F_0 livers. A stronger response on hepatic transcriptomic profiles was found in the progeny, where F_1 DEGs were related to methionine cycle, transsulfuration pathway, estrogen signaling, and lipid and retinoid metabolism by PPARα/RXRα playing a central role. Several links were found between parental metabolic and both transcriptomic and DNA methylation patterns of progeny livers. Identified upstream regulators (CNR1, RORA, PPARA, PPARGC1A and ESR2) based on F_1 DEGs were also linked to differential DNA methylation in the livers of the adult progeny. Those regulators function in *de novo* lipogenesis through PPAR downstream signalling, possibly affecting energy metabolism and estrogen signalling. An effect on F_0 body weight, but not length was observed at 44 DPF, but growth at 91 DPF was equal for both feed groups. No differences in mature body weight was observed between dietary groups in both generations.

Conclusions. Results from metabolic profiling revealed a general shift in lipid profiles, signs of lipid peroxidation and an oxidised and pro-inflammatory environment, which led to an anti-inflammatory and anti-oxidative response to high dietary ARA in the fish. High dietary ARA levels did not affect the body weight of zebrafish in this study. Changes in liver DNA methylation and gene expression profiles in adult male progeny were associated with the parental diet, whereas the effect on gene expression was less strong in parents than in progeny. Several links were found between the metabolic profiles in parents and both DNA methylation and gene expression of the progeny, which suggested an impact of parental diet on the progeny perhaps during early embryonic development. Embryonic development can be influenced and adult hepatic gene expression patterns possibly primed through either epigenetic mechanisms or modulation of nutrient composition in the yolk.

List of publications

Paper I

Adam AC, Lie KK, Moren M, Skjaerven KH. *High dietary arachidonic acid levels induce changes in complex lipids and immune-related eicosanoids and increase levels of oxidised metabolites in zebrafish (Danio rerio).* Br J Nutr. 2017 May 09:1-11.

Paper II

Anne-Catrin Adam, Kaja Helvik Skjærven, Paul Whatmore, Mari Moren, Kai Kristoffer Lie. *Parental high dietary arachidonic acid levels modulated the hepatic transcriptome of adult zebrafish (Danio rerio) progeny.* Submitted.

Paper III

Anne-Catrin Adam, Kai Kristoffer Lie, Paul Whatmore, Lars Martin Jakt, Mari Moren, Kaja Helvik Skjærven. *Profiling DNA methylation patterns of zebrafish liver associated with parental high dietary arachidonic acid.* Manuscript.

Abbreviations

ALA	Alpha-linolenic acid (18:3n-3)
ARA	Arachidonic acid (20:4n-6)
CpG	5’-Cystosine-phosphate-guanine-3’ sequence in the DNA
DEG	Differentially expressed gene
DHA	Docosahexaenoic acid (22:6n-3)
DML	Differentially methylated locus
DNA	Deoxyribonucleic acid
DPF	Days post fertilization
EPA	Eicosapentaenoic acid (20:5n-3)
FA	Fatty acid
HETE	Hydroxy-eicosatetraenoic acid
LA	Linoleic acid (18:2n-6)
mRNA	Messenger ribonucleic acid
MspI	A restriction endonuclease
n-3	Omega-3
n-6	Omega-6
PCR	Polymerase chain reaction
PPARα	Peroxisome proliferator-activated receptor alpha
PUFA	Polyunsaturated fatty acid
RNA	Ribonucleic acid
RXRα	Retinoic X receptor alpha
TFA	Total fatty acids

1. Introduction

1.1 Changes in dietary n-3 and n-6 PUFA profiles

Global aquaculture is growing (Figure 2A) along with an increased demand for fish as safe and nutritious food for a rapidly expanding world population (Figure 2B) [FAO 2016]. A key goal of increasing sustainability in fish farming has involved reducing the dependence on marine feed ingredients, which in turn has driven an increasing need for plant-based alternatives in fish feed [Turchini et al. 2009; Olsen 2011]. Plant-based materials cannot fully replace marine feed ingredients due to their limited ability to meet nutritional requirements for most carnivorous farmed fish species [Olsen 2011; Torrecillas et al. 2017]. However, new research has shown that extensive micronutrient supplementation of plant-based feeds can adjust for the lack of marine based feed ingredients for Atlantic salmon (*Salmo salar*) [Hemre et al. 2016]. One of today's challenges in aquaculture is a robust domestication of farmed fish over generations to select for fish than can tolerate high levels of plant-based ingredients [Ulloa et al. 2014]. In addition, the aquaculture industry substitute marine oil with increasing amounts of plant oils in the feed for farmed fish. Because of this, extensive research has focused on the effect of plant oils on farmed fish [Leaver et al. 2008; Olsen 2011; Teoh et al. 2016; Torrecillas et al. 2017]. Compared to marine oils, plant oils are relatively poor sources of omega-3 polyunsaturated fatty acids (n-3 PUFAs) and especially lack long-chain n-3 PUFAs such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). They are rich in n-6 and n-9 PUFAs such as linoleic acid (LA, 18:2n-6) and oleic acid (18:1n-9). Inclusion of both plant proteins and plant oil as partial replacement for fish meals and fish oil has been shown to give adverse effects on growth, and intestinal inflammation in fish [Torstensen et al. 2000; Mundheim et al. 2004; Espe et al. 2006; Torstensen et al. 2008; Uran et al. 2008; Krogdahl et al. 2010]. Broodstock feed ingredients and composition might be a potential for improvement of performance, robustness and welfare of the next generation in farmed fish.

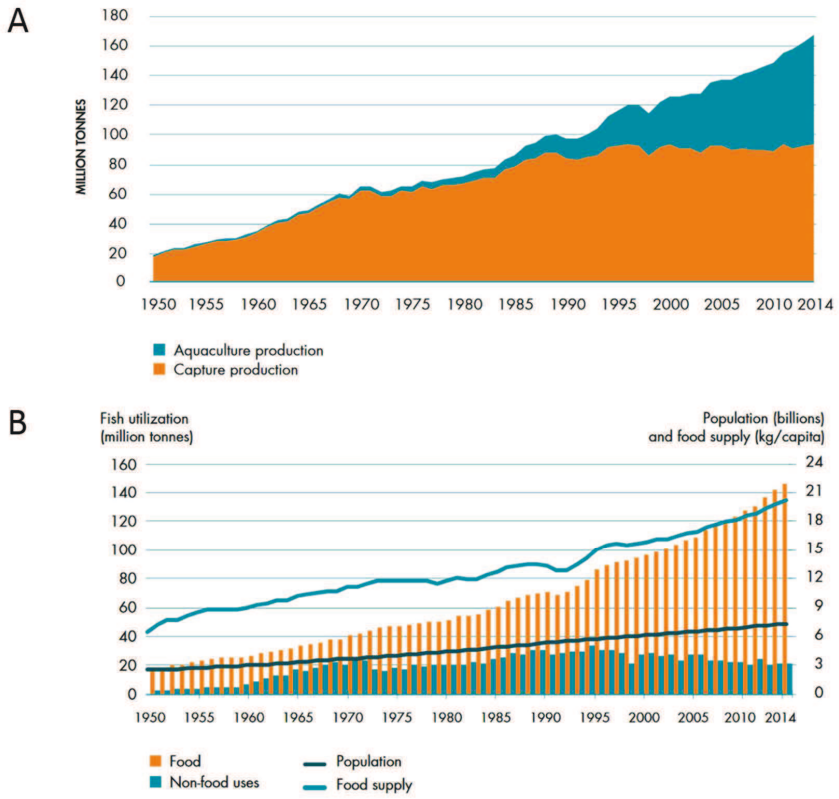

Figure 1 - Development of world capture fisheries and aquaculture production (A) and world fish utilization and supply (B) from 1950 to 2014. © FAO [FAO 2016].

The above mentioned concerns regarding increased dietary n-6 PUFAs in cultured fish is also applicable to current nutritional habits of humans. Generally, we observe a loss of omega-3 PUFA in today's Western diet [Sanders 2000; Cordain et al. 2005; Blasbalg et al. 2011], which is a result of increased amounts of dietary vegetable oils combined with a higher consumption of animal products. Such dietary habits additionally involve an increased intake of n-6 PUFAs such as LA, a precursor of the more biological active ARA. The western diet, is considered to be deficient in n-3 PUFAs, with an average PUFA ratio of about 1:16 compared to an estimated ratio of 1:1 of the hunter-gatherer diet (Figure 2) [Simopoulos 2006]. A decreased dietary n-3/n-6 PUFA ratio is associated with a variety of diseases, of which most are related to inflammation

[Simopoulos 1996; Simopoulos 2006]. Inflammation is often addressed to ARA as precursor of a wide range of biologically important eicosanoids [Patterson et al. 2012]. In addition, high total fat intake increases the risk for health problems according to the 2008 FAO/WHO report [FAO 2008].

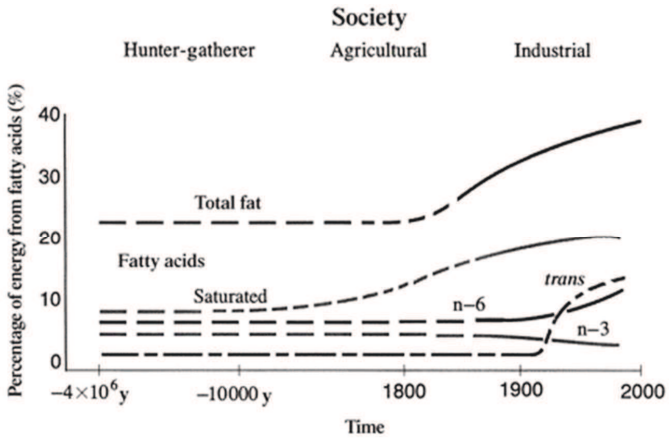

Figure 2 - Hypothetical scheme of the relative percentages of fat and different fatty acid families intake in human nutrition and their putative changes during the preceding 100 years. Permission for reprint from American Society of Nutrition [Simopoulos 1999].

1.2 n-6 PUFAs and ARA in mammals and fish

n-6 PUFAs and their counterparts. All vertebrates, including fish, require dietary intake of n-3 and n-6 PUFAs as they cannot synthesize any PUFA from monounsaturated FAs [Das 2006]. The essentiality of n-3 and n-6 PUFAs primarily involves α-linolenic acid (ALA, 18:3n-3) and LA, which cannot get synthesised in vertebrates and are required for the conversion to highly unsaturated FAs such as EPA and ARA (conditionally essential), respectively [Hastings et al. 2001; Nakamura et al. 2004]. ARA, as well as its n-3 counterparts EPA and DHA, play important roles as precursors of the eicosanoid signalling molecules in vertebrates [Nakamura et al. 2004]. LA, the precursor for ARA, occurs naturally in seeds and vegetable oils and the most highly consumed PUFA in the Western diet. Whereas ARA, the principal n-6

PUFA, naturally occurs in animal tissues as an important structural component of phospholipids in cell membranes, especially abundant in the brain, muscles and livers. However, a question still exists whether high dietary LA results in LA being converted into ARA and thus pro-inflammatory lipid mediators [Fritsche 2008; Rett et al. 2011]. In fish, requirements of dietary PUFAs vary qualitatively and quantitatively with developmental stages and species such as marine and freshwater fish [Tocher 2010]. They can generally be classified by their requirement of either higher n-3 or n-6 PUFAs, or equal amounts of both [Watanabe 1982]. Differences also exist in the conversion patterns of C18 (ALA and LA) to C20 PUFAs (EPA and ARA) between marine and freshwater species [Tocher et al. 2006]. Freshwater species are able to convert them, whereas marine fish have a reduced ability to synthesize long chain PUFAs from their C18 precursors [Hastings et al. 2001; Turchini et al. 2009]. Zebrafish (*Danio rerio*) belongs to the group of fishes that require higher amounts of n-6 PUFA [Watanabe 1982]. It has been shown that growth and fertilization rates in zebrafish were positively correlated with the level of dietary n-6 PUFAs [Meinelt et al. 1999; Meinelt et al. 2000].

Physiological functions. n-3 and n-6 PUFAs are not interconvertible and exert often opposing physiological functions. Besides serving as an energy source, they also have a potential to control gene expression through activating nuclear receptors, and to change cellular phenotypes by changing membrane phospholipid composition, which closely depends on the dietary fatty acid pool [Kliewer et al. 1997; Jump 2004; Schmitz et al. 2008; Wahli et al. 2012]. Cell-type specific metabolism, quantity and type of dietary lipids, transcription factors and abundance of nuclear receptors and membrane receptors determine the physiological response of dietary fatty acids [Jump 2004; Varga et al. 2011]. Fatty acids regulate gene expression in the liver by controlling key transcription factors such as PPAR, SREBP, RXR and LXR [Jump et al. 2005]. For example, regulation of PPARα by direct PUFA binding induces β-oxidation and can thereby control hepatic lipid composition and also impact body lipid composition [Jump et al. 2005]. β-oxidation, the catabolism of FAs, takes place in either mitochondria or peroxisomes (highly unsaturated FAs) leading to energy production.

ARA derived eicosanoids. ARA is often discussed in context of inflammatory processes as it is precursor for a variety of bioactive compounds like eicosanoids [Patterson et al. 2012]. Free ARA can be either absorbed from the diet or released by phospholipase A_2 from cell membranes into the fatty acid pool. In mammals, the metabolic conversion of ARA is determined by three major pathways resulting in the production of signalling molecules such as prostaglandins, lipoxins, leukotrienes, thromboxanes, prostacyclines and hydroperoxy fatty acids, collectively known as eicosanoids [Harizi et al. 2008]. The synthesis to eicosanoids relies on three families of enzymes like cyclooxygenase (COX), lipoxygenase (LOX), cytochrome P450 and non-enzymatic pathways. COX produce prostaglandins, prostacyclins, and thromboxanes, whereas LOX produces leukotrienes and hydroxy-FA (HETE, HODE, HEPE e.g.) [Brash 1999; Smith et al. 2000]. ARA can be further metabolized by cytochrome P450 to hydroxy-FA and epoxy-FA (EpOME, EpETrE, EpETE, EpODE e.g.) [Arnold et al. 2010]. Even though these enzyme classes show substrate, region and stereo-specificity, they can metabolize both, n-3 and n-6 PUFAs likewise [Arnold et al. 2010; Schuchardt et al. 2013]. Especially EPA and ARA compete for COX [Schmitz *et al.* 2008], whereas ARA is a preferred substrate in teleosts and in mammals they show equal affinity to ARA and EPA [Vecchio et al. 2010; Furne et al. 2013].

n-6 PUFAs and ARA in health and disease. The fatty acid pool of an organism reflects both composition of a diet and endogenous synthesis. The physiological response to dietary fatty acids, particularly PUFA, is dependent on the dietary intake (quantity) and the type and balance (ratio) of n-3 and n-6 PUFAs. As vertebrates require specific essential FAs, deficiencies or disproportionate intake can have negative effects on health. Epidemiological studies indicate that higher intake of ω-6 PUFAs may contribute to health problems like metabolic syndrome, obesity, cardiovascular diseases, cancer and other chronic inflammatory or autoimmune diseases in human [Simopoulos 2008; Candela et al. 2011; Thomas et al. 2016]. Inflammation is often addressed to ARA as the principal precursor of a wide range of certain biologically important eicosanoids, which are traditionally considered as pro-inflammatory [Patterson et al. 2012]. However, controversy exists over generally labelling n-6 PUFA derived eicosanoids as pro-inflammatory as n-6 PUFA intake can cause both anti- and

pro-inflammatory responses [Farvid et al. 2014; Harris et al. 2014; Torrecillas et al. 2017; Tortosa-Caparros et al. 2017]. ARA and its eicosanoids have been widely studied in the context of inflammation, pain, fever and blood pressure [Funk 2001]. The functional range of eicosanoids involves apoptosis, cellular differentiation, platelet aggregability, chemotaxis, inflammatory processes and the recruitment of the immune system [Harizi et al. 2008]. ARA directed research in fish focused on the effects on bone metabolism, growth, reproduction, stress resistance, immune response, morphogenesis and fatty acid metabolism [Sorbera et al. 2001; Furne et al. 2013; de Vrieze et al. 2014; Montero et al. 2015; Lie et al. 2016; Shahkar et al. 2016; Norberg et al. 2017; Tian et al. 2017; Xu et al. 2017].

1.3 DNA methylation and basic concepts of epigenetics

1.3.1 Determinants of the epigenome

The epigenome and epigenotype. Differential gene expression is the reason cells become structurally and functionally heterogeneous even though they have the same genomic information [Jaenisch et al. 2003]. The *epi*genome (Greek prefix *epi-* means ἐπι: "over, outside of, around") as major contributor serves as an *additional* layer to genetic information while it does not change the DNA sequence itself. The term ‘epigenetics’ describes different mechanisms that allow multiple phenotypic outcomes from a single genotype. This was first introduced by Conrad Hal Waddington more than 75 years ago [Waddington 2012]. Epigenetics is the study of mitotically or meiotically stable alterations in single gene expression potential that arises during development and cell differentiation, by random changes or through environmental stimuli, which do not entail a change in the DNA sequence itself [Wu *et al.* 2001; Jaenisch et al. 2003]. Epigenetics was intended to describe the gap between genotype and phenotype, the so-called epigenotype (Figure 3) as a collection of epigenetic mechanisms [Holliday 2006; Jamniczky et al. 2010]. Essentially, epigenetic mechanisms affect whether genes in the genome get transcribed (expressed) and subsequently translated into proteins.

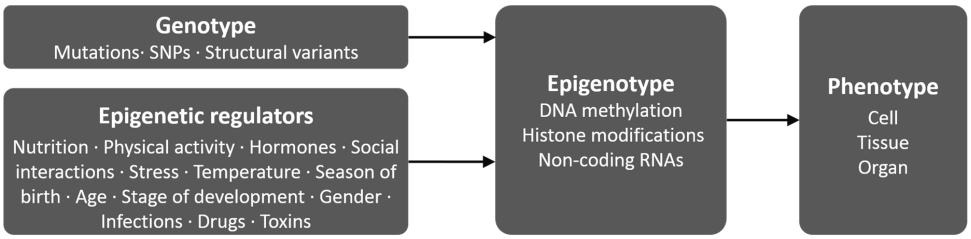

Figure 3 - Interactions between epigenetic regulators and genotype characteristics determining the epigenotype and therewith the phenotype. Modified after [Dauncey 2013].

Epigenetic mechanisms. To regulate gene expression, the genome utilizes multiple regulatory layers such as the genome sequence itself, epigenetic marks and nuclear organization [Fedorova et al. 2008; Pombo et al. 2015]. As far as we understand it today, DNA methylation, histone tail modifications, non-coding RNAs and chromatin remodelling are part of the epigenetic machinery regulating the genome (Figure 4). Post-translational modifications to histone tails can influence the packaging of the DNA between heterochromatin (tightly packed, "closed" state) and euchromatin (loosely packed, "open" state). Non-coding RNAs can degrade mRNAs through interaction with the translational machinery. Recent discoveries revealed chemical tags on RNA that might influence translation and RNA stability thus adding more complexity in the understanding of gene regulation [Dominissini 2014; Willyard 2017]. The close interplay and cross-talk between the epigenetic mechanisms leads to different 'packaging' of the chromatin in order to determine the repression or permission for gene transcription [Cedar et al. 2009; Wong et al. 2011; Molina-Serrano et al. 2013; Matzke et al. 2014]. Epigenetic mechanisms and modifications are changing during development but also in a tissue-specific manner which makes the epigenome immensely dynamic on the one hand, and gives stability and diversity to the cellular phenotypes on the other hand [Laird 2010]. DNA methylation, involved in a variety of biological processes, is one of the best characterized epigenetic mechanisms and is regarded as a key player in the epigenetic regulation of transcription [Jin et al. 2011].

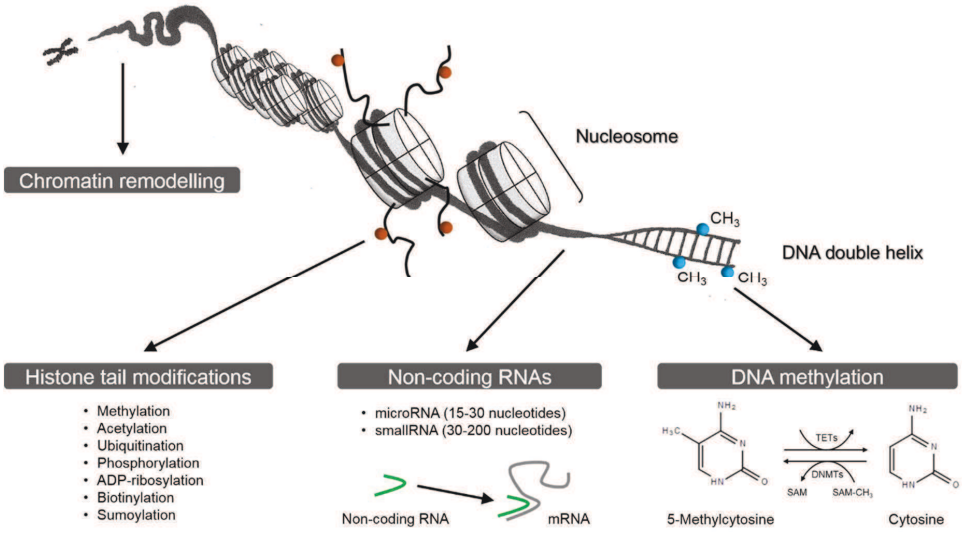

Figure 4 - Epigenetic features involved in gene expression regulation. ADP: Adenosine diphosphate; Sumoylation: 'small ubiquitin-like modifier'-ylation; CH_3: Methyl group; TETs: Ten-eleven translocation enzymes; DNMTs: DNA methyltransferases; SAM: S-Adenosyl methionine. Modified after [Milagro et al. 2013].

1.3.2 Properties and the biological impact of DNA methylation

Occurrence and biological importance. DNA methylation is a post-replication modification where the nucleotide cytosine gets methylated by adding methyl groups to form 5-methylcytosine from cytosine (C) [Jaenisch et al. 2003]. DNA methylation occurs typically when it is positioned close to guanine (G) in the context of CpG dinucleotides (CpGs), but can also occur in non-CpG context (CHG, CHH). DNA methylation is symmetrical to the other DNA strand and symmetry can be maintained through cell division. CpG islands, which are regions with high frequency of CpG sequences, are often associated with promoter regions of a gene [Ng et al. 1999; Jones 2012]. The non-random distribution of methylation differs during development, among tissues, cell types, genomic regions [Meissner et al. 2008; Suzuki et al. 2008; Lokk et al. 2014; Spruijt et al. 2014; Chatterjee et al. 2015; Sabet et al. 2016] and varies also with gender and age [Boks et al. 2009; Hall et al. 2014; Chatterjee et al. 2016]. DNA methylation has implications for physiologic and pathologic processes as it plays critical roles in many biological functions such as gene expression regulation, genomic

imprinting, X-chromosome inactivation, stem cell differentiation and embryogenesis [Li et al. 1993; Newell-Price et al. 2000; Bird 2002; Cotton et al. 2015]. Methylation patterns are regulated by several DNA methyltransferases (DNMTs) and DNA methylation changes in temporal, spatial and cell-type-specific manners [Liu et al. 2016; Edwards et al. 2017]. Abnormalities in the patterns are often observed in several diseases [Chen et al. 2006; Yang et al. 2014]. In vertebrates, there is a division between maintenance DNMTs (DNMT1), *de novo* DNMTs (DNMT3), and ten eleven translocation (TET) enzymes, which are involved in removing DNA methylation [Oliveros 2007-2015; Voisin et al. 2015; Zhao et al. 2016; Zenk et al. 2017].

Inheritance of DNA methylation. DNA methylation can get inherited through cell divisions both mitotically (somatic cells) and meiotically (germ cells). The latter may involve a transgenerational inheritance of DNA methylation. Changes to locus-specific DNA methylation patterns are considered as transgenerational (multigenerational) inherited, if an established effect in F_0 remains persistent up to the F_3 generation as the first not directly exposed generation. For example, exposure in F_0 affects the developing F_1 and also F_2 (being present as germline in F_1) are considered as directly exposed [Skinner 2008]. In fish though, adult environmental exposure in F_0 results in directly exposed F_1, and F_2 is the first not directly exposed generation. This is because in fish the germ cells of F_1 (i.e. the actual F_2 cells) start to develop after external fertilization [Raz et al. 2002; Aguero et al. 2017]. However, the developing F_2 cells are exposed to the F_0 environment through the composition in the oocyte. The key node for the inheritance of methylation changes to the next generation is the zygotic reprogramming of the germline epigenome, e.g. DNA methylations being consistently copied. It has been reported that the germline can get epigenetically programmed during gametogenesis [Trasler 1998; Allegrucci et al. 2005; McCarrey et al. 2005].

Even though uncertainties surrounding the epigenetic effects of environment on transgenerational inheritance still exists [Schmidt 2013], inheritance of phenotypic changes due to environmental stimuli has been observed in several studies [Rakyan et al. 2003; Skinner et al. 2013; Sen et al. 2015; Beck et al. 2017; Carvan et al. 2017]. Nutrition has been shown to have transgenerational effects in mice, where feeding

pregnant females with a methyl-donor rich diet correlated with the methylation status of transposable element responsible for the coat-color resulting in a changed phenotype of the progeny [Wolff et al. 1998]. Furthermore, progeny of male mice fed a low-protein diet revealed increased hepatic expression of genes involved in lipid and cholesterol biosynthesis and changes in DNA methylation associated with the paternal diet, particularly in likely enhancers for PPARα [Carone et al. 2010]. Paternal high-fat-diet and phenotype programmed metabolic profiles in female progeny, was found to impair glucose tolerance and insulin secretion [Ng et al. 2010]. These studies suggest the transmission of epigenetic factors that can be associated with dietary interventions of the parental generation and result in altered phenotypes or risk of disease in the progeny.

DNA methylation changes during embryonic development. In mammals, the embryonic development after fertilization until birth undergoes special methylation dynamics and patterns. Global demethylation followed by remethylation are essential for the pluripotent state of cells that allows differentiation and embryogenesis [Santos et al. 2002]. Shortly after fertilization an active whole genome demethylation takes place, except on imprinted genes [Ivanova et al. 2012]. A decrease in global DNA methylation level in the paternal genome takes places actively, whereas maternal genome demethylation is passively and slower. New methylation patterns that are species specific and cell type specific are established after fertilization at different developmental stages depending on the species [Santos et al. 2002; Allegrucci et al. 2005; Potok et al. 2013; Liu et al. 2016]. Embryonic development represents a sensitive period where methylation and gene expression patterns get established and perturbations in those patterns can have life-long consequences such as phenotypic effects and risk for disease [Burdge et al. 2010]. A study on mice revealed that acute dietary zinc deficiency before ovulation changed oocyte methylation patterns that led to an impaired *in vivo* fertilization and blastocyst formation [Tian et al. 2013]. In teleost, DNA methylation is highly sensitive to environmental exposures. For instance, early stages of teleost development are highly sensitive to temperature fluctuations and changes DNA methylation and/or associated genes in both zebrafish [Campos et al.

2012] and Atlantic cod (*Gadus morhua*) [Skjaerven et al. 2014]. Recently, it was shown that early temperature exposures in European sea bass (*Dicentrarchus labrax*) changes the DNA methylation at locus specific sites dependent on temperature treatment [Anastasiadi et al. 2017]. In addition, European sea bass early exposures to increased temperature changes the sex ratios and the promoter DNA methylation of gonadal aromatase (*cyp19a*) [Navarro-Martin et al. 2011].

Regulation of the gene expression potential. Two plausible mechanisms has been proposed on how DNA methylation can influence gene expression [Bird 2002]. The first proposes the exclusion of proteins and transcription factors by enable them to bind to their recognition site by direct interference of the methyl group with the protein. The second mechanism involves attraction of methyl-CpG-binding proteins that result in inhibiting of gene expression. The position of the methylation within the transcriptional unit and outside of a gene body plays an important role in gene regulation [Suzuki et al. 2008; Aran et al. 2011; Brenet et al. 2011; Jones 2012; Schubeler 2015].

1.3.3 Intergenerational regulation of gene expression

In addition to epigenetic mechanisms, non-genetic factors can also play important roles in the transmission of diet effects. New research has shown that an organism's phenotype is not only determined by its own genome and environment, but also by parental environment. Maternal and paternal dietary habits can program progeny health, but the mechanism need further elucidation [Cerf 2011; Sinclair et al. 2013]. The parental environment has been associated with differential gene expression in the progeny, in both embryonic stages and mature tissues [Cannon et al. 2014; Skjaerven et al. 2016]. Considerable research has been done to investigate how epigenetic regulation of gene expression is linked to parental impact on the next generation. The node of the transmission of such environmental cues from parents to progeny, is through the germline (meiosis). Gamete maturation from germ cells is dependent on its environment and thus its regulation, such as through the diet, which can influence nutrient supply, metabolic profile and physiology in the cells that in turn can affect the oocyte maturation process [Gu et al. 2015]. Nutrition has been shown to affect oocyte

nutrient composition and reproductive success (fecundity) in various species [Jaya-Ram et al. 2008; Wonnacott et al. 2010; Warzych et al. 2011; Migaud et al. 2013; Dunning et al. 2014; Newman et al. 2016; Norberg et al. 2017]. Diet effects on the progeny can possibly be mediated through non-genetic (non-mendelian) mechanisms such as 1) nutrient composition of the oocyte and 2) maternal or paternal mRNA deposited in the gametes [Abrams et al. 2009; Aanes et al. 2011; Migaud et al. 2013].

Transcripts deposited in the oocyte. Maternal-to-zygotic transition (Figure 5), or mid-blastula transition, represents the period where the transcriptome of the zygote starts to be active after a couple of cleavage cycles [Schier 2007]. This period varies in time across species [Tadros et al. 2009]. Before the mid-blastula stage (transcriptionally inactive zygote), the newly fertilized egg cell relies on a repertoire of both maternal and paternal [Ostermeier et al. 2004; Nanassy et al. 2008; Liebers et al. 2014] encoded gene products (mRNAs, miRNA tRNA, rRNA) generated during gamete maturation, deposited and thus inherited to the zygote. In zebrafish these gene products regulate the first cleavage cycles until zygotic transcription replaces this function in two major waves [Kimmel et al. 1995; Abrams et al. 2009; Tadros et al. 2009]. The maternal-to-zygotic transition is followed by gastrulation and the formation of the three germ layers [Kimmel et al. 1995]. Non-coding RNAs such as microRNAs are involved in the regulation of embryogenesis, and both maternal and paternal microRNAs has been shown to play important embryonic development roles in mice [Tang et al. 2007; Nanassy et al. 2008; Rodgers et al. 2015]. However, no maternal microRNAs have yet been identified that play an indispensable role in zebrafish embryogenesis [Schier et al. 2006; Abrams et al. 2009].

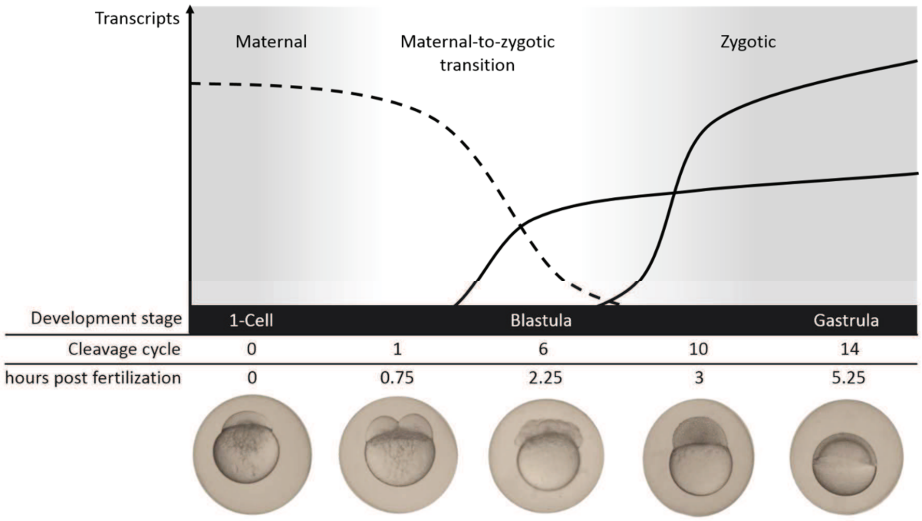

Figure 5 – Maternal-to-zygotic transition in zebrafish. Maternal mRNA deposition that affects the early development of the next generation while the embryonic genome is transcriptionally inactive. During the maternal-zygotic transition (zygotic gene activation), zygotic genes start be transcribed and maternal mRNA gets degraded. Key embryonic stages are visualized below the indicated development stage, cleavage cycle and hours post fertilization. The dashed black line illustrates maternal transcripts and continuous black lines show minor and major waves of zygotic genome activation. Staging of embryonic development at 28±1°C after [Kimmel et al. 1995]. Scheme modified after [Tadros et al. 2009; Aanes et al. 2011]. Photos are taken by Anne-Catrin Adam, 2013.

2. Methodological consideration

2.1 'Multi-omics' approach

High-throughput biotechnology approaches, collectively termed as 'omics', promote the understanding of biological systems on the different levels between the genotype to phenotypic alterations. In the central dogma of 'multi-omics', fields such as genomics, transcriptomics, epigenomics, proteomics and metabolomics are combined within a single study. This allows investigation of an entire biological system from its DNA, epigenetic marks to RNA, through to proteins and metabolites (Figure 6). For nutritional studies, this approach aims to increase the understanding of the role of dietary nutrients in shaping the phenotype by detecting genes (genomics) that are transcribed to mRNA (transcriptomics) and influenced by epigenetic marks (epigenomics), translated to proteins (proteomics) and generating metabolites (metabolomics). Generally speaking, sequencing technologies are applied to DNA and RNA analysis, and mass spectrometric quantifications to protein and metabolite analysis [Buescher et al. 2016]. Utilization of such high-throughput genomic tools in nutrition research helps to increase the fundamental knowledge on the complexity and dynamics between diet and health. Previous knowledge from former studies help to build networks in order to understand the way nutrition can influence metabolic pathways in health and disease.

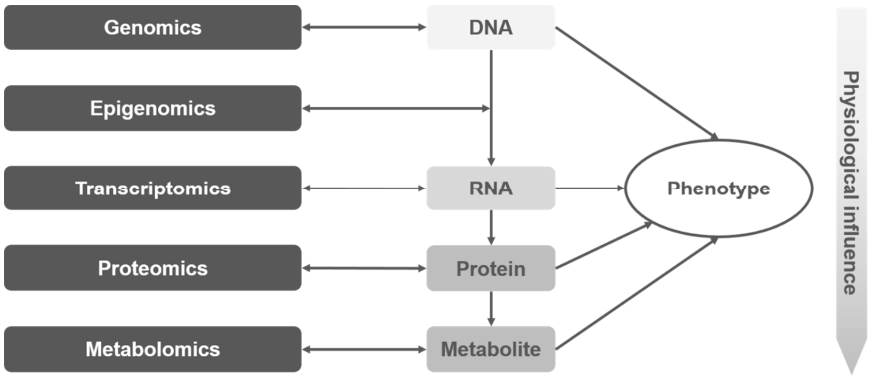

Figure 6 - **The central 'multi-omics' dogma.** Modified after [Sales et al. 2014].

Different high-throughput technologies to screen metabolic, transcriptomic and DNA methylation signatures associated with high dietary ARA levels were applied in this PhD project (Figure 7). Protocols were described in three publications: Metabolomics is addressed in **paper I**, RNA-sequencing is described in **paper II** and reduced representation bisulfite sequencing (RRBS) is presented in **paper III**.

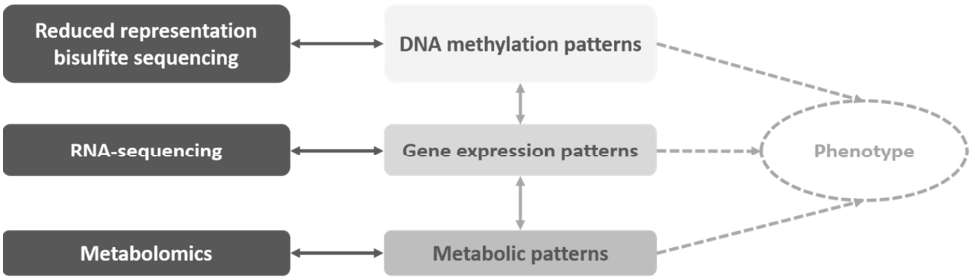

Figure 7 - Scheme illustrating the methodological strategy applied to this PhD project.

Reduced representation bisulfite sequencing (RRBS). Several different techniques are used to study DNA methylation that are based either on affinity purification of methylated DNA, digestion with methylation-sensitive restriction enzymes or bisulfite conversion of the DNA using methylation insensitive enzymes such as MspI [Shen et

al. 2007; Altun et al. 2010; Bock et al. 2010]. Among these approaches that enable mapping of methylated cytosine profiles [Harris et al. 2010], RRBS (a bisulfite sequencing method) has been become popular [Nagarajan et al. 2014; Seiliez et al. 2017]. The major difference between RRBS and other techniques is that DNA is treated with bisulfite and enriched (through enzymatic digestion) for CpG-rich fragments, which are mostly present in promoter regions [Gu et al. 2011]. RRBS identifies global alteration of DNA methylation on a single nucleotide resolution that makes it more attractive than enrichment-based methods. Sequence data produced by RRBS is limited in that it only covers approximately 5% of the genome (reduced representation), but is optimal for non-targeted studies focused on screening (**Paper III**). This is in contrast to whole-genome bisulfite sequencing (WGBS) which performs ~95% genome coverage and can assess nearly every CpG site, including low-CpG-density regions. However, for examination of genome-wide methylation patterns RRBS is an efficient and cost-effective alternative to WGBS [McRae et al. 2014]. In addition, RRBS enables efficient identification of CpGs as it captures 85% of CpG islands, 60% of promoters and requires little input sample [Gu et al. 2011]. Common challenges in methylation data analysis are related to inter-individual, age and cell-type-characteristic variation in methylation patterns [Bock 2012].

RNA-sequencing. Transcriptomics is the study of gene expression profiles (transcriptome) of a genome across a variety of biological situations using high-throughput methods, such as RNA-sequencing (RNA-Seq) [Wang et al. 2009]. RNA-Seq allows selective sequencing of total mRNA present in a system that reflects the genes that are actively expressed at any given moment. Comparing gene expression patterns allows an identification of differential expressed genes in cells, tissues or in response to environmental stimuli, such as nutrition (**Paper II**). It enables an understanding of regulation and molecular mechanisms of genes under certain conditions. Functions and biological mechanisms of differentially expressed genes can be determined by looking at their enrichment for gene ontology terms [2017] or biological pathways using KEGG (Kyoto Encyclopedia of Genes and Genomes) [Kanehisa et al. 2012].

Metabolomics. The metabolome is a set of metabolites (small-molecule chemicals) under defined conditions in a biological system (cell, organism, tissue). It is the final downstream product of the interaction between genome and environment and the closest link to the phenotype [Fiehn 2002; Kosmides et al. 2013]. Metabolomics aims to study global metabolite profiles in a system (cell, organism, tissue) under a given set of conditions such as the impact of a diet on global metabolic fingerprint (**Paper I**). Many laboratories offer different commercial analysis usually using mass spectrometry technology with their own set of internal standards. That introduced a degree of limitation to our data as analysis-coverage of metabolites is dependent on the platforms and metabolites standards provided by the service we used.

2.2 Zebrafish as a vertebrate model

Zebrafish (*Danio rerio*) is a tropical freshwater teleost fish belonging to the family *Cyprinidae* native to Southeast Asia that is phylogenetically closely related to goldfish and carp [Sabet et al. 2016]. Zebrafish has become an attractive research tool to investigate molecular biological process in order to understand health and disease in vertebrates, in particular both human and aquaculture fish species [Ingham 1997; Grunwald et al. 2002; Ward et al. 2002; Dahm et al. 2006; Seth et al. 2013; Ribas et al. 2014; Ulloa et al. 2014; Yen et al. 2014]. Among many favourable features that zebrafish have, a fundamental advantage is the considerable amount of genetic identity with humans [Howe et al. 2013], similar anatomy and physiological processes they have in common with other vertebrates due to phylogenetic close relationships [Dahm et al. 2006; Sabet et al. 2016]. Zebrafish are small (4-5 cm), robust and cheap to maintain. They became a favored model in research as they are appreciated for their virtually transparent and large quantities of eggs (100-200 eggs per female), rapid embryonic development (in 24h all major organs have been set) and quick generation time (2-3 months to reach reproductive age). Zebrafish has been used to evaluate the importance of nutrition in outcomes related to development, health and disease by investigating physiological, cellular, and molecular processes [Schlegel et al. 2007; Watts et al. 2012; Skjaerven et al. 2016; Watts et al. 2016]. Zebrafish are omnivorous

and though they have been used as a model species to understand biological functions and mechanisms for many decades, there hasn't as yet been established a completely defined standardized diet for zebrafish [Kaushik et al. 2011; Penglase et al. 2012]. Furthermore, zebrafish is as also a widely used model for studying functional genomics [Alestrom et al. 2006], organ function [Ackermann et al. 2003], behavior [Moody et al. 2017], toxicology [Williams et al. 2014; Kamstra et al. 2015], endocrine disruption [Segner 2009], nutritional and environmental epigenomics [Murphy et al. 2016; Kamstra et al. 2017].

The zebrafish methylome. Zebrafish is an excellent model for understanding DNA methylation and its role in regulation of gene expression as they share similar DNA methylation machinery similar to mammals [Goll et al. 2011; McGaughey et al. 2014]. The first single-nucleotide resolution DNA methylome has been provided for zebrafish brain and liver [Chatterjee et al. 2013; Chatterjee et al. 2014]. Global remodeling of the parental methylome is thought to generate the state of totipotency as an important basis for cell fate determination [Surani et al. 2007; Hackett et al. 2013]. DNA methylation has been shown to be involved in pre-patterning of the gene expression potential in zebrafish embryos consistent with a transmission of methylation states from gametes to early embryos [Andersen et al. 2012]. Interestingly, reprogramming of the methylome during embryo development in zebrafish is different from the classical model in mammals [Morgan et al. 2005; O'Neill 2013].

The zebrafish liver. My target organ for the transcriptional and DNA methylation analysis in zebrafish was the liver as an essential metabolic organ that plays a central role in the regulation of lipid metabolism. Liver tissue originates from endoderm cells already early starting at 6 hours post fertilization in zebrafish [Tao et al. 2009; Gilbert 2016]. The liver of a zebrafish has mature hepatocytes by 5 DPF. Zebrafish liver is similar to human livers in terms of biological functions such as metabolism, detoxification and homeostasis, but its structural organization differs from that of rodents or humans [Menke et al. 2011]. Except for hepatic immune cells such as Kupffer cells, zebrafish do have all other cell types of the mammalian liver [Kryvi 1997; Goessling et al. 2015]. Hepatocytes (Figure 8) make up the majority of the liver

cell types, which are arranged in tubules with bile ductules coursing in between them. Endothelial cells lining the sinusoids and hepatic stellate cells are present in zebrafish liver [Goessling et al. 2015].

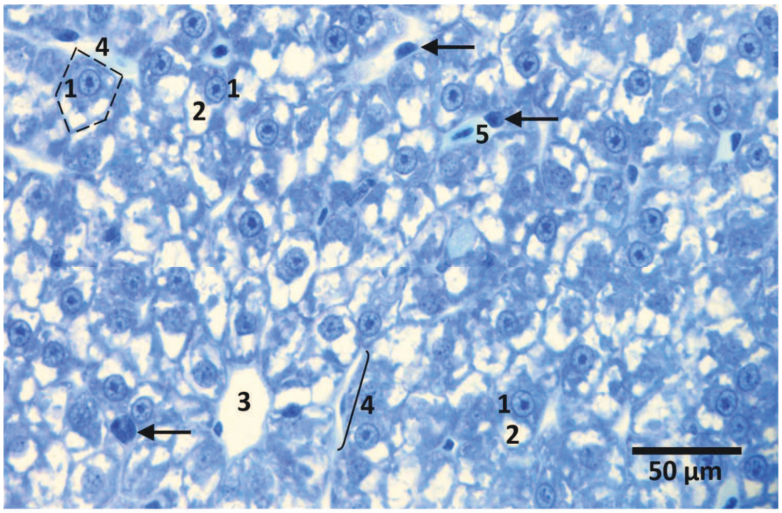

Figure 8 - Histological image of male zebrafish liver. **1:** Hepatocyte with large round nucleus and distinct centrally located nucleolus, **2**: Fat vacuole located in a hepatocyte, **3**: Bile ductile, **4:** Sinusoid, **5:** Erythrocyte with flat nucleus. **Three arrows** point at other liver tissue cells than hepatocytes, most likely macrophages. Livers (142 DPF) were rinsed in 1x PBS, fixed in 4% paraformaldehyde overnight at 4°C followed by dehydration, infiltration and embedding in Technovit 7100 following the manufacturers' protocol (Kulzer Technik, Germany). 1µm semi-thin sections were cut on a microtome (Leica, model #RM2155) and stained with toluidine blue (stains proteins, nucleic acids and membranes; non-stained tissues are lipids, solutes and the lumen of the bile canaliculi and sinusoids). Image provided by Kaja Skjærven (NIFES, 2014).

2.3 Experimental design

In order to study the impact of the parental diet on the progeny, we performed a transgenerational feeding trial where zebrafish were fed a plant-based diet low in ARA (control) and high in ARA (high ARA) in the first generation (Figure 9). The progeny was given only the control diet in order to reveal an effect of the parental diet in the next generation. We analyzed metabolic profiles in whole F_0 fish. Gene expression and DNA methylation profiles were analyzed in both parental and progeny male livers as

main metabolizing organ responding sensitively to nutritional changes. Diet composition and experimental design of the feeding trial has been described for F_0 generation in paper I and for F_1 generation in paper II.

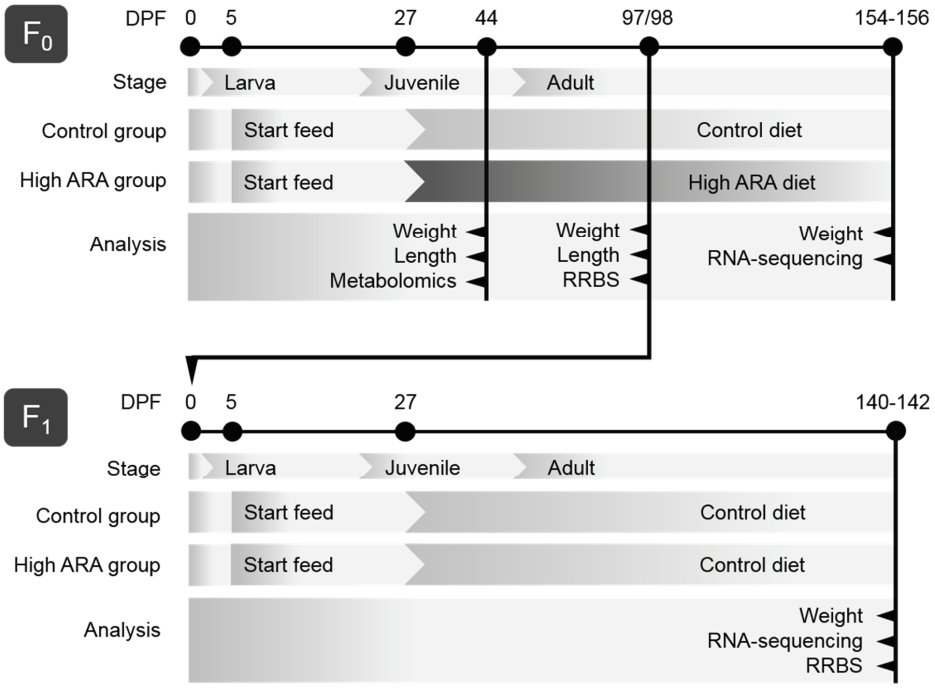

Figure 9 - Design of the zebrafish feeding trial over two generations. Fish were fed Gemma micro® (Skretting, Norway) and *Artemia nauplii* (Silver Star *Artemia*, USA) as start feed from 5 and 7 days post fertilization (DPF) until 26 DPF in both generations, respectively. The experimental diet (control and high ARA) was given from 27 DPF until sampling. Adult F_0 were mated at 97 DPF to generate F_1 progeny. Both groups in F_1 were fed the control diet from 27 DPF until sampling. Body weight and length records, whole fish sampling for metabolomics and male liver sampling for reduced representation bisulfite sequencing and RNA-sequencing were performed at different stages as indicated in the figure above. DPF: days post fertilization; RRBS: Reduced representation bisulfite sequencing. Figure modified after Paper I and Paper II.

The experimental diets, control and high ARA, were equal in their ingredient composition, except for the oily ingredients, where an ARA-rich oil was added in 12 times higher amounts to the high ARA diet to decrease the dietary n-3/n-6 PUFA ratio compared to the control diet. We chose to add ARA as the physiologically more

important n-6 PUFA in order to provoke an ARA associated effect on overall metabolism and thereby strengthen a transcriptional and potential transgenerational effect. To minimize an effect through lower dietary n-3 PUFA levels while increasing n-6 PUFA levels, we aimed to keep levels of potent n-3 PUFAs, DHA and EPA, in both diets similar. There is need for a standardized zebrafish diet [Penglase et al. 2012]. Although little is known about the fatty acid requirement in zebrafish, the ARA levels were based on previous studies [Watanabe 1982; Meinelt et al. 1999; Meinelt et al. 2000; Boglino et al. 2012; de Vrieze et al. 2014] with the intention to avoid deficient and toxic levels. Other nutrient levels were based on carp requirements [NRC 2011] and ARRAINA diet compositions [Hemre et al. 2016].

3. Research aims

This study was designed to investigate the impact of high dietary ARA levels in the next generation (Figure 10). Based on growing evidence, alterations in DNA methylation patterns may be involved in the epigenetic gene regulation. Changes in gene expression may thereby affect metabolic and phenotypic alterations of an organism, and presumably of the next generation. 'Omics' technologies were used to investigate differences in hepatic gene expression and DNA methylation signatures. Metabolomics was chosen to profile key metabolites affected by the diet in the first generation. We followed three specific aims:

- Determine the alterations in the metabolic fingerprint of high dietary ARA compared to lower dietary ARA levels in zebrafish (F_0).
- Elucidate the changes to the gene expression profiles in both F_0 and F_1 male livers associated with dietary ARA levels in the first generation.
- Investigate the differences in the DNA methylation profiles in both F_0 and F_1 male livers associated with dietary ARA levels in the first generation.

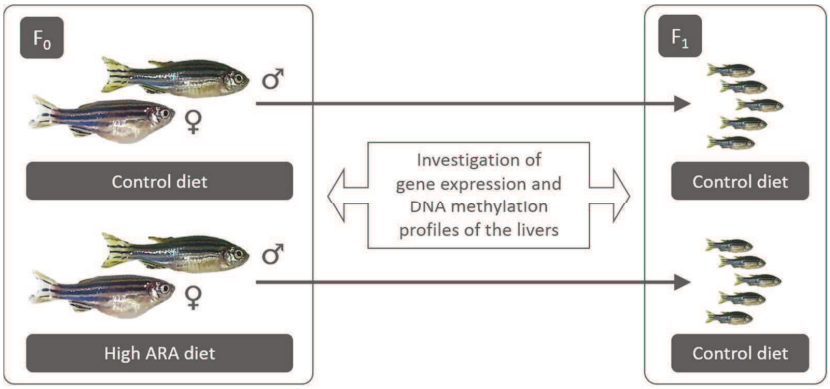

Figure 10 - Scheme illustrating the main aim of the transgenerational feeding trial. F_0 generation of zebrafish was given a diet either low (control) or high in arachidonic acid (high ARA), and the progeny was given only the control diet in order to reveal an effect of the parental diet on transcriptomic and DNA methylation profiles in the progeny livers.

4. Research questions

Do high dietary ARA levels compared to lower dietary ARA levels alter the metabolic signature of zebrafish?

Hypotheses:

- High dietary ARA changes the fatty acid profiles in zebrafish (**Paper I**).
- High dietary ARA changes the metabolic profile in zebrafish (**Paper I**).

Do dietary ARA levels affect hepatic gene expression and DNA methylation patterns?

Hypotheses:

- High dietary ARA changes the gene expression profiles in F_0 livers (**Paper II**).
- High dietary ARA changes the DNA methylation profiles in in F_0 livers (**Paper III**).

Do dietary ARA levels affect the next generation?

Hypotheses:

- Parental high dietary ARA changes the hepatic gene expression profiles of adult progeny (**Paper II**).
- Parental high dietary ARA changes the hepatic DNA methylation profiles of adult progeny (**Paper III**).

5. Summary of the results

Paper I – "High dietary arachidonic acid levels induce changes in complex lipids and immune-related eicosanoids and increase levels of oxidised metabolites in zebrafish (*Danio rerio*)"

1. **Design:**

   Zebrafish (F_0) were fed a diet either low (control, 1.5% ARA/TFA) or high in ARA (high ARA, 17.3% ARA/TFA) from 27 DPF until sampling for metabolic profiling (44 DPF) and until weight and length measurement (44 DPF, 91 DPF).

2. **Major findings:**

   - High dietary ARA affected weight but not length of 44 DPF zebrafish, but at 91 DPF the growth was equal for both feed groups
   - High dietary ARA levels resulted in a lower n-3/n-6 PUFA ratio and a general shift in lipid profiles at 44 DPF after feeding experimental diets for 17 days
   - At 44 DPF, a total of 153 out of 566 detected metabolites differ between the two dietary groups
   - High dietary ARA increased dicarboxylic acids, ARA-derived eicosanoids (HETEs), and oxidized lipids and amino acids
   - High dietary ARA affected levels of metabolites with known anti-oxidative properties such as glutathione, urate, carnosine, ascorbate and endocannabinoids

3. **Conclusions:**

   Results indicated oxidative stress and lipid peroxidation in the fish after feeding experimental diets for 17 days with the high ARA diet. Increased pro-inflammatory eicosanoids, endocannabinoids, and oxidized lipids and amino acids suggest an inflammatory and oxidised environment, which led to an anti-inflammatory and anti-oxidative response in the fish.

Paper II – "Parental high dietary arachidonic acid levels modulated the hepatic transcriptome of adult zebrafish (*Danio rerio*) progeny"

1. **Design:**

   Analysis of changes in the hepatic transcriptomic pattern of mature male zebrafish in the first (F_0, 154-156 DPF) and second (F_1, 140-142 DPF) generation using RNA-Seq. The parental fish were fed a diet either low (control, 1.5% ARA/TFA) or high in ARA (high ARA, 17.3% ARA/TFA). The progeny was given only the control diet.

2. **Major findings:**

   - No differences in mature body weight between dietary groups in both generations
   - Few genes were differentially expressed between dietary groups in F_0 livers compared to F_1 livers
   - F_0 DEGs were involved in β-oxidation, RXR and PPAR signalling
   - Main F_1 gene expression changes were related to methionine cycle, transsulfuration pathway, estrogen signalling, and lipid and retinoid metabolism by PPARα/RXRα playing a central role

3. **Conclusions:**

   Results demonstrate that the dietary n-3/n-6 PUFA ratio was more strongly associated with hepatic differential gene expression in adult progeny than in the parents as we found surprisingly few DEGs in F_0 livers. The observed transcriptomic changes in F_1 were related to methionine, transsulfuration, retinoid, lipid and estrogen signalling pathways. Several links were found between parental metabolic (Paper I) and transcriptomic patterns of the progeny (Paper II).

Paper III – "Profiling DNA methylation patterns of zebrafish liver associated with parental high dietary arachidonic acid"

1. **Design:**

   Investigation of hepatic DNA methylation profiles of mature male zebrafish in the first (F_0, 98 DPF) and second (F_1, 140-142 DPF) generation using RRBS. The parental fish were fed a diet either low (control, 1.5% ARA/TFA) or high in ARA (high ARA, 17.3% ARA/TFA). The progeny was given only the control diet. Comparison of genes connected to differentially methylated loci (DMLs) with differentially expressed genes (DEGs) and upstream regulators was performed.

2. **Major findings:**

   - Strong difference in hepatic DNA methylation between the dietary groups in both generations
   - DMLs were more frequent in introns (gene bodies) and intergenic regions than in promoters for both generations. For parental livers, hypermethylation was dominating these genomic regions
   - None of the F_0 or F_1 genes linked to DMLs were enriched in KEGG pathways or Gene Ontology terms, but 12.5% of the genes linked to DMLs of parental and progeny livers were common
   - 5 genes linked to DMLs were concordant to DEGs in F_0, while for F_1 generation 37 concordant DML/DEG genes were involved in methionine cycle, lipid and estrogen signalling
   - CNR1, RORA, PPARA, PPARGC1A and ESR2 were identified upstream regulators based on F_1 DEGs and were also linked to a DML in F_1 livers

3. **Conclusions:**

   Parental dietary ARA levels changed hepatic DNA methylation profiles in the progeny. Identified upstream regulators were linked to differential gene expression and differential methylation in the F_1 livers. They function in *de novo* lipogenesis

through PPAR downstream signalling, possibly affecting energy metabolism and estrogen signalling. Links to F_0 metabolic profiles (Paper I) and F_1 gene expression (Paper II) were made. The results indicate that parental high ARA levels might have affected the developing embryo possibly through epigenetic mechanisms.

6. General discussion

6.1 Dietary ARA levels alter the metabolic signature of zebrafish

The metabolome is reflecting the metabolites of cellular regulatory processes in response to environmental and genetic changes [Fiehn 2002]. We screened juvenile zebrafish for changes in metabolism after feeding them experimental diets composed of high n-6 PUFA, particularly ARA, for 17 days. Through screening whole sets of metabolites, we were able to investigate a wide range of compounds and to profile the complex effect of diet composition.

The FA composition of an organism is depending on different factors such as dietary FA composition, digestibility, uptake, transport, elongation, desaturation and β-oxidation [Turchini et al. 2009]. Our results demonstrate that n-3 and n-6 PUFA profiles in a plant-based diet given to zebrafish were associated with the PUFA profiles observed in juveniles, where particularly dietary n-3/n-6 PUFA ratios (control: 0.6, high ARA: 0.2) were reflected in the fish (**Paper I**). Others have shown that dietary PUFA profiles from different lipid sources were also reflected in different tissues in zebrafish [Tocher et al. 2001; Jaya-Ram et al. 2008]. High dietary ARA fed to Atlantic cod showed a correlation of dietary ARA with plasma and liver ARA levels [Norberg et al. 2017]. Furthermore, we observed changes in ARA-derived eicosanoids, in levels of oxidized lipids and amino acids, and changes in the phospholipid profiles suggesting highly incorporation of n-6 PUFAs, particularly ARA into membranes. Collectively, these results emphasize that dietary n-3 and n-6 PUFA profiles gave a strong response on a wide range of metabolites, where increased dietary n-6 PUFAs, particularly ARA, suggested to cause a change in the physiological state to oxidized and pro-inflammatory (**Paper I**).

Interestingly, zebrafish fed high ARA levels showed a slight difference in body weight, but not in length compared to the control group at 44 DPF (**Paper I**), but adult fish were not different in weight either at 91 DPF (**Paper I**) or 154-156 DPF (**Paper II**).

However, other studies have shown that dietary n-6 PUFA levels were correlating positively with growth and fertilization rates in zebrafish [Meinelt et al. 1999; Meinelt et al. 2000].

The underlying aim of the metabolic analysis in juveniles was to elucidate the dietary effect on global metabolism. It is possible that the effect of a diet with a low n-3/n-6 PUFA ratio might be even stronger in one or another tissue as screening the whole body could have averaged those differences. Tissues or organs with varying functions and cell-type specific expression pattern utilize metabolites as fuels differently. However, our downstream analysis focused on liver, an essential metabolic organ, as most compounds absorbed by the intestine will pass through liver tissue, which in turn regulates, metabolizes and distributes compounds to other tissues.

6.2 Dietary ARA levels affect hepatic transcriptomic and DNA methylation profiles

Changes in gene expression, which is defined by the reactions that control gene product (RNA) abundance, are often invoked to explain metabolic differences [Raser et al. 2005]. As stated before, feeding experimental diets for 17 days with increased ARA levels, had a strong effect on the metabolic fingerprint of juvenile fish (**Paper I**). Surprisingly few DEGs (20) were found in adult male livers (**Paper II**). Considering, that the fish were fed on a high ARA diet, one would assume that the metabolic effect observed at the juvenile stage would to some degree be reflected in hepatic gene expression changes in the mature fish. However, from this study, among the few DEGs, some were involved in β-oxidation, RXR and PPAR signalling (**Paper II**), which can be linked to the differences found in the lipid and fatty acid profiles of juvenile fish. It is conceivable that the missing correlation between metabolic and transcriptomic profiles is caused by differences in age (juvenile vs mature) and tissue types (whole fish vs male liver). In addition, possible explanation could also be linked to gene expression profiles being sensitive to the standardization of liver dissections like sampling time, temperature, circadian rhythm, starvation time or other unknown factors [Raser et al. 2005]. It is also plausible that the dietary response for F_0 was stronger in

other tissues such as muscle or intestine of the fish causing a difference in metabolite composition in the whole fish which is not reflected in the liver transcriptome.

Despite less differential expression in F_0 livers, we found a strong response to high dietary ARA levels on liver DNA methylation profiles. A total of 2338 DMLs were found in male F_0 livers (**Paper III**). Comparing the gene expression and DNA methylation results, we found an overlap for five genes in the F_0 generation (0.2%, Figure 11). A link has been made to the metabolic profiles, particularly lysophospholipid profiles in juveniles (**Paper I and III**).

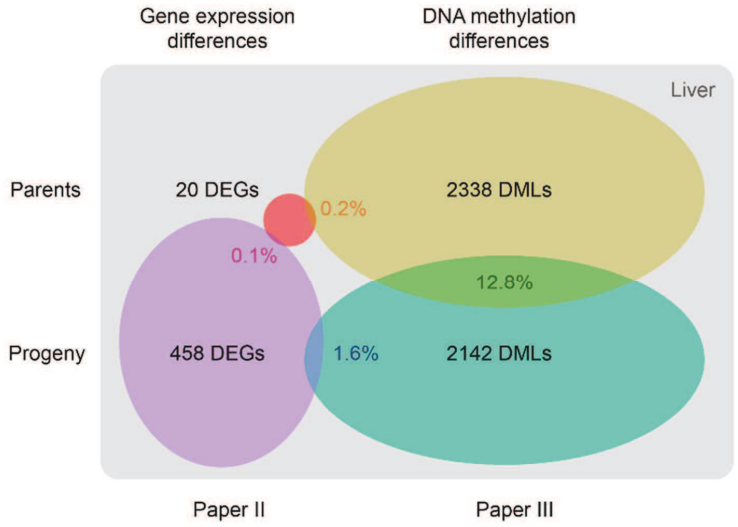

Figure 11 - Schematized overall differences in DNA methylation and gene expression between high ARA and control group in F_0 and F_1 generation. Percentage of overlap between concordant genes to differentially methylated loci (DMLs) and differentially expressed genes (DEGs) is given. Proportions of the circles illustrate the number of DMLs and DEGs between high ARA and control group.

6.3 Dietary ARA levels affect the next generation

Regulation of gene expression by parental diet

It is hypothesised that environmental stimuli, such as diet, can exert transgenerational effects through altering epigenetic mechanisms, such as DNA methylation that

potentially carry information from one generation to the next generation [Burdge et al. 2010]. One example demonstrated, feeding pregnant mice with varying amounts of methyl-donors was associated with changes in the methylation status of a transposable element responsible for the coat-colour of the progeny [Wolff et al. 1998]. Later, other studies have shown that nutrition can influence DNA methylation resulting in altered phenotypes [Waterland et al. 2003; Amarasinghe et al. 2015]. This PhD project was driven by that idea that alterations in the dietary n-6 PUFA profile, particularly of ARA, can influence epigenetic regulation of gene expression, which can be passed on to the following generation. The present results showed, that both hepatic gene expression and DNA methylation profiles of progeny were altered due to the parental high ARA diet (**Paper III**). Comparing the hepatic gene expression and DNA methylation results of the F_1 generation, an overlap has been found for 37 genes (1.6%, Figure 11). One possibility in which an effect of the parental diet may be mediated, is through metabolic influences during gamete maturation. Broodstock nutrition is an important aspect of aquaculture production insuring good egg quality and larvae health. ARA has been studied especially in relation to reproductive performance, egg and larval quality in fish [Furuita et al. 2003; Mazorra et al. 2003; Norambuena et al. 2013; Parma et al. 2015]. Dietary PUFAs can alter the nutrient composition in the ovaries and thereby also the yolk composition, of which the development of the embryo is depending on [Sorbera et al. 2001; Jaya-Ram et al. 2008; Asil et al. 2017; Norberg et al. 2017]. Another possibility of how parental diet can effect adult progeny constitutes the period of developmentally programming of the transcriptome during early embryonic development. Before the zygotic genome gets activated (maternal-to-zygotic transition), the development of the fertilized egg is regulated by maternal transcripts such as mRNAs and non-coding RNAs [Pelegri 2003; Tadros et al. 2009; Liebers et al. 2014]. A recent study using zebrafish, showed that parental diet has the potential to significantly alter the embryonic gene expression pattern during organogenesis [Skjaerven et al. 2016]. Furthermore, it is possible that alterations in the transcript "package" deposited in the egg can impact the development of the progeny beyond the embryonic stage and thereby priming adult transcription and potentially physiology [Waterland et al. 2004]. The data here is limited, however, further studies of yolk

nutrient composition and transcriptomic analysis of newly fertilized eggs or embryos, and mature liver tissue composition might be useful to provide answers to these speculations.

Liver transcriptome profiles of F_0 and F_1 generation

The principal component analysis of liver transcriptomic patterns of the control groups of F_0 and F_1 revealed less overlap than expected (**Paper II**). One would expect the gene expression of the F_0 and F_1 control groups to cluster closer together than observed in the present study. Similar patterns were seen for the DNA methylation patterns and one might wonder if the DNA methylation profiles were more directly responsive to dietary PUFA levels, as methylation profiles in F_1 (control diet) were more similar to each other than the profiles in F_0 (two experimental diets) (**Paper III**). Many factors could have contributed, such as age difference when sampling mature livers and inter-individual genetic differences. One might however speculate that the diet given to the parents of the present F_0 generation has contributed to these observations as our in-house standard diet fed to wild-type zebrafish has a different diet composition than our experimental diet. Based on that idea, it is plausible that the F_0 generation was in turn influenced by the diet of their parents. That could also possible explain why both dietary groups in F_0 revealed few differentially expressed genes as their parents were fed the same diet. As a consequence, these results suggest the period of early development as major contributor to the regulation of the transcriptome in adult zebrafish livers.

Feeding the parents dietary high ARA levels altered the hepatic DNA methylation profiles in both parents and offspring, whereas the response in gene expression was stronger in progeny livers (Figure 11). Studying mature liver, we found a low correlation between DEGs and DMLs, and it is difficult to claim whether the changes in hepatic DNA methylation constitute a mechanistic link between the parental diet and gene expression in the progeny. However, it is fascinating to observe, that parental diet left its ‘marks’ in the livers of the progeny possibly through programming of transcriptome and methylome at early life stages or in other tissues than the liver.

Looking at the few concordant DEG and DML genes, we found consistency in the pathways involved (**Paper III**). The overlapping genes relate to pathways indicating *de novo* lipogenesis through PPAR downstream signalling, affecting energy, fatty acid metabolism and estrogen signalling as discussed in **paper III**. Interestingly, the metabolic profiles of the F_0 juveniles pointed to an increased β-oxidation (**Paper I**) in the fish, whereas hepatic gene expression suggested *de novo* lipogenesis (**Paper III**). Feeding grass carp (*Ctenopharyngodon idellus*) with moderate ARA levels decreased lipogenic gene expression and lipid accumulation [Tian et al. 2014]. However, in our experimental feeding trial, zebrafish were not different in their body weights and we did not observe any phenotypic differences between the groups. Analysis of metabolites of the liver would add further insight into downstream effects of the observed changes in DNA methylation and gene expression patterns.

Does the parental high ARA diet constitute a concern for the next generation's health?

Adult health or disease can have its origin in critical periods such as early embryonic development where gene expression rapidly changes and where environmental exposure may "set" physiological functions and may induce persistent changes in an organism's phenotype [Waterland et al. 2004; Burdge et al. 2010; Parlee et al. 2014; Vickers 2014]. Maternal poor nutrition (Dutch famine 1944-1945) during pregnancy and before conception has been shown to be associated with increased risk for cardiovascular disease, high lipid profiles and obesity in the progeny in adult life [Ravelli et al. 1976; Lumey 1992; Painter et al. 2006]. In-utero undernutrition associated metabolic phenotype has been even shown to influence the second-generation progeny [Jimenez-Chillaron et al. 2009]. The present gene expression and DNA methylation pattern results from zebrafish are suggesting an impact of parental diet on adult liver gene expression most likely through programming during early embryonic development. We did not observe phenotypic effects in the progeny, however it is possible that the DNA methylation pattern affected gene expression to a higher degree at other developmental stages and/or other tissues than the liver.

6.4 The zebrafish model in nutrition research

Zebrafish is proposed as model for nutrition and growth studies in aquaculture fish species due to its large number of progeny, its rapid development and short generation time [Alestrom et al. 2006; Dahm et al. 2006; Ulloa et al. 2014]. The increasing interest of using zebrafish in aquaculture nutrition research lays in the preliminary evaluation and improvement of diets with less time effort and lower costs [Dahm et al. 2006; Ulloa et al. 2014]. In the present study, we investigated the metabolic signature of fish fed a plant-based diet with increased n-6 PUFAs (low n-3/n-6 PUFA ratio). We demonstrated in zebrafish, that the dietary n-3/n-6 PUFA ratio was reflected in the fish and main changes were observed in PUFA-derived eicosanoids and complex lipid profiles (**Paper I**). Several studies have shown that replacing fish oil with plant oils in aquaculture diets does not compromise health or growth, whereas increased plant proteins reduced the growth in some species [Turchini et al. 2009; Hemre et al. 2016]. Zebrafish has been suggested as useful model for studying lipid and fatty acids metabolism at a molecular biological and genetic level [Tocher et al. 2001]. Except small differences in essential fatty acid requirements between marine and freshwater fish species, most of the essential lipid metabolism underlying basic pathways including digestion and absorption, transport, lipogenesis and β-oxidation are the same in fish as they are in mammals [Tocher 2010; NRC 2011].

It is essential to determine molecular mechanisms by which physiological processes are changed in response to diet [Ulloa et al. 2014]. Diet can affect gene expression through epigenetic regulation that can persist throughout life or across multiple generations. The potential of dietary PUFAs to alter epigenetic mechanisms and to bring intergenerational effects [Burdge et al. 2014], evoked the interest in transgenerational effects of plant oil based fish feed. Zebrafish was suggested as a useful model employed in nutritional genomics (relationship between diet and genome), nutrigenomics (relationship between dietary ingredients and gene expression) and nutritional epigenomics (relationship between diet and epigenetic mechanisms) to pre-evaluate new diet ingredients, to optimize growth, to program fish health while reducing stress and disease in multiple generations [Mutch et al. 2005;

Jimenez-Chillaron et al. 2012; Ulloa et al. 2014; Moghadam et al. 2015; Metzger et al. 2016]. However, the knowledge for farmed fish is still limited [Moghadam et al. 2015]. The present results emphasize the potential of the dietary PUFA composition affecting gene expression and epigenetic mechanisms in adult progeny most likely through early metabolic programming by parental diet (**Paper III**). There have been a few studies demonstrating in zebrafish and rainbow trout (*Oncorhynchus mykiss*), that early life nutrition can program metabolism [Geurden et al. 2007; Fang et al. 2014; Geurden et al. 2014]. Studies in rainbow trout and gilthead sea bream (*Sparus aurata*) demonstrated that broodstock diet has the potential to influence metabolism of the progeny and to improve utilization of very low fish meal and fish oil diets, respectively [Izquierdo et al. 2015; Seiliez et al. 2017]. Future efforts need to highlight the potential and benefits of controlling nutrients in broodstock diets in order to improve growth rates, to maintain fish health and performance by preventing fish diseases, to ensure an optimal nutrient content of the yolk for the developing embryo and to sustainable produce both healthy and nutritious fish.

7. Conclusions

Zebrafish fed high dietary ARA levels (high ARA diet), compared to fish fed low ARA levels (control diet) were investigated for their metabolic signature at juvenile stage, hepatic gene expression and DNA methylation at adult stage and in their adult progeny. Based on the results obtained from the transgenerational feeding trial, we can conclude that:

- The dietary ARA levels did not affect body weight when comparing control and high ARA group for both adult fish and their adult progeny.
- High dietary ARA levels compared to lower dietary ARA levels altered the n-3/n-6 PUFA ratio and the complex lipid profiles in juvenile zebrafish.
- High dietary ARA levels compared to lower dietary ARA levels were associated with an inflammatory and oxidized environment resulting in an anti-inflammatory and anti-oxidative response in juvenile zebrafish.
- Parental high dietary ARA changed hepatic DNA methylation and gene expression profiles in both mature parents and progeny, whereas the response on the hepatic gene expression level was less strong in the parents.
- We observed links between the hepatic gene expression and DNA methylation profiles: Genes encoding transcription regulators as well as nuclear and steroid receptors were connected to differential gene expression in the progeny and differentially methylated loci in the livers of both parents and progeny.
- Results from hepatic gene expression analysis from both parents and progeny suggest that parental diet prior to fertilization (nutrient composition of the gametes) and during maternal-to-zygotic transition in early embryonic development was priming adult gene expression.

8. Future perspectives

Based upon the results from hepatic gene expression and DNA methylation analysis of both F_0 and F_1 zebrafish, it is conceivable that parental diet was "programming" adult gene expression patterns through different plausible nodes, such as nutrient composition of the gametes or early embryonic development. Considering the unexpected small similarity of the hepatic transcriptomic patterns between parents and progeny that both received the same diet, it is tempting to speculate that previous dietary treatments of the parents from the F_0 generation also influenced the results. Consequently, future feeding experiments should consider the dietary impact of generations previous to an experimental F_0 generation as nutrition is often an overlooked factor. Furthermore, analysis of transgenerational inheritance of dietary effects should be investigated also in F_2 and F_3 generation.

The present study demonstrated interesting results from hepatic transcriptomic and DNA methylation analysis, which have the potential to give new insight into the metabolic and thus phenotypic effects of increased dietary ARA. However, further investigations should elaborate metabolic profiles on different tissues such as liver, muscle, brain, gonads, intestine and other immunological organs in order to investigate physiological, thus phenotypic effects associated with the parental diet.

Based on our experiment, the link between hepatic gene expression profiles and DNA methylation is weaker than expected when studying livers. Comparison of DNA methylation patterns of more tissues with the liver methylome would give us an idea of how dynamic or conserved the DNA methylation patterns are between different tissues. We would also like to study changes in DNA methylation profiles during different developmental stages, and elucidate at what stage of development and in which tissue the DNA methylation changes the gene expression profile. As such, future studies are required to understand whether DNA methylation changes demonstrate a causal relationship between dietary profiles and gene expression regulation. Furthermore, an investigation of nutritional profiles, and both gene expression and

methylation patterns during development seems indispensable in order to achieve an understanding of mechanisms involved in transgenerational diet effects. It is plausible that both maternal and paternal dietary profiles contribute to the development of the progeny in one way or another. Further studies should investigate a gender-dependent transmission of dietary effects through either nutritional composition of the fertilized eggs or epigenetic mechanisms that allow to discriminate maternal or paternal contribution to the embryonic development. In order to increase the knowledge on the complexity of dietary effects, an investigation of other epigenetic mechanisms involved in gene expression regulation such as histone tail modification, chromatin remodelling or non-coding RNAs would be useful.

Source of data

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Paper I

Adam AC, Lie KK, Moren M, Skjaerven KH.

High dietary arachidonic acid levels induce changes in complex lipids and immune-related eicosanoids and increase levels of oxidised metabolites in zebrafish (*Danio rerio*)

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High dietary arachidonic acid levels induce changes in complex lipids and immune-related eicosanoids and increase levels of oxidised metabolites in zebrafish (*Danio rerio*)

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Abstract

This study explores the effect of high dietary arachidonic acid (ARA) levels (high ARA) compared with low dietary ARA levels (control) on the general metabolism using zebrafish as the model organism. The fatty acid composition of today's 'modern diet' tends towards higher *n*-6 PUFA levels in relation to *n*-3 PUFA. Low dietary *n*-3:*n*-6 PUFA ratio is a health concern, as *n*-6 PUFA give rise to eicosanoids and PG, which are traditionally considered pro-inflammatory, especially when derived from ARA. Juvenile zebrafish fed a high-ARA diet for 17 d had a lower whole-body *n*-3:*n*-6 PUFA ratio compared with zebrafish fed a low-ARA (control) diet (0·6 in the control group *v.* 0·2 in the high-ARA group). Metabolic profiling revealed altered levels of eicosanoids, PUFA, dicarboxylic acids and complex lipids such as glycerophospholipids and lysophospholipids as the most significant differences compared with the control group. ARA-derived hydroxylated eicosanoids, such as hydroxy-eicosatetraenoic acids, were elevated in response to high-ARA feed. In addition, increased levels of oxidised lipids and amino acids indicated an oxidised environment due to *n*-6 PUFA excess in the fish. To conclude, our results indicate that an ARA-enriched diet induces changes in complex lipids and immune-related eicosanoids and increases levels of oxidised lipids and amino acids, suggesting oxidative stress and lipid peroxidation.

Key words: Metabolomics: Zebrafish: Arachidonic acid: Eicosanoids: Oxidative stress

Today's 'modern diet', with an increased consumption of saturated fat, meat and vegetable oil, and a decreased consumption of fish and fresh vegetables, has led to a selective loss of *n*-3 PUFA in favour of *n*-6 PUFA, which results in a decreased *n*-3:*n*-6 PUFA ratio[1,2]. The physiological effects of a decreasing *n*-3:*n*-6 PUFA ratio are diverse, and epidemiological studies indicate that a disproportionally high intake of *n*-6 PUFA may contribute to health problems like the metabolic syndrome, diabetes, obesity, CVD, cancer and other inflammatory, neurodegenerative or autoimmune diseases[3–8]. Generally, high total fat intake increases the risk for health problems according to the 2008 FAO/WHO report[9,10].

The general view is that the above-mentioned health effects of high-*n*-6 PUFA intake are caused by the potent bioactive metabolic products of PUFA. Essential PUFA, like arachidonic acid (ARA) and EPA are converted to numerous bioactive lipid classes, collectively known as oxylipins (oxidation products of ARA and EPA)[11]. Oxylipin and ARA levels can be influenced by the diet directly; however, ARA conversion to eicosanoids is a rate-limiting enzymatic process[12]. Biological functions of those oxylipins, and especially eicosanoids, are traditionally considered anti-inflammatory when derived from *n*-3 PUFA and pro-inflammatory when derived from *n*-6 PUFA. This knowledge evoked focus on the risks and benefits of PUFA consumption[13,14]. On the contrary, Calder[15] emphasised that labelling ARA-derived eicosanoids as pro-inflammatory is an oversimplification because of the fact that consumption of *n*-6 PUFA can have variable effects on physiology, with both anti- and pro-inflammatory responses[16,17]. ARA-derived PG (2-series) induce inflammation, inhibit pro-inflammatory leukotrienes and cytokines, and induce anti-inflammatory lipoxins[18,15].

ARA-derived eicosanoids have been studied intensively, and ARA is widely discussed in the context of signalling cascades regulating inflammation, pain, fever and other homoeostatic actions such as blood pressure, bone metabolism, growth and reproduction[19–23]. These biological functions are traditionally attributed to the immunomodulating lipid mediators such as ARA-derived hydroxy-eicosatetraenoic acids (HETE)[24], PG, thromboxanes and leukotrienes[6,15]. The variety of lipid mediators that regulate physiological functions makes it difficult

Abbreviations: 4-HNE, 4-hydroxy-nonenal; ARA, arachidonic acid; HETE, hydroxy-eicosatetraenoic acids.

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to elucidate their individual biological roles[11]. Fatty acids and eicosanoids exert their biological function by changing cell membrane composition and by controlling gene expression through nuclear receptors like PPAR, hepatocyte nuclear factor 4α and liver X receptor, and through transcription factors such as NF-κB and sterol-regulatory-element-binding protein[13,24–27]. These nuclear receptors are regulated by direct fatty acid- and eicosanoid-binding, or by the regulation of G-protein-linked cell surface receptors, thereby activating signalling cascades[24,26–28].

The zebrafish is an omnivorous, tropical, freshwater fish, and a well-suited model organism for understanding vertebrate metabolism at a molecular and genetic level[29–32]. Knowledge gained from zebrafish studies are highly relevant to both humans[33] and fish[34,35]. The focus on the essential fatty acid ARA in fish nutrition is rising, and its impact on health performance and reproduction in both marine and saltwater species is gaining more attention[36–40]. Most of the studies on the nutritional effects of ARA on zebrafish and other fish have focused on stress response, survival, bone development, deformities, reproduction and growth performance[22,23,41–43]. These studies demonstrate the importance of appropriate dietary ARA levels for maintaining optimal growth, reproduction and overall health, with special emphasis on larval fish requirements[36]. Powell *et al.*[44] investigated the role of ARA and different dietary *n*-3:*n*-6 PUFA ratios in inflammation in zebrafish. They measured key inflammatory markers, growth and body fat in adult zebrafish in response to dietary *n*-3:*n*-6 PUFA ratios. These results indicate that a low *n*-3:*n*-6 PUFA ratio can impact health through metabolic changes when high levels of ARA are provided through diet.

In the present study, we fed zebrafish a diet high or low in ARA. We elucidated the metabolic changes in zebrafish induced by a dietary shift in PUFA composition. The dietary levels of ARA fed to the high-ARA group were chosen to provoke the metabolism. We aimed to study the metabolic processes that could explain the effect that others have shown when dietary *n*-3:*n*-6 PUFA ratio changes. Thereby, we can point the effect to ARA and not its precursors. We used metabolomics to investigate the manifoldness of changes in response to feeding high dietary ARA levels for 17 d during the extensive growth period from the larval stage at 27 d post fertilisation (DPF) until juvenile stage (44 DPF). We found that high ARA levels contribute to a strong shift in lipid metabolism involving significant lipid mediators, which suggests an impact on physiological functions and challenges the redox environment in the fish.

Methods

Ethical considerations

The feeding experiment was approved by the Norwegian Animal Research Authority and was conducted according to current animal welfare regulations in Norway: FOR-1996-01-15-23. Facilities for zebrafish husbandry were optimally equipped to ensure refinement of breeding, accommodation and care. Handling and treatment of the fish ensured reduction of any possible pain, distress or lasting harm to the fish.

Table 1. Feed composition

Ingredients	Control (g/kg DM)	High-ARA (g/kg DM)
Protein blend*	767·9	767·9
Agar†	1·0	1·0
Fish oil‡	8·0	8·0
Rape seed oil§	48·0	20·0
Flax seed oil§	20·0	4·0
Cargill's ARA-rich oil‖	4·0	48·0
Dextrin†	46·17	46·17
Cellulose¶	19·3	19·3
Lecithin**	20·0	20·0
Mineral mix††	50·0	50·0
Vitamin mix‡‡	10·0	10·0
Methionine§§	2·5	2·5
Cyanocobalamin(1 %)‖‖	0·99998	0·99998
Folic acid (97 %)‖‖	0·0111	0·0111
Pyridoxine hydrochloride‖‖	0·0199	0·0199
Astaxanthin¶¶	0·3	0·3
Sucrose†	1·0	1·0
Tocopherol mix***	0·75	0·75

ARA, arachidonic acid.
* BioMar AS products: fishmeal, 5 %; krill meal, 1 %; soya protein concentrate, 6·2 %; maize, 5 %; wheat, 7·5 %; wheat gluten, 13 %; pea protein, 49·8 %; field peas, 12·5 %.
† Dissolved in 200 ml heated Milli-Q water; Sigma Aldrich Norway AS.
‡ Cod liver oil; Møllers, Axellus AS.
§ Rømer Produkt.
‖ Donated by Cargill (40 % ARA; Alking Bioengineering).
¶ Sigma-Aldrich.
** Alfa Aesar.
†† Merck; ingredients (g/kg of diet): $CaHPO_4 \cdot 2H_2O$, 30; $CoCl_2 \cdot 6H_2O$, 0·007; $CuSO_4 \cdot 5H_2O$, 0·02; K_2SO_4, 15; KI, 0·05; $MgSO_4 \cdot 7H_2O$, 5; $MnSO_4 \cdot H_2O$, 0·05; NaCl, 2·873; Se-yeast, 0·2; $ZnSO_4 \cdot 7H_2O$, 0·5; $FeSO_4 \cdot 7H_2O$, 0·6.
‡‡ Obtained from Vilomix Norway AS, Norway; without cyanocobalamin, folic acid and pyridoxine hydrochloride (vitamin B_6) because of the trial set up with two directions (mg/kg of diet): vitamin A, 20; vitamin D, 4; vitamin E (50 %, acetate), 200; vitamin K (50 %), 10; vitamin C (35 %, phosphate), 350; choline, 1000; ascorbic acid, 1000; thiamine hydrochloride, 15; riboflavin (80 %), 19; nicotinamide, 200; inositol, 400; calcium pantothenate, 60; biotin (2 %), 50; filler (protein blend), 6672.
§§ Sigma-Aldrich.
‖‖ Normin AS.
¶¶ Dissolved in the agar solution; provided as a gift from G.O. Johnsen AS.
*** Provided as a gift from BASF.

Formulation and preparation of diets

The feeding experiment and diets were made as previously described in detail[45]. In short, we fed zebrafish a control diet with low ARA levels (ARA 0·19 % of DM), or an experimental diet with high ARA levels (ARA 2·10 % of DM), referred to as high-ARA diet. The ARA levels were chosen on the basis of previous studies on fish to avoid deficient levels in the low-ARA group[22,46]. Protein, mineral and vitamin blends as well as oil composition are provided in Table 1. Initially, all feed ingredients were mixed (protein, mineral and vitamin blends, and oil including fish oil, rape seed oil, flax seed oil and Cargill's ARA-rich oil) with a solution of dissolved agar until a smooth texture was achieved. Astaxanthin was added to the agar solution before mixing with other feed ingredients. The feed paste was dried at 42°C for 72 h, ground and sieved into fractions of different feed-pellet sizes and stored at–20°C until feeding. The feeding regimen was as follows: <200 µm fed 27–43 DPF, 350 µm fed 44–57 DPF and 560 µm fed 58–90 DPF.

Experimental setup

Zebrafish AB strain (*Danio rerio*) were handled and fed as previously described[45]. In short, zebrafish embryos were

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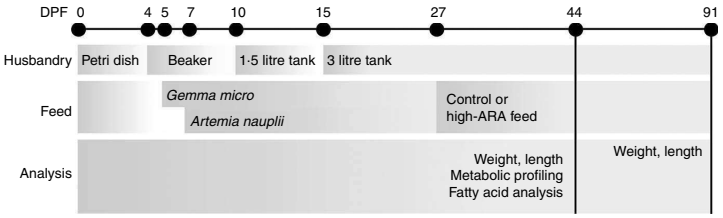

Fig. 1. Experimental design. Zebrafish were fed *Gemma micro* and *Artemia nauplii* as start feed from 5 and 7 d post fertilisation (DPF), respectively. The experimental feeds, control or high arachidonic acid (ARA), were given to ten replicate tanks for each feed from 27 DPF onwards. Weight and length were measured at 44 and 91 DPF. Metabolic profiling and fatty acid analysis were performed at 44 DPF.

collected randomly and incubated in Petri dishes. At 4 DPF they were transferred to beakers with sixty larvae (Fig. 1). Zebrafish were fed twice a day from 5 DPF with dry feed (Gemma micro®; Skretting) in addition to *Artemia nauplii* (*Artemia*; Silver Star) from 7 DPF until 27 DPF. At 15 DPF, larvae were randomly transferred into 3 litre tanks in a reverse osmosis water treatment system (Aquatic Habitats recirculation system). For each diet group we assigned ten sex-mixed 3 litre tanks containing sixty fish. Each tank represents one biological replicate. Both the control and the high-ARA diet were given from 27 DPF onwards, twice a day, until 90 DPF. Fish were fed *ad libitum* from 27–43 DPF, and thereafter from 44 DPF with a restrictive diet of 7 % of the tank total biomass[45]. Fish were kept under steadily monitored standard conditions with 28 ± 1°C, 14 h light–10 h dark period, conductivity of 500 µS and pH 7·5.

Sampling and growth measures

Fish were deprived of food 18 h before sampling. In all, 44 and 91 DPF zebrafish were anaesthetised with 0·05 % tricaine methanesulfonate (MS-222; Metacain) before weighing, standard length measuring and sampling. Anaesthetised whole fish were snap-frozen with liquid N_2 and stored at –80°C for fatty acid and metabolic profiling.

Fatty acid analysis in feed and fish

Fatty acid composition of the dried diets and 44 DPF whole fish was determined on isolated fatty acid fractions as previously described by Jordal *et al.*[47], modified by Lie & Lambertsen[48] using GLC. Quantification of fatty acids was done using 19 : 0 as the accredited internal standard and integration of peak areas was done using Dionex Chromeleon (version 7.1.3.2425). Fatty acid quantification was done in whole-44-DPF-fish homogenates, where three parallels of twenty pooled individuals were made by combining two tanks.

Metabolic profiling

Targeted metabolic analysis involving high-throughput characterisation of all detectable metabolites was performed by Metabolon®, Inc. in order to examine the effect of higher ARA levels on the general metabolism in 44 DPF zebrafish. Six parallels consisting of forty pooled individuals in each parallel were sent for analysis. Targeted metabolic profiling measures defined groups of characterised metabolites in a quantitative manner using internal standards. All applied methods used Waters ACQUITY Ultra HPLC and Thermo Scientific Q-Exactive high-resolution/accurate MS interfaced with a heated electrospray ionisation source and Orbitrap mass analyser operated at 35 000 mass resolution. Sample preparation, extraction and metabolite identification was as described by Skjærven *et al.*[45]. Compounds were identified by comparison with library entries of purified standards by Metabolon®. Values of detected compounds of known identity were normalised to the Bradford protein concentration, log-transformed and described as intensity scale.

Statistical analysis

Data visualisation and statistical significance testing of weight, length and fatty acid analysis data were performed using GraphPad Prism version 6.00. Weight and length data were analysed using a non-parametric test (Mann–Whitney test) as none of the data showed Gaussian distribution, except weight data at 91 DPF, which was analysed by an unpaired, parametric *t* test with Welch's correction. Fatty acid levels are presented as mean values and standard deviations and tested with an unpaired *t* test to reveal significant differences between the feed groups. Statistical significance was generally accepted at $P < 0{\cdot}05$.

For metabolic profiling, each value got rescaled to set the median = 1, and got described as scaled intensity in tables and figures. Missing values in one sample were assumed to be below the detection limit and were imputed with the minimum value from other samples for subsequent statistical analysis. Welch's two-sample *t* test (ArrayStudio; Omicsoft) was used on log-transformed data to identify significant ($P < 0{\cdot}05$) metabolites with pairwise comparison between the two feed groups. Calculation of the false discovery rate (*q* value) took into account multiple comparisons that occur in metabolic-based studies by using a cut-off point ($q \leq 0{\cdot}05$) for indication of high confidence in a result. Relative fold changes, termed as mean ratios (MR), were calculated from high-ARA to control group using group averages of the scaled intensity values. Scaled data are presented in the online Supplementary Table S1 as a pathway heat-map including group averages of the scaled intensity values, MR, *P* and *q* values from statistical testing. Data visualisation was done using GraphPad Prism. The MetaboLync

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Cytoscape Plugin was used to calculate sub-pathway enrichment. Pathway enrichment scores determine the number of statistically significant regulated compounds (*k*) relative to all detected compounds (*m*) in a pathway, compared with the total number of significant regulated compounds (*n*) relative to all detected compounds (*N*) in the analysis: (*k*/*m*)/(*n*/*N*).

Results

High dietary arachidonic acid affected weight but not length

Dietary high ARA levels had a slight effect only at 44 DPF, where the high-ARA group was significantly lighter ($P=0{\cdot}04$) compared with the control group (Table 2). At this stage, both feed groups show a large weight variation. At 91 DPF we observed no differences in weight and length (Table 2).

High dietary arachidonic acid affected fatty acid profiles

Feeding of experimental diets for 17 d changed the fatty acid profiles in 44 DPF zebrafish. The ratios of *n*-3:*n*-6 PUFA with control and high-ARA feed were 0·6 and 0·2, respectively (Table 3). GLC analysis revealed six times higher ARA concentrations in the high-ARA group compared with the control group, accompanied by significantly elevated levels of its elongated and desaturated products adrenate (22 : 4*n*-6) and *n*-6 DPA (22 : 5*n*-6). Fatty acid levels are presented in Table 3 (full list of analysed fatty acids is given in the online Supplementary Table S2 (feed) and Table S3 (fish)). Oleic acid (18 : 1*n*-9) and linoleic acid (18 : 2*n*-6) were the most abundant fatty acids in both feed groups. Feeding the high-ARA diet, which consisted of relatively lower amounts of rape-seed and flax-seed oil, compared with the control feed (control 6·8 %; high ARA 2·4 %), resulted in lower levels of α-linolenic acid

Table 2. Weight and length measures†
(Mean values and standard deviations)

	Weight (mg)							Length (cm)						
	Control			High-ARA				Control			High-ARA			
DPF	Mean	SD	*n*‡	Mean	SD	*n*‡	*P*	Mean	SD	*n*‡	Mean	SD	*n*‡	*P*
44	50·72	27·43	57	40·23	25·26	48	0·04*	1·29	0·28	57	1·23	0·28	48	0·27
91	265·0	99·0	49	266·4	92·3	48	0·94	2·35	0·29	49	2·35	0·28	48	0·89

ARA, arachidonic acid; DPF, days post fertilisation.
Statistically significant: * $P<0{\cdot}05$.
† Statistical significance analysis was done by non-parametric Mann–Whitney test, except for 91 DPF (weight) which was analysed by a parametric *t* test with Welch's correction. 44 DPF weight and length data and 91 DPF length data do not follow a Gaussian distribution.
‡ *n* are individual fish originated from different populations (tanks) which got summarised within the feed group for subsequent statistical analysis.

Table 3. Fatty acid profiles (selected) of feed and zebrafish fed for 17 d with either control or high-arachidonic acid (ARA) feed
(Mean values and standard deviations)

	Feed (mg fatty acid/g feed)‡		Zebrafish (mg fatty acid/g fish)†				
			Control		High-ARA		
	Control	High-ARA	Mean	SD	Mean	SD	*P*
18 : 1*n*-9 oleic acid	43·32	27·24	18·59	4·14	12·31	0·35	0·058
18 : 2*n*-6 linoleate	31·27	26·80	10·61	2·13	8·58	0·21	0·177
18 : 3*n*-3 α-linolenate	17·50	6·43	4·17	0·88	1·56	0·06	0·007**
18 : 4*n*-3 stearidonate	0·25	0·25	0·16	0·05	0·07	0·01	0·035*
20 : 3*n*-6 dihomo-linolenate	0·17	1·79	0·48	0·1	0·87	0·02	0·002**
20 : 4*n*-6 ARA	1·87	20·66	1·04	0·16	5·74	0·13	<0·001***
20 : 5*n*-3 EPA	1·26	1·3	0·47	0·08	0·32	0·02	0·038*
22 : 4*n*-6 adrenate	0·05	0·14	0·06	0·01	0·35	0·0	<0·001***
22 : 5*n*-6 docosapentaenoate (*n*-6 DPA)	0·05	0·04	0·09	0·03	0·56	0·03	<0·001***
22 : 5 *n*-3 docosapentaenoate (*n*-3 DPA)	0·15	0·15	0·14	0·03	0·12	0·01	0·148
22 : 6 *n*-3 DHA	1·42	1·37	2·13	0·27	1·63	0·05	0·034*
Sum unidentified	1·27	1·55	0·61	0·1	0·61	0·01	0·996
Sum identified	121·00	118·00	52·77	10·86	47·3	1·08	0·435
Sum SFA	17·40	25·20	10·47	2·12	11·4	0·26	0·491
Sum MUFA	49·30	31·90	22·00	4·81	15·1	0·44	0·069
Sum PUFA	54·60	60·50	20·40	3·93	20·8	0·46	0·870
Sum EPA + DHA	2·68	2·67	2·60	0·35	1·94	0·06	0·033*
Sum *n*-3 PUFA	20·90	9·75	7·57	1·41	4·08	0·11	0·013*
Sum *n*-6 PUFA	33·60	50·70	12·67	2·52	16·60	0·36	0·055
n-3:*n*-6	0·6	0·2	0·6	0·0	0·2	0·0	

Statistically different mean values between the control and the high-ARA group were determined using unpaired *t* test using GraphPad Prism. * $P<0{\cdot}05$, ** $P<0{\cdot}01$, *** $P<0{\cdot}001$.
† Mean is calculated for three biological replicates consisting of twenty pooled 44 DPF zebrafish/replicate.
‡ Data are expressed as the mean of two technical replicates.

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(ALA; 18 : 3*n*-3) in the high-ARA group compared with the control group. Although EPA and DHA (22 : 6*n*-3) levels were balanced in the two feeds, there was a difference in EPA and DHA levels between the control and the high-ARA group. High dietary ARA levels contributed to an altered *n*-3:*n*-6 PUFA ratio of 0·6 in the control group compared with 0·2 in the high-ARA group, indicating higher *n*-6 PUFA levels and lower *n*-3 PUFA levels in the high-ARA group.

General functional characterisation of detected metabolites from metabolic profiling

In total, 153 out of 566 detectable compounds from metabolic profiling were statistically different between the feed groups, using Welch's two-sample *t* test ($P<0{\cdot}05$; $q<0{\cdot}05$; online Supplementary Table S1). Principal component analysis shows a clear separation of the samples according to the feed groups (online Supplementary Fig. S1). The majority of metabolites affected by ARA belong to the lipid and amino acid main pathway. The lipid main pathway shows the highest count (65 %) for statistically significant metabolites among all significantly different biochemicals (Fig. 2(a)). The high-ARA group yielded a shift in lipid metabolism, especially in PUFA, complex lipids and ARA-derived eicosanoids. Moreover, a total of six sub-pathways related to lipid metabolism were enriched in the high-ARA group (Fig. 2(b)). The entire set of enriched sub-pathways is given in the online Supplementary Table S4.

High dietary arachidonic acid affected lipid metabolism

The high-ARA group revealed higher levels of eicosanoids, which obtained the maximum enrichment score (Fig. 2(b)). Among those, ARA-derived HETE like 5-HETE (3-fold increase), 12-HETE (6·5-fold increase) and 5-keto-eicosatetraenoic acid (5-KETE; 4-fold increase) were significantly elevated in the high-ARA group (Fig. 3(a) and 4), whereas 5-hydroxy-EPA (Fig. 3(a) and 4) was four times decreased. The latter derives from the *n*-3 PUFA EPA (20 : 5*n*-3), which was decreased, as well as its precursors ALA (in the online Supplementary Table S1 referred to as salt of ALA: *α*-linolenate; 18 : 3*n*-3) and stearidonate (18 : 4*n*-3). DHA (22 : 6*n*-3) levels detected in metabolic profiling were not different between feed groups (Fig. 3(a) and (b)), whereas GLC analysis revealed decreased DHA levels in the high-ARA group compared with the control group (Table 3). The high-ARA group exhibited decreased *n*-3 PUFA and increased *n*-6 PUFA levels compared with the control group. The high-ARA group showed a 5-fold increase in ARA (in the online Supplementary Table S1 referred to as salt of ARA: arachidonate) and adrenate and six times higher *n*-6 DPA (22 : 5*n*-6) levels compared with the control group (Fig. 3(a) and (c)).

The high-ARA group showed increased levels of arachidonoyl-containing glycerophospholipids, plasmalogens and lysophospholipids, which are arachidonoyl-containing complex lipids. Linoleoyl (18 : 2*n*-6) as well as linolenoyl-containing (18 : 3*n*-3) glycerophospholipids and lysophospholipids were decreased in the high-ARA group. One glycerophospholipid, named 1-linoleoyl-2-arachidonoyl-GPC (18 : 2/20 : 4*n*-6), was predominant among all detected phospholipids as it exhibited twelve times higher levels in the high-ARA group. Sphingolipids, especially sphingomyelins showed significantly lower levels in the high-ARA group. Dicarboxylic acids such as 2-hydroxyadipate, maleate, suberate, azelate, sebacate and pimelate, and the ketone body 3-hydroxybutyrate were significantly increased in the high-ARA group. Higher ARA levels decreased both carnitine and acetylcarnitine as well as several acylcarnitines, but also increased *cis*-4-decenoyl carnitine and stearoylcarnitine.

High dietary arachidonic acid affected redox environment, vitamins, cofactors, carbohydrate and energy metabolism

ARA-derived endocannabinoids and monoacylglycerols like 2-arachidonoyl-glycerol and its inactive analogue 1-arachidonoyl-glycerol showed statistically higher levels in the high-ARA group.

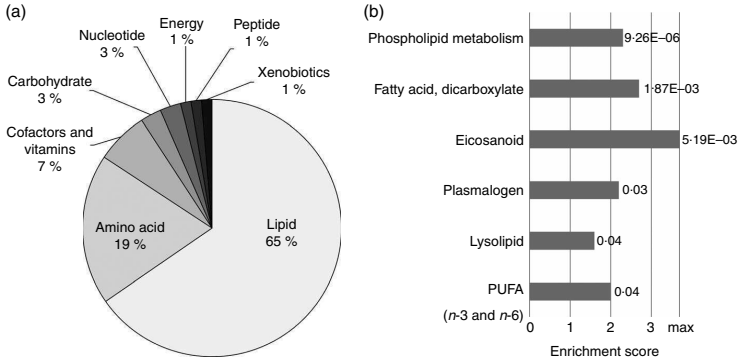

Fig. 2. Metabolic profiling revealed complex changes in lipid metabolism. (a) Proportional clustering shows statistically different metabolites (*n* 153, $P<0{\cdot}05$) affiliated to their main pathway. (b) Enrichment analysis revealed six significantly enriched sub-pathways. Scores underlie an enrichment of significantly different metabolites of total detected metabolites within a sub-pathway in relation to all significant metabolites to all detected metabolites. Graph shows statistically significant enriched ($P<0{\cdot}05$) sub-pathways according to their calculated enrichment scores with indicated *P* values. The maximum achievable enrichment score is 3·7 (marked with max), if all detected metabolites in a sub-pathway are described as statistical significant different. For all sub-pathway enrichment scores see the online Supplementary Table S4.

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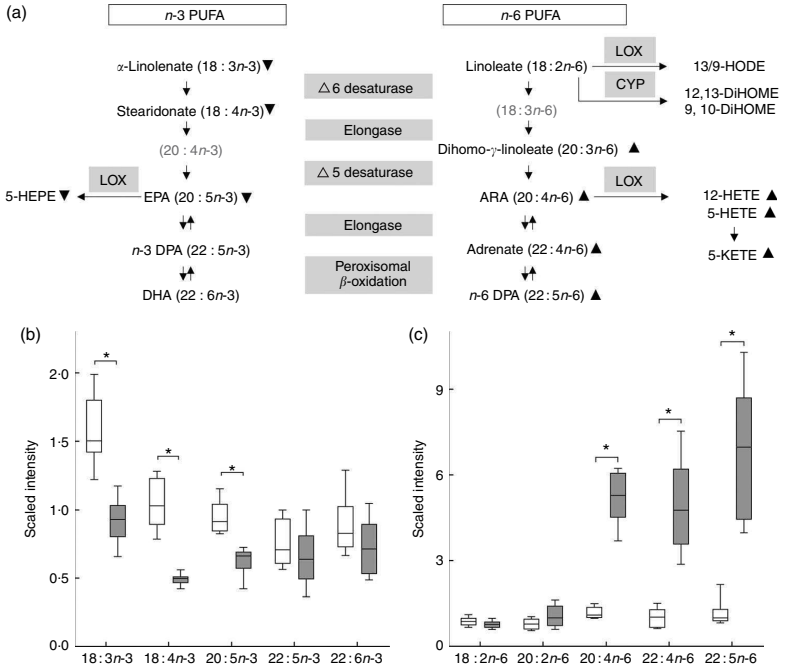

Fig. 3. Metabolic profiling revealed changes in PUFA synthesis. (a) Metabolites illustrated in the PUFA synthesis pathway with lipoxygenase (LOX) and cytochrome P450 (CYP)-derived eicosanoid classes. ▼, ▲, Statistically significant lower and higher metabolite levels in the high-arachidonic acid (ARA) group compared with the control group. Grey highlighted metabolites were not detected. (b) and (c) Box plots of normalised data expressed as scaled intensity of single *n*-3 and *n*-6 PUFA, respectively. □, Control; ▨, high-ARA; ARA, 20 : 4*n*-6; DHA, 22 : 6*n*-3; EPA, 20 : 5 *n*-3; *n*-3 DPA, 22 : 5 *n*-3; *n*-6 DPA, 22 : 5 *n*-6; 5-HEPE, 5-hydroxy-EPA; 5-HETE, 5-hydroxy-eicosatetraenoic acid; 12-HETE, 12-hydroxy-eicosatetraenoic acid; 5-KETE (5-oxo-ETE), 5-keto-eicosatetraenoic acid (5-oxo-eicosatetraenoic acid); 13/9-HODE, 13/9-hydroxy-octadecadienoic acid; 12,13-DiHOME, 12,13-dihydroxy-octadecenoic acid; 9,10-DiHOME, 9,10-dihydroxy-octadecenoic acid. * Significant difference ($P < 0{\cdot}05$) between feed groups (Welch's two-sample *t* test).

Moreover, *N*-stearoyl-taurine and *N*-palmitoyl-taurine, known as aminoacyl-endocannabinoids, had higher levels in response to higher *n*-6 PUFA levels as well. 7-hydroxy-cholesterol as well as oxidised products of amino acids and peptides like methionine-sulfoxide and *N*-acetyl-methionine-sulfoxide (oxidised products of methionine), cysteine *s*-sulfate (oxidised product of cysteine), cysteine-glutathione-disulfide and 4-hydroxy-nonenal (4-HNE)-glutathione (oxidised products of glutathione) were elevated in the high-ARA group. Cystine, the disulfide form of cysteine, was increased in the high-ARA group compared with the control group. Cysteine for glutathione synthesis was significantly decreased in the high-ARA group, whereas both GSH and GSSG decreased slightly (not significant; $0{\cdot}05 < P < 0{\cdot}1$) in the high-ARA group. In addition, ascorbate (ascorbic acid) and its oxidised derivatives like threonate and oxalate were increased in the high-ARA group. Urate levels were three times decreased and carnosine levels were 2·5 times decreased in the high-ARA group. *δ*-tocopherol levels were decreased, whereas *α*-/*β*-/γ-tocopherol levels were unaffected in the high-ARA group. Concurrently, pyridoxate and pyridoxamine showed lower levels in the high-ARA group, whereas pyridoxal and pyridoxamine phosphate were not different. Central metabolites related to glycolysis and gluconeogenesis (glucose-6-phosphate) and the pentose phosphate way (ribose-5-phosphate) showed lower levels in the high-ARA group. Concerning the TCA cycle, *α*-ketoglutarate and succinylcarnitine were significantly decreased, and malate was slightly (not significant; $P < 0{\cdot}1$) decreased in the high-ARA group.

Discussion

Metabolic profiling of zebrafish fed high-ARA levels revealed changes in complex lipids, fatty acid metabolism and immune-related eicosanoids, and suggests a challenged redox environment (Fig. 5).

Lipid metabolism

As predicted, increasing ARA and *n*-6 PUFA levels in the feed resulted in a lower *n*-3:*n*-6 PUFA ratio in the fish. Increased ARA levels gave rise to its elongated metabolites adrenate (22 : 4*n*-6) and *n*-6 DPA (22 : 5*n*-6), which is suggestive of increased peroxisomal *β*-oxidative degradation of very-long-chain fatty acids[49]. *β*-Oxidation is the main catabolic pathway for long-chain fatty acids; however, when capacity is overwhelmed, minor and alternative catabolic pathways such as

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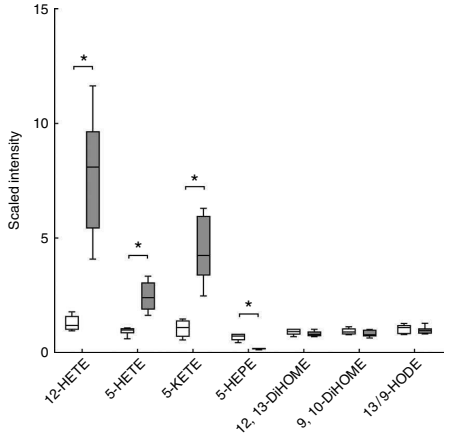

Fig. 4. Metabolic profiling revealed changes in eicosanoids derived from linoleic acid, EPA and arachidonic acid (ARA). Box plots of normalised data are expressed as the scaled intensity of single eicosanoids. 12-HETE, 12-hydroxy-eicosatetraenoic acid; 5-HETE, 5-hydroxy-eicosatetraenoic acid; 5-KETE (5-oxo-ETE), 5-keto-eicosatetraenoic acid (5-oxo-eicosatetraenoic acid); 5-HEPE, 5-hydroxy-EPA; 12,13-DiHOME, 12,13-dihydroxy-octadecenoic acid; 9,10-DiHOME, 9,10-dihydroxy-octadecenoic acid; 13/9-HODE, 13/9-hydroxy-octadecadienoic acid; □, control; ▨, high-ARA. * Significant difference ($P < 0{\cdot}05$) between feed groups (Welch's two-sample *t* test).

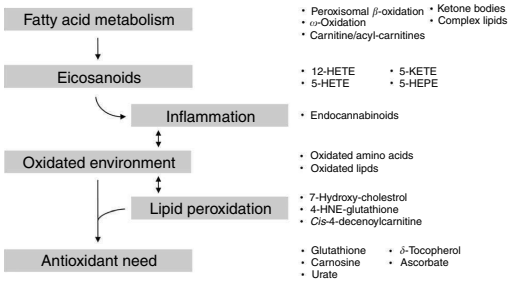

Fig. 5. High dietary arachidonic acid changed the metabolic fingerprint in zebrafish. Changes are characterised not only by a general change in lipid profiles and eicosanoids, but also by changed metabolites indicating inflammation and lipid peroxidation and changes in the antioxidant status. Arrows indicate the suggestive physiological conditions in the fish. Metabolites to which reference is made are given on the right side. 12-HETE, 12-hydroxy-eicosatetraenoic acid; 5-KETE (5-oxo-ETE), 5-keto-eicosatetraenoic acid (5-oxo-eicosatetraenoic acid); 5-HETE, 5-hydroxy-eicosatetraenoic acid; 5-HEPE, 5-hydroxy-EPA; 4-HNE-glutathione, 4-hydroxy-nonenal-glutathione.

ω-oxidation may become more important[50]. Little research has been done on the biological significance of fatty acid *ω*-oxidation as described by Miura [51]. Intermediates formed by *ω*-oxidation, such as dicarboxylic acids, accumulated in the high-ARA group, suggesting an overwhelmed *β*-oxidation. Furthermore, increased ketogenesis (3-hydroxybutyrate) and decreased carnitine and acetylcarnitine levels, as observed in the high-ARA group, are indicative of a challenged *β*-oxidation as well.

High dietary ARA levels affected fatty acid metabolism by favouring *n*-6 PUFA, but our results also highlight changes in complex lipid profiles (glycerophospholipids, plasmalogens and lysophospholipids). These changes are particularly characterised by increased levels of arachidonoyl-containing complex lipids following the high dietary ARA intake and suggesting subsequent incorporation of dietary fatty acids into complex lipids[52]. Changing the fatty acid composition of phospholipids can impact membrane and immune cell function in the fish. Composition of phospholipids can affect their affinity as substrates for enzymes which generate signalling molecules, and could contribute to the alteration of immune cell responsiveness as suggested by Calder & Grimble[53].

Eicosanoids

ARA can be metabolised by a variety of enzymes resulting in a complex mixture of biologically active derivatives (eicosanoids) with distinct functions[24]. The properties and precise roles of those eicosanoids are not fully understood in mammals, and even less is known for fish. In the present study, high dietary ARA gave rise to intermediate lipoxygenase products such as 5-HETE, 5-KETE and 12-HETE in the fish. 5-HETE can reversibly be converted to 5-KETE through an oxidation reaction, and oxidative stress could increase the conversion to 5-KETE[24,54]. ARA-derived eicosanoids (HETE) show a diversity in biological functions under different physiological and pathophysiological conditions[24]. As for PG and leukotrienes, HETE has also been described in context of inflammatory responses[15]. Several studies have shown that a disproportionally high intake of *n*-6 PUFA can promote inflammation, resulting in pathophysiological effects in humans[13,14,24]. However, it is suggested that *n*-6 PUFA and their metabolites are involved in both pro- and anti-inflammatory signalling pathways in mammals[15,16]. In the present study, abundant ARA could have contributed to the promotion of metabolically triggered inflammation, as suggested by others[55–57]. Particularly, altered fatty acid profiles can change eicosanoid production and can thereby impact physiological functions by altering the range of inflammatory and immune cell responses[53,58].

In the present study, we observed elevated levels of endocannabinoids such as *N*-stearoyltaurine, *N*-palmitoyltaurine and 2-arachidonoyl-glycerol[59]. These endocannabinoids are understood as anti-inflammatory molecules[60] induced by stress; especially, 2-arachidonoyl-glycerol plays an active role in ameliorating inflammation[61]. Similarly, Alvheim *et al.*[62] observed elevated levels of 2-arachidonoyl-glycerol in mice fed high levels of linoleic acid, an ARA precursor. Powell *et al.*[44] observed a decrease in chronic inflammatory response genes (C-reactive protein, serum amyloid A and vitellogenin) in zebrafish with decreasing dietary *n-3:n-6* PUFA ratio. Whether an ARA-stimulated increase in anti-inflammatory endocannabinoids in zebrafish and mice is part of a compensatory action induced by an increase in pro-inflammatory eicosanoids is not known. Despite these divergent observations, our results suggest an inflammatory challenged metabolism in response to a low dietary *n-3:n-6* PUFA ratio.

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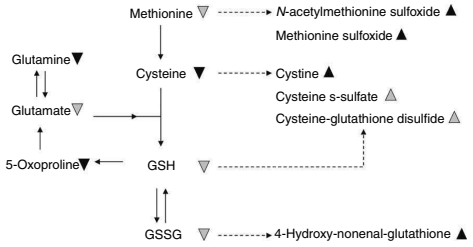

Fig. 6. High dietary arachidonic acid (ARA) affected the redox environment, characterised by increased oxidised amino acids in zebrafish. The observed effect suggests changes in the oxidation–reduction state, indicating oxidative stress and lipid peroxidation. ▼, ▲, Statistically significant ($P < 0{\cdot}05$) lower and higher metabolite levels in the high-ARA group compared with the control group. ▽, △, Lower and higher metabolites levels, which narrowly missed the statistical cut-off point for significance ($0{\cdot}05 < P < 0{\cdot}1$) in the high-ARA group.

Oxidative and antioxidative response

An inflammatory environment is often associated with antioxidative events as a consequence of a changing oxidised environment. In the high-ARA group, we observed distinct changes in the redox environment (Fig. 6) compared with the control group. Oxidised products of lipids (7-hydroxy-cholesterol), amino acids and peptides (*cis*-4-decenoylcarnitine, methionine-sulfoxide, *N*-acetylmethionine-sulfoxide, cysteine-*S*-sulfate, cysteine-glutathione-disulfide, 4-HNE-glutathione) were increased. Especially, increasing levels of cystine and decreasing levels of cysteine reflect changes in the oxidation–reduction state in the high-ARA group. Furthermore, increased methionine-sulfoxide levels resulting from methionine oxidation can have profound functional consequences for target proteins, especially when signalling protein residues are affected[63]. These results indicate that increasing dietary levels of *n*-6 PUFA resulted in changes in oxidising conditions in the fish. Oxidative stress can potentiate the possibility of systemic inflammation[7], which in turn affects the susceptibility for inflammation-underlying diseases[5,6].

Increased levels of oxidised lipids in the high-ARA group, like 7-hydroxy-cholesterol, *cis*-4-decenoylcarnitine and 4-HNE-glutathione, have been previously connected to oxidative stress and radical-mediated lipid peroxidation in other experiments[64–68]. There is evidence that an increasing fatty acid unsaturation (PUFA) correlates positively with peroxidisability of lipids[69]. In the present study, increased levels of 4-HNE-glutathione, which results from 4-HNE detoxification[70,71], suggest lipid peroxidation in the high-ARA group. 4-HNE is the major end product of reactive oxygen species-mediated peroxidation of membrane *n*-6 PUFA like ARA and linoleic acid in inflammation-related events[64,72].

Formation of these oxidised products triggered an antioxidant demand to prevent increasing oxidation as indicated in the high-ARA group. Interestingly, some metabolites with known antioxidative properties such as glutathione, carnosine, δ-tocopherol and urate[73] were decreased, whereas ascorbate[74] was increased in the high-ARA group. Zebrafish, like humans, depend on dietary uptake of ascorbate[75,76], which suggests an increased uptake from the feed rather than an increased endogenous synthesis of ascorbate in the high-ARA group. At the same time, decreased glutathione levels are consistent with an increasing demand for 4-HNE detoxification through glutathione. In cell-culture studies, increased conversion of ARA into HETE showed an increase in oxygen free radicals accompanied by glutathione depletion (GSH) that was leading to cellular damage[77]. Taken together, increasing *n*-6 PUFA availability in high-ARA fish led to an antioxidative response due to an enrichment of several oxidative products originating from enzymatic and non-enzymatic oxidation, indicating increasing lipid-peroxidation.

Zebrafish growth

We observed a slight weight difference in juvenile zebrafish (only 44 DPF) between the feed groups. Developmental processes during larval and juvenile stages are especially sensitive to a dietary imbalance[78]. Higher susceptibility to dietary imbalances during larval and early juvenile stages might have contributed to the growth effect we observed in 44 DPF fish that disappeared later at the adult stage. Although we do not know the mechanisms behind the growth recovery, compensatory growth has been demonstrated in fish following food deprivation[79]. Lie *et al.*[23] and de Vrieze *et al.*[22] observed no growth effect on cod larvae and zebrafish, respectively, in response to high dietary ARA levels. Boglino *et al.*[46] showed that both too-high and too-low *n*-6 PUFA levels did affect the growth of Senegalese sole larvae. Meinelt *et al.*[42] showed a positive correlation of higher dietary *n*-6 PUFA levels with growth in zebrafish, just as other animal and human studies show an association between higher *n*-6 PUFA intake and weight gain[46,80]. Different nutritional composition of the diets, like single fatty acid balance, magnitude of the dietary *n*-3:*n*-6 PUFA ratio, minerals and vitamins, might explain the difference in growth.

Conclusions

We have shown that high dietary ARA levels dramatically affect *n*-3:*n*-6 PUFA profiles, especially ARA-derived eicosanoids, which can greatly impact physiologic outcomes in the fish. Lipid peroxidation and an oxidised and pro-inflammatory environment, as implicated by our results, may result from both high *n*-6 PUFA availability and a shift in ARA-derived pro- and anti-inflammatory eicosanoids in zebrafish. The effect was characterised not only by a general change in lipid profiles and eicosanoids, but also by changed metabolites, indicating lipid peroxidation, oxidation of amino acids and changes in antioxidant status. However, the link between a low dietary *n*-3:*n*-6 PUFA ratio, elevated eicosanoid and endocannabinoid levels, and the regulation of the redox and immune system needs to be further studied, which is required to elucidate the underlying mechanisms. To our knowledge, the present study is the first to use metabolomics to reveal the metabolic fingerprint of high dietary ARA levels in teleosts. Previous studies using metabolic profiling have focused on the involvement of ARA pathways in vascular endothelial cells and CVD[81–83]. We find that zebrafish can be a useful vertebrate model to study the impact of nutrients on the manifoldness of the metabolic fingerprint.

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Our results from juvenile zebrafish highlight the metabolic fingerprint shaped by a specific diet.

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K. K. L., K. H. S. and M. M. designed the study. K. H. S., K. K. L. and M. M. performed the experiment. A.-C. A., K. H. S. and K. K. L. performed the analyses. A.-C. A. analysed the data. A.-C. A., K. K. L., K. H. S. and M. M. wrote the article.

None of the authors has any conflicts of interest to declare.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114517000903

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Supplementary material for:

High dietary arachidonic acid levels induce changes in complex lipids and immune-related eicosanoids and increase levels of oxidised metabolites in zebrafish (*Danio rerio*).

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Table S1. Pathway heat-map of scaled metabolic profiling data

Please visit http://vedlegg.uib.no/?id=4782d82b2ac351fcd673fcf4ad466dc7 to access the file.

Table S2. Fatty acid profile (mg fatty acid/g feed) of control and high ARA feed given to juvenile zebrafish from 27 until 90 DPF measured by GLC

Fatty acids (mg fatty acid/g feed)	Control feed [a]	High ARA feed [a]
06 : 0	0·01	0·01
08 : 0	0·01	0·01
10 : 0	0·03	0·04
12 : 0	0·01	0·01
14 : 0	0·69	0·84
14 : 1*n*-9	0·02	0·02
15 : 0	0·13	0·17
16 : 0	11·14	11·84
16 : 1*n*-9	0·09	0·06
16 : 1*n*-7	0·97	0·95
17 : 0	0·11	0·16
16 : 2*n*-4	0·07	0·07
18 : 0	3·78	5·24
16 : 3*n*-3	0·08	0·08
18 : 1*n*-11	0·16	0·01
18 : 1*n*-9	43·32	27·24
18 : 1*n*-7	2·40	1·45
16 : 4*n*-3	0·08	0·05
18 : 2*n*-6 (LA)	31·27	26·80
18 : 3*n*-6	0·10	1·06
20 : 0	0·58	0·67
18 : 3*n*-3 (ALA)	17·50	6·43
20 : 1*n*-11	0·08	0·08
20 : 1*n*-9	1·52	1·28
20 : 1*n*-7	0·03	0·03
18 : 4*n*-3	0·25	0·25
20 : 2*n*-6	0·11	0·22
20 : 3*n*-9	0·01	0·01
20 : 3*n*-6	0·17	1·79
22 : 0	0·37	1·61
20 : 3*n*-3	<0·01	<0·01
20 : 4*n*-6 (ARA)	1·87	20·66
22 : 1*n*-11	0·53	0·56
22 : 1*n*-9	0·12	0·04
20 : 4*n*-3	0·06	0·07
20 : 5*n*-3 (EPA)	1·26	1·30
24 : 0	0·58	4·62
22 : 4*n*-6	0·05	0·14
21 : 5*n*-3	0·04	0·02
24 : 1*n*-9	0·09	0·14
22 : 5*n*-6	0·05	0·04
22 : 5*n*-3	0·15	0·15
22 : 6*n*-3 (DHA)	1·42	1·37

24 : 5*n*-3	0·03	0·02
24 : 6*n*-3	0·01	0·01
Sum unidentified	1·27	1·55
Sum identified	121	118
Sum fatty acids	123	119
Sum saturated	17·4	25·2
Sum 16 : 1	1·06	1·02
Sum 18 : 1	45·9	28·7
Sum 20 : 1	1·63	1·40
Sum 22 : 1	0·643	0·596
Sum monounsaturated	49·3	31·9
Sum EPA + DHA	2·68	2·67
Sum *n*-3 PUFA	20·9	9·75
Sum *n*-6 PUFA	33·6	50·7
Sum polyunsaturated	54·6	60·5
(*n*-3) / (*n*-6)	0·6	0·2

[a] Data expressed as mean from two technical replicates.

Table S3. Fatty acid profile in 44 DPF zebrafish measured by GLC

Fatty acid (mg fatty acid/g fish)	Control		High ARA		Control vs. High ARA
	Mean	SD	Mean	SD	*P*
06 : 0					
08 : 0					
10 : 0					
12 : 0	0·017	0·006	0·020	0·000	0·373901
14 : 0	0·343	0·080	0·383	0·015	0·443939
14 : 1*n*-9	0·027	0·006	0·023	0·006	0·518519
15 : 0	0·140	0·026	0·140	0·000	1
16 : 0	7·323	1·528	7·370	0·205	0·96069
16 : 1*n*-9	0·313	0·057	0·260	0·010	0·184849
16 : 1*n*-7	0·897	0·196	0·813	0·025	0·504673
17 : 0	0·230	0·046	0·233	0·006	0·906554
16 : 2*n*-4	0·133	0·031	0·113	0·006	0·327635
18 : 0	2·153	0·392	2·580	0·060	0·13591
16 : 3*n*-3	0·080	0·020	0·073	0·006	0·608653
18 : 1*n*-11	0·060	0·000	0·040	0·000	
18 : 1*n*-9	18·593	4·136	12·310	0·349	0·0586939
18 : 1*n*-7	1·297	0·252	0·960	0·010	0·0815588
16 : 4*n*-3					
18 : 2*n*-6 (LA)	10·610	2·132	8·583	0·205	0·176561
18 : 3*n*-6	0·230	0·066	0·347	0·012	* 0·0385952
20 : 0	0·103	0·015	0·103	0·006	1
18 : 3*n*-3 (ALA)	4·167	0·884	1·557	0·055	** 0·00695511
20 : 1*n*-11	0·097	0·025	0·080	0·010	0·34649
20 : 1*n*-9	0·500	0·105	0·380	0·017	0·12343
20 : 1*n*-7					
18 : 4*n*-3	0·163	0·050	0·070	0·010	* 0·0345059
20 : 2*n*-6	0·167	0·032	0·170	0·010	0·872158
20 : 3*n*-9					
20 : 3*n*-6	0·480	0·095	0·870	0·020	** 0·00227562
22 : 0	0·083	0·012	0·207	0·006	*** 7·81238E-05
20 : 3*n*-3	0·010	0·000	0·077	0·006	*** 0·000584356

20 : 4*n*-6 (ARA)	1·043	0·155	5·743	0·127	*** 2·19531E-06
22 : 1*n*-11	0·123	0·021	0·193	0·015	** 0·00933784
22 : 1*n*-9	0·017	0·006	0·013	0·006	0·518519
20 : 4*n*-3	0·230	0·050	0·100	0·000	* 0·010795
20 : 5*n*-3 (EPA)	0·467	0·081	0·320	0·020	* 0·0379966
24 : 0	0·077	0·015	0·347	0·015	*** 2·69348E-05
22 : 4*n*-6	0·060	0·010	0·350	0·000	*** 9·40091E-07
21 : 5*n*-3	0·027	0·006	0·040	0·000	* 0·0161301
24 : 1*n*-9	0·043	0·006	0·040	0·000	0·373901
22 : 5*n*-6	0·087	0·025	0·557	0·031	*** 3·30109E-05
22 : 5*n*-3	0·143	0·025	0·117	0·006	0·148148
22 : 6*n*-3 (DHA)	2·127	0·269	1·627	0·049	* 0·0338107
24 : 5*n*-3	0·047	0·006	0·033	0·006	* 0·0474207
24 : 6*n*-3	0·113	0·021	0·063	0·006	* 0·016011
Sum unidentified	0·606	0·099	0·607	0·012	0·995674
Sum identified	52·767	10·862	47·300	1·082	0·434632
Sum fatty acids	53·400	10·967	47·900	1·082	0·436115
Sum saturated	10·467	2·120	11·400	0·265	0·491322
Sum 16 : 1	1·211	0·260	1·070	0·010	0·401807
Sum 18 : 1	19·933	4·406	13·333	0·321	0·0608359
Sum 20 : 1	0·596	0·133	0·463	0·032	0·169408
Sum 22 : 1	0·145	0·028	0·210	0·017	* 0·0275717
Sum monounsaturated	22·000	4·812	15·100	0·436	0·0687074
Sum EPA + DHA	2·597	0·350	1·943	0·064	* 0·0334292
Sum *n*-3 PUFA	7·570	1·412	4·080	0·111	* 0·0129635
Sum *n*-6 PUFA	12·667	2·517	16·600	0·361	0·0552434
Sum polyunsaturated	20·400	3·934	20·800	0·458	0·869649
(*n*-3) / (*n*-6)	0·600	0·000	0·200	0·000	

* $P<0·05$, ** $P<0·01$, *** $P<0·001$. Statistical different mean values were determined using unpaired *t*-test using GraphPad Prism. Fatty acid levels under detection limit (<0·01 mg/g homogenate) were not stated (empty data). Means are calculated of 3 biological replicates consisting of 20 pooled 44 DPF zebrafish per replicate.

Table S4. Significant different detected metabolites from metabolic screening between control and high ARA group according to their sub-pathway affiliation and their enrichment score from enrichment analysis

Sub-pathway	Enrichment score	*P*	Significant (k)	Detected (m)	Significant metabolites
Bacterial/fungal	3·7	0·27	1	1	tartronate (hydroxymalonate)
Eicosanoid	**3·7**	**5·19E-03**	**4**	**4**	**12-HETE, 5-HEPE, 5-HETE, 5-KETE**
Fatty acid metabolism (acyl choline)	3·7	0·27	1	1	palmitoylcholine
Fatty acid, amino	3·7	0·27	1	1	2-aminoheptanoate
Fatty acid, keto	3·7	0·27	1	1	1-dihomo-linoleoylglycerol (20:2)
Hemoglobin and porphyrin metabolism	3·7	0·27	1	1	heme
Ketone bodies	3·7	0·27	1	1	3-hydroxybutyrate (BHBA)
Phosphatidylserine (PS)	3·7	0·27	1	1	1-stearoyl-2-arachidonoyl-GPS (18:0/20:4)
Thiamine metabolism	3·7	0·27	1	1	5-(2-Hydroxyethyl)-4-methylthiazole
Ascorbate and aldarate metabolism	2·8	0·06	3	4	ascorbate (Vitamin C), oxalate (ethanedioate), threonate
Fatty acid, dicarboxylate	**2·7**	**1·87E-03**	**8**	**11**	**2-hydroxyadipate, 2-hydroxyglutarate, azelate (nonanedioate), maleate, pimelate (heptanedioate), sebacate (decanedioate), suberate (octanedioate), undecanedioate**
Phospholipid metabolism	**2·3**	**9·26E-06**	**22**	**36**	**1,2-dilinoleoyl-GPC (18:2/18:2), 1,2-dioleoyl-GPC (18:1/18:1)*, 1,2-distearoyl-GPC (18:0/18:0), 1-linoleoyl-2-arachidonoyl-GPC (18:2/20:4*n*-6)*, 1-oleoyl-2-linoleoyl-GPC (18:1/18:2)*, 1-oleoyl-2-linoleoyl-GPE (18:1/18:2)*, 1-palmitoleoyl-2-linoleoyl-GPC (16:1/18:2)*, 1-palmitoyl-2-arachidonoyl-GPC (16:0/20:4), 1-palmitoyl-2-arachidonoyl-GPE (16:0/20:4)*, 1-palmitoyl-2-linolenoyl-GPC (16:0/18:3)*, 1-palmitoyl-2-linoleoyl-GPC (16:0/18:2), 1-palmitoyl-2-linoleoyl-GPE (16:0/18:2), 1-palmitoyl-2-linoleoyl-GPS (16:0/18:2), 1-palmitoyl-2-oleoyl-GPC (16:0/18:1), 1-palmitoyl-2-oleoyl-GPE (16:0/18:1), 1-stearoyl-2-arachidonoyl-GPC (18:0/20:4), 1-stearoyl-2-arachidonoyl-GPE (18:0/20:4), 1-stearoyl-2-arachidonoyl-GPI (18:0/20:4), 1-stearoyl-2-linoleoyl-GPC (18:0/18:2)*, 1-stearoyl-2-linoleoyl-GPE (18:0/18:2)*, phosphoethanolamine, trimethylamine N-oxide**
Plasmalogen	**2·2**	**0·03**	**6**	**10**	**1-(1-enyl-palmitoyl)-2-arachidonoyl-GPC (P-16:0/20:4)*, 1-(1-enyl-palmitoyl)-2-linoleoyl-GPC (P-16:0/18:2)*, 1-(1-enyl-palmitoyl)-2-linoleoyl-GPE (P-16:0/18:2)*, 1-(1-enyl-palmitoyl)-2-palmitoyl-GPC (P-16:0/16:0)*, 1-(1-enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4)*, 1-(1-enyl-stearoyl)-2-linoleoyl-GPE (P-18:0/18:2)***
Polyunsaturated fatty acid (*n*-3 and *n*-6 PUFA)	**2**	**0·04**	**7**	**13**	**adrenate (22:4*n*-6), arachidonate (20:4*n*-6), dihomo-linolenate (20:3*n*-3 or *n*-6), docosapentaenoate (*n*-6 DPA; 22:5*n*-6), eicosapentaenoate (EPA; 20:5*n*-3), linolenate [alpha or gamma; (18:3*n*-3 or *n*-6)], stearidonate (18:4*n*-3)**
Carnitine metabolism	1·8	0·47	1	2	carnitine
Diacylglycerol	1·8	0·3	2	4	1-oleoyl-2-linoleoyl-glycerol (18:1/18:2), 1-oleoyl-3-linoleoyl-glycerol (18:1/18:2)
Dipeptide derivative	1·8	0·47	1	2	carnosine
Fatty acid metabolism (also BCAA metabolism)	1·8	0·47	1	2	butyrylcarnitine
Fatty acid, monohydroxy	1·8	0·3	2	4	2-hydroxypalmitate, 2-hydroxystearate
Medium chain fatty acid	1·8	0·47	1	2	10-undecenoate (11:1*n*-1)
Pterin metabolism	1·8	0·47	1	2	pterin
Vitamin B6 metabolism	1·8	0·3	2	4	pyridoxamine, pyridoxate
Lysolipid	**1·6**	**0·04**	**13**	**30**	**1-arachidonoyl-GPA (20:4), 1-arachidonoyl-GPC (20:4*n*-6)*, 1-arachidonoyl-GPE (20:4*n*-6)*, 1-arachidonoyl-GPI (20:4)*, 1-lignoceroyl-GPC (24:0), 1-linolenoyl-GPC (18:3)*, 1-linoleoyl-GPC (18:2), 1-linoleoyl-GPE (18:2)*, 1-linoleoyl-GPS (18:2)*, 1-oleoyl-GPC (18:1), 1-oleoyl-GPE (18:1), 1-palmitoyl-GPI (16:0)*, 1-stearoyl-GPA (18:0)**
Sphingolipid metabolism	1·5	0·11	9	22	lactosyl-N-palmitoyl-sphingosine, N-palmitoyl-sphingosine (d18:1/16:0), sphingomyelin (d18:1/20:0, d16:1/22:0)*, sphingomyelin (d18:1/21:0, d17:1/22:0, d16:1/23:0)*, sphingomyelin (d18:1/22:1, d18:2/22:0, d16:1/24:1)*, sphingomyelin (d18:1/24:1, d18:2/24:0)*, sphingomyelin (d18:2/23:0, d18:1/23:1, d17:1/24:1)*, sphingomyelin

					(d18:2/24:1, d18:1/24:2)*, tricosanoyl sphingomyelin (d18:1/23:0)*
Sterol	1·5	0·41	2	5	7-hydroxycholesterol (alpha or beta), campesterol
Glutathione metabolism	1·4	0·37	3	8	4-hydroxy-nonenal-glutathione, 5-oxoproline, S-methylglutathione
Long chain fatty acid	1·3	0·32	5	14	arachidate (20:0), erucate (22:1*n*-9), myristate (14:0), nonadecanoate (19:0), stearate (18:0)
Glycine, serine and threonine metabolism	1·2	0·46	3	9	dimethylglycine, N-acetylthreonine, threonine
Leucine, isoleucine and valine metabolism	1·2	0·33	7	21	2-methylbutyrylcarnitine (C5), alpha-hydroxyisovalerate, beta-hydroxyisovalerate, beta-hydroxyisovaleroylcarnitine, isovalerate, N-acetylvaline, tiglylcarnitine
Riboflavin metabolism	1·2	0·61	1	3	flavin mononucleotide (FMN)
Tryptophan metabolism	1·2	0·51	2	6	5-hydroxyindoleacetate, C-glycosyltryptophan
Fatty acid metabolism (acyl carnitine)	1·1	0·48	4	13	acetylcarnitine, cis-4-decenoyl carnitine, myristoleoylcarnitine*, stearoylcarnitine
Methionine, cysteine, SAM and taurine metabolism	1	0·56	5	18	cysteine, cystine, hypotaurine, methionine sulfoxide, N-acetylmethionine sulfoxide
Monoacylglycerol	1	0·62	5	19	1-arachidonylglycerol (20:4), 1-dihomo-linolenylglycerol (20:3), 2-arachidonoylglycerol (20:4), 2-myristoylglycerol (14:0), 2-palmitoylglycerol (16:0)
Endocannabinoid	0·9	0·68	2	8	N-palmitoyltaurine, N-stearoyltaurine
Lysine metabolism	0·9	0·73	3	13	5-aminovalerate, glutarate (pentanedioate), saccharopine
Lysoplasmalogen	0·9	0·72	1	4	1-(1-enyl-oleoyl)-GPE (P-18:1)*
Pentose phosphate pathway	0·9	0·72	1	4	ribose 5-phosphate
Phenylalanine and tyrosine metabolism	0·9	0·73	3	13	3-methoxytyrosine, 4-hydroxyphenylpyruvate, N-acetylphenylalanine
Tocopherol metabolism	0·9	0·72	1	4	delta-tocopherol
TCA cycle	0·8	0·75	2	9	alpha-ketoglutarate, succinylcarnitine
Glutamate metabolism	0·7	0·84	2	11	4-hydroxyglutamate, glutamine
Purine metabolism, (hypo)xanthine/inosine containing	0·7	0·84	2	11	allantoic acid, urate
Fructose, mannose and galactose metabolism	0·6	0·85	1	6	mannose-6-phosphate
Aminosugar metabolism	0·5	0·92	1	8	glucosamine-6-phosphate
Food component/plant	0·4	0·94	1	9	ergothioneine
Glycolysis, gluconeogenesis, and pyruvate metabolism	0·4	0·96	1	10	glucose 6-phosphate
Purine metabolism, guanine containing	0·4	0·94	1	9	guanosine 3'-monophosphate (3'-GMP)
Gamma-glutamyl amino acid	0·3	0·98	1	12	gamma-glutamylglutamine
Purine metabolism, adenine containing	0·3	0·99	1	14	adenosine 3'-monophosphate (3'-AMP)
Urea cycle; arginine and proline metabolism	0·2	1	1	17	pro-hydroxy-pro
Advanced glycation end-product	1	1	0	1	
Alanine and aspartate metabolism	1	1	0	6	
Biotin metabolism	1	1	0	1	
Chemical	1	1	0	5	
Creatine metabolism	1	1	0	4	
Dipeptide	1	1	0	13	
Drug	1	1	0	1	
Fatty acid synthesis	1	1	0	1	
Fatty acid, amide	1	1	0	3	
Fatty acid, branched	1	1	0	3	
Fatty acid, dihydroxy	1	1	0	2	
Glycerolipid metabolism	1	1	0	3	
Glycogen metabolism	1	1	0	5	
Guanidino and acetamido metabolism	1	1	0	2	
Histidine metabolism	1	1	0	13	
Inositol metabolism	1	1	0	3	
Mevalonate metabolism	1	1	0	1	
Nicotinate and nicotinamide metabolism	1	1	0	7	
Nucleotide sugar	1	1	0	6	
Oxidative phosphorylation	1	1	0	2	
Pantothenate and CoA metabolism	1	1	0	1	
Pentose metabolism	1	1	0	5	

Polyamine metabolism	1	1	0	5
Primary bile acid metabolism	1	1	0	4
Purine and pyrimidine metabolism	1	1	0	1
Pyrimidine metabolism, cytidine containing	1	1	0	8
Pyrimidine metabolism, orotate containing	1	1	0	2
Pyrimidine metabolism, thymine containing	1	1	0	5
Pyrimidine metabolism, uracil containing	1	1	0	11
Secondary bile acid metabolism	1	1	0	1
Steroid	1	1	0	1
Tetrahydrobiopterin metabolism	1	1	0	2
SUM			153	566

* Indicates compounds that have not been officially confirmed based on a standard, but we are confident in its identity.

Data is shown according to the pathway enrichment scores in descending order. Bold data represents significant enriched sub-pathways (P<0·05). Enrichment scores determine the number of statistically significant regulated compounds (k) relative to all detected compounds (m) in a pathway, compared with the total number of significant regulated compounds (n=153) relative to all detected compounds (N=566) in the analysis: (k/m)/(n/N). The maximum achievable enrichment score is 3·7, if all detected metabolites in a sub-pathway are described as statistical significant different (P<0·05).

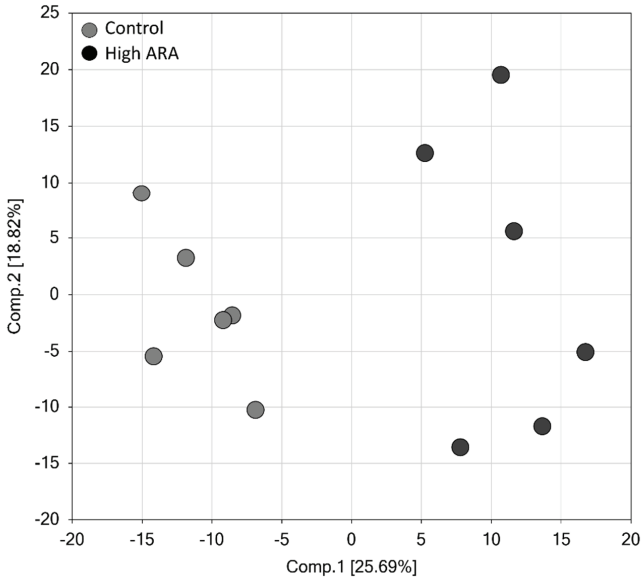

Fig. S1. Principle component analysis of metabolic profiling from 44 DPF zebrafish fed either control or high ARA feed from 27 DPF until sampling.

Paper II

Anne-Catrin Adam, Kaja Helvik Skjærven, Paul Whatmore, Mari Moren, Kai Kristoffer Lie.

Parental high dietary arachidonic acid levels modulated the hepatic transcriptome of adult zebrafish (*Danio rerio*) progeny

Submitted.

Parental high dietary arachidonic acid levels modulated the hepatic transcriptome of adult zebrafish (*Danio rerio*) progeny

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Abstract

Disproportionate high intake of n-6 polyunsaturated fatty acids (PUFAs) in the diet is considered as a major human health concern. The present study examines changes in the hepatic gene expression pattern of adult male zebrafish progeny associated with high levels of the n-6 PUFA arachidonic acid (ARA) in the parental diet. The parental generation (F_0) was fed a diet which was either low (control) or high in ARA (high ARA). Progenies of both groups (F_1) were given the control diet. No differences in body weight were found between the diet groups within adult stages of either F_0 or F_1 generation. Few differentially expressed genes were observed between the two dietary groups in the F_0 in contrast to the F_1 generation. Several links were found between the previous metabolic analysis of the parental fish and the gene expression analysis in their adult progeny. Main gene expression differences in the progeny were observed related to lipid and retinoid metabolism by PPARα/RXRα playing a central role in mediating changes to lipid and long-chain fatty acid metabolism. The enrichment of genes involved in *β*-oxidation observed in the progeny, corresponded to the increase in peroxisomal *β*-oxidative degradation of long-chain fatty acids in the parental fish metabolomics data. Similar links between the F_0 and F_1 generation were identified for the methionine cycle and transsulfuration pathway in the high ARA group. In addition, estrogen signalling was found to be affected by parental high dietary ARA levels, where gene expression was opposite directed in F_1 compared to F_0. This study shows that the dietary n-3/n-6 PUFA ratio can alter gene expression patterns in the adult progeny. Whether the effect is mediated by permanent epigenetic mechanisms regulating gene expression in developing gametes needs to be further investigated.

Introduction

In today's dietary pattern, we observe a selective decrease of n-3 polyunsaturated fatty acids (PUFAs) in favour of n-6 PUFAs. This results in a decreased n-3/n-6 PUFA ratio [1, 2]. Physiological effects of a decreasing n-3/n-6 PUFA ratio are diverse, but taken together, studies indicate that a disproportional high intake of n-6 PUFAs may contribute to health problems [3-7]. In the past decades, several studies have shown the benefit of increased n-3 PUFA levels in the diet [8, 9], and focus has been directed on n-3 PUFAs such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). Arachidonic acid (ARA, 20:4n-6), an n-6 PUFA that competes for the same enzymes and therefore is interlinked, has been less studied. ARA and its derivatives, have vital roles in growth and various signalling cascades regulating inflammatory processes, bone metabolism and reproduction as observed in different species [10-14]. n-3 and n-6 PUFAs have the potential to change cellular phenotypes by changing membrane lipid composition and controlling gene expression through activating nuclear receptors [15-17]. In addition, n-3 and n-6 PUFAs can also affect DNA methylation patterns [18-20]. Recently, we demonstrated that high dietary ARA levels fed to zebrafish affected the levels of oxidized amino acids and lipids, and changed the immune-related eicosanoids and lipid metabolism [21]. More and more studies indicate that diet also affect following generations in terms of long-term health of the progeny [22-26]. Here, we investigate whether high ARA given to the parents' generation can impact the progeny's transcriptome. However, little is known about how changes in the parental dietary n-3 and n-6 PUFA composition impact the adult progeny.

The period of oocyte and spermatozoa maturation displays a sensitive window, where parental nutrition has metabolic influence on future fertilized eggs [27]. Another way how parental diet effects can be mediated is through transcripts deposited in the newly fertilized egg that regulate early embryonic development and thus determine future gene expression patterns, growth and physiology [28-30]. It has been shown that dietary micronutrient status of the parents can influence gene expression patterns of the embryos using zebrafish [31, 32]. Furthermore, nutritional induced obesity of the parents has shown to affect fertility (egg production) and gene expression of zebrafish eggs [33].

Studies on different vertebrate species have demonstrated that the maternal dietary n-3 and n-6 PUFA profile influenced oocyte composition, embryonic development and health of progeny [9, 34-37]. In teleost, dietary n-3 and n-6 PUFA composition was found to affect oocytes and reproductive performance [38-42], but little is known about the changes in gene expression profiles in adult progeny.

Zebrafish (*Danio rerio*), a tropical freshwater teleost fish, is an acknowledged vertebrate model organism. It has been widely used in research to increase our understanding of gene function and the importance of nutrition in outcomes related to development, health and disease in vertebrates [43-50]. In the present study, we fed parental zebrafish either a control diet (low in ARA) or a diet high in ARA, whereas progeny from both dietary groups were fed the control diet until adulthood. We wanted to investigate the impact of parental high dietary ARA levels on transcriptomic patterns in adult progeny.

Materials and methods

Ethical considerations

This zebrafish feeding trial was conducted in accordance with the Norwegian Animal Research Authority and approved by the Norwegian Food Safety Authority (division no. 54, reference 2012/145126) according to the current Regulation on Animal Experimentation (FOR 1996-01-15 no. 23).

Feeding trial and zebrafish husbandry

Standardized operating procedures for mating, handling and feeding for both F_0 and F_1 generation of wildtype AB zebrafish (*Danio rerio*) has previously been reported [31]. Briefly, F_0 embryos were collected randomly and larvae were fed with Gemma micro® (Skretting, Norway) as a start feed from 5 days post fertilization (DPF) and *Artemia* nauplii (Silver Star *Artemia,* USA) from 7 DPF until 26 DPF (Fig 1). The experimental diets were given twice a day from 27 DPF onwards. Control and high ARA diet composition can be found in S1 File [21]. Progeny (F_1 generation), from both parental diet groups, were fed as the F_0 control fish with the experimental control diet from 27 DPF until sampling. Fish were kept in 10 gender mixed tanks (containing 60 fish each until 44

DPF and thereafter reduced to 20 fish each) per diet group. All fish were kept under steadily monitored standard conditions with 28±1^{0}C, 14 h light-10 h dark period, conductivity of 500 µS, 6 ppm (mg/L) dissolved oxygen and pH 7.5 in tanks in a reverse osmosis water treatment system (Aquatic Habitats® recirculation system, MBKI Ltd, GBR). F_0 generation was mated at 97 DPF.

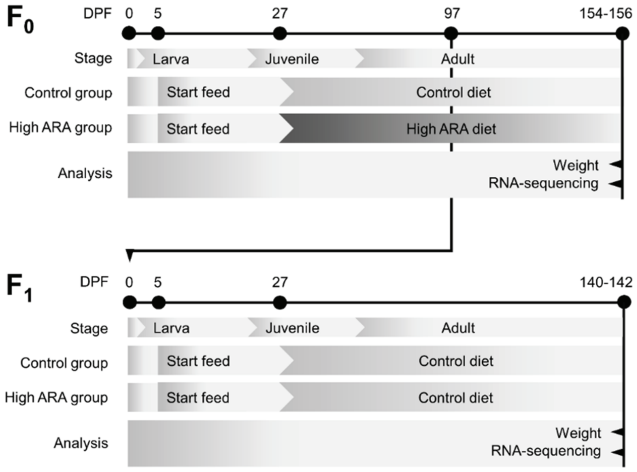

Fig 1. Experimental design of the transgenerational zebrafish feeding trial.
F_0 zebrafish from both control and high ARA group were fed a start feed containing Gemma micro® and *Artemia* nauplii from 5 DPF until 26 DPF. The two experimental groups were given either a control or high ARA diet from 27 DPF onwards until sampling. F_0 fish were mated at 97 DPF to produce F_1 generation. Both groups in the F_1 generation were fed the control diet from 27 DPF until sampling. F_0 and F_1 body weight (grams) and liver tissue sampling for transcriptome analysis (RNA-sequencing) were performed at 154-156 DPF (F_0) and 140-142 DPF (F_1).

Liver sampling and RNA extraction

Prior to dissection, fish were deprived of food for 18 h, anesthetized with 0.05% Tricaine Methane Sulphonate (PHARMAQ AS, Norway), blotted dry on tissue paper prior to weighing, euthanized by cutting the cardinal vein and the liver was dissected subsequently. Livers were sampled in random order between 154-156 DPF (F_0) and 140-142 DPF (F_1) due to simultaneous sampling for other analyses connected to this trial. Six biological replicates representing six different tanks for each of the dietary groups, where

one replicate is a pool of six male livers from one tank. Livers were briefly rinsed in 1x PBS, snap frozen with liquid nitrogen and stored at -80^{0}C for transcriptome analysis (RNA-sequencing). Total RNA was extracted using QIAzol Lysis Reagent (Qiagen, Germany) and RNA samples were DNase treated with the Ambion™ DNA-*free*™ DNA Removal Kit (Invitrogen, USA) in order to avoid remaining genomic DNA. RNA quantity was verified using NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, USA). RNA integrity (RIN) was determined using an Agilent 2100 Bioanalyser (RNA 6000 Nano LabChip kit, Agilent Technologies, USA). RIN values were on average 9.06±0.39.

RNA high-throughput sequencing and data processing

The Norwegian Sequencing Centre (NSC) performed RNA-sequencing (RNA-seq) and library preparation using TruSeq™ Stranded mRNA Library Prep Kit (Illumina, Inc, USA). Libraries were sequenced on the NextSeq500 platform (Illumina, Inc, USA) to generate single-end 75bp reads. Sequence quality was assessed using FastQC v0.11.5. Finding high quality (Phred scores almost universally above 30) and close to zero adapter contamination on the raw reads, we decided that mapping untrimmed reads to the genome was the optimal strategy, instead allowing the mapping software to exclude errors through discarded mismatches [51]. An average of 10 047 201 reads per sample were mapped to the GRCz10 (Genome Reference Consortium Zebrafish Build 10) assembly based on both RefSeq (GCF 000002035.5 GRCz10) and Ensembl [52] using the default parameters of HISAT2 [53] resulting in an average of 76.29% of reads unambiguously assigned to RefSeq genes and 82.22% of reads unambiguously assigned to Ensembl genes. Read counts per gene were quantified using featureCounts [54] and pre-filtered to exclude combined mean read counts smaller than 10.

Bioinformatic analysis

Differential gene expression was estimated using DESeq2 [55]. By default, internal normalization was performed to correct for variable sequencing depth and library size. Wald-test was used for significance testing and Benjamini-Hochberg for p-value false discovery correction (adjusted p). DESeq2 analysis and visualisation of data were performed in R (http://cran.rproject.org/). Mapping against different reference genomes

can produce variable expression values and differentially expressed genes (DEGs) identified [56]. The annotated DEG lists from both RefSeq and Ensembl reference genomes and both generations are listed in S1, S2, S3 and S4 Tables. However, enrichment and downstream analyses were based on concordant DEGs between both reference genome annotations (S5 and S6 Tables).The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [57] and are accessible through GEO Series accession number GSE104692 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE104692).

Concordant gene symbols of DEGs (adjusted p-value<0.05) of F_1 generation were sent to Ingenuity® Pathway Analysis software suite (IPA®, Ingenuity Systems, USA) for downstream analysis. DEGs with corresponding adjusted p-value and log2 fold change (log2FC) were imported into IPA® as human orthologues (S7 Table). An overlap p-value (right-tailed Fisher`s Exact test, $p<0.05$) and an activation z-score for the correlation between the imported RNA-seq dataset and the Ingenuity® Knowledge Base is calculated. IPA® integrates direct DEG changes to predict an upregulation or downregulation of canonical pathways and biological functions in F_1 high ARA livers using z-scores.

Validation of RNA-sequencing by real-time qRT-PCR

Real-time quantitative reverse transcription PCR (real-time qRT-PCR) was performed as previously described [58] for validating the RNA-seq data. Reverse transcription and PCR of standard curve and individual samples was run with the Gene Amp 9700 PCR machine (Applied Biosystems, USA). Real-time qRT-PCR was performed starting with a 2 min template incubation and denaturation step at 94^0C, followed by 45 cycles divided in 10 s denaturation at 95^0C, 10 s annealing at 60^0C and 10 s synthesis at 72^0C using the LightCycler® 480 Real-Time PCR Systems (Roche Applied Sciences, Germany) with the LightCycler® 480 SYBR Green I Master kit (Roche Applied Science, Germany). Samples were amplified in triplicates and the mean was used for further calculations. Normalised expression of target genes was determined using the geNorm algorithm [59] based on the geometric mean of 3 stable reference genes: *eef1a1/1* (elongation factor 1-alpha) [60], *uba52* (Ubiquitin A-52 Residue Ribosomal Protein Fusion Product 1) [60]

and *rpl13a* (Ribosomal Protein L13) [61] (S2 File). We investigated gene transcription for *acaca* (acetyl-CoA carboxylase alpha) and *map2k6* (mitogen-activated protein kinase kinase 6) that were exclusively differentially expressed in either F_1 or F_0 generation, respectively.

Statistical analysis

Differences in body weight between the dietary groups are presented as mean with standard deviation (SD) and an unpaired, two-tailed *t*-test was used for significance testing (p-value<0.05). Validation of the RNA-seq results by real-time qRT-PCR was tested by Pearson correlation coefficient calculation based on 6 replicates for each comparison and a two-tailed *t*-test for group differences. Statistical significance analysis of F_0 and F_1 body mass and Pearson correlation analysis for RNA-seq validation was performed with GraphPad Prism 6 software (GraphPad Software, Inc, USA).

Results

Body weight of F_0 and F_1 zebrafish

We observed no changes in body weight between the diet groups in both F_0 and F_1 generation (Table 1).

Table 1. Body weight of F_0 zebrafish and their progeny (F_1).

	Control (g)			**High ARA** (g)			
	Mean	SD	n [1]	Mean	SD	n [1]	p [2]
F_0 fish (154-156 DPF)	0.44	0.05	36	0.42	0.06	36	0.19
F_1 fish (140-142 DPF)	0.35	0.04	36	0.34	0.06	35	0.40

Fish age is given in days post fertilization (DPF).
[1] n are individual fish originated from six tanks.
[2] An unpaired, two-tailed *t*-test (GraphPad) was used for significance testing (p<0.05).

Liver gene expression patterns

Principal component analysis (Fig 2) and volcano plot comparison (Fig 3) showed a clear separation between the dietary groups in F_1 compared to F_0 generation. We found 20

DEGs (adjusted p<0.05) in F_0 generation (Fig 3A, S3 File) and 458 DEGs in F_1 generation (Fig 3B, S3 File).

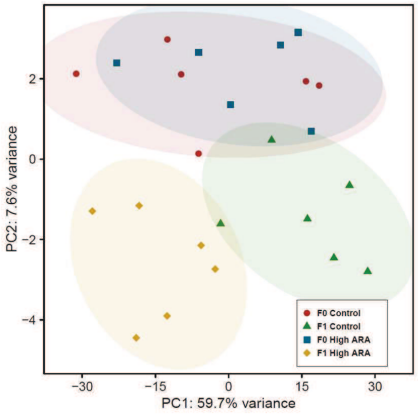

Fig 2. Principal component analysis of RNA-sequencing data from male livers in F_0 generation fed either a control or high ARA diet and their F_1 progeny fed the control diet. The plot displays high similarity in the transcriptome of F_0 control and high ARA livers compared to clearer separation in gene expression patterns between the F_1 diet groups. The magnitude of variation between replicates within a diet group were similar among all groups. Plot shows data underlying log-transformed read counts based on RefSeq reference genome mapping.

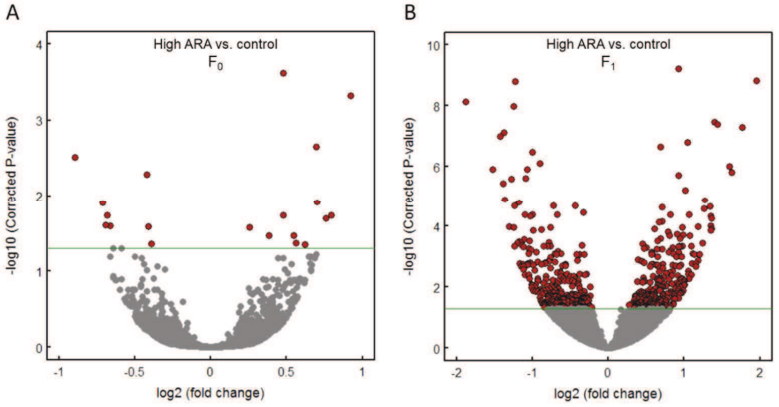

Fig 3. Volcano plot of RNA-sequencing data from male livers in F_0 generation fed either a control or high ARA diet and their F_1 progeny where both groups received the control diet. Presented data represents overlapping genes from both RefSeq and Ensembl reference genome mapping (GRCz10). Red spots represent overlapping differentially expressed genes (DEGs) in F_0 (**A**) and F_1 (**B**) generation. The green line denoted the significance threshold (adjusted p<0.05) for DEGs.

ARA induced modulation of the parental (F_0) liver transcriptome

No functional enrichment analysis was performed due to low DEG count in F_0 generation. However, among the 20 DEGs (S5 Table), we found two genes relevant for lipid metabolism affected: *ncoa3* (nuclear receptor coactivator 3) involved in co-activation of different nuclear receptors like retinoid x receptors (RXRs) and peroxisome proliferator-activated receptors (PPARs), and *bbox1* (gamma-butyrobetaine hydroxylase 1) involved in the biosynthesis of carnitine, which is essential for fatty acid supply in *β*-oxidation.

ARA induced modulation of the progeny (F_1) liver transcriptome

DEGs (adjusted $p<0.05$) from F_1 generation were sent to downstream analysis using IPA® (S7 Table). Parental high ARA diet was associated with differential expression of genes coding for a diverse set of genes clustering in several diverse canonical pathways of which 'eIF2 signalling pathway' was predicted to be the most significantly pathway (Table 2).

The top most significantly enriched lipid metabolism related biological functions are shown in Table 3. The full list is given in S4 File where various biological functions related to phospholipid, steroid, long chain fatty acid and cholesterol metabolism were enriched by the F_1 DEGs. 'LXR/RXR activation' canonical pathway is predicted to be downregulated (z=-2; Table 2). 'PPARα/RXRα Activation' was found to be a significantly enriched canonical pathway in the high ARA group. *Pparaa* showed higher expression levels (p=0.005, S4 Table) as shown in Fig 4. 'Oxidation of fatty acids' is one of the top most enriched biological functions (Table 3). For *acox1* being involved in the first enzymatic step during peroxisomal *β*-oxidation and acting downstream of PPARα, showed upregulated expression in high ARA livers (Fig 4). *Helz2* which encodes a nuclear transcriptional co-activator for PPARα, was downregulated. High ARA livers showed an upregulated expression of genes involved in the long-chain fatty acid biosynthesis (*acaca, fasn* and *srebf1*) compared to livers arriving from control group (Fig 4). *Elovl4b,* which is involved in very long-chain fatty acid elongation was found to be significantly downregulated in high ARA livers. We found an upregulated gene expression for *dagla* that is involved in the synthesis of 2-arachidonoyl-glycerol, an

endocannabinoid. Genes like *prkcq* and *dgkza* play roles in lipid signalling pathways like T cell receptor signalling and showed higher gene expression levels in high ARA group compared to control group.

'Metabolism of retinoid' was one of the enriched biological functions among lipid metabolism (S4 File). Transcripts of genes encoding enzymes involved in retinaldehyde synthesis from beta-carotene (*bco1*) and subsequent synthesis to retinoic acid (*aldh1a2*) as well as retinol saturation (*retsat*) were found to be downregulated in the high ARA group (Fig 4). *Rxraa* that is transcriptionally regulated by stereoisomers of retinoic acid, was shown to be downregulated in livers associated with parental high ARA diet. Two transcripts encoding transporters for beta-carotene (*scarb1*) and retinol (*rbp2b*) were downregulated.

Among other enriched canonical pathways (Table 2), we found significantly upregulated transcripts of *mat1a*, *prmt1*, *cbsb*, *cth* and *got1* that clustered in 'cysteine biosynthesis III (mammalian)', 'cysteine biosynthesis/homocysteine degradation' and 'superpathway of methionine degradation' (Fig 5). Cysteine is provided through the transsulfuration pathway (*cbcb*, *cth*) for glutathione metabolism where *gsta.1* involved in glutathione detoxification was found to be downregulated in the high ARA group. *gls2b* and *glud1a* that are related to glutamate metabolism showed decreased expression in high ARA livers (Fig 5). *aldh1l1*, which is involved in the folate cycle, was downregulated in high ARA livers compared to control livers.

We observed increased gene expression of the estrogen receptor 1 (*esr1*) in livers associated with parental high ARA levels. 'Concentration of progesteron', 'metabolism of estrogen', 'sulfation of beta-estradiol' and different steroid metabolism related function annotations were suggested to be enriched by IPA® (Table 3 and S4 File). Among the unmapped IDs, vitellogenin 5 (*vtg5*, no human orthologue) expression was found to be upregulated in high ARA livers compared to the control. In F_0 high ARA livers, both *vtg5* ($p=0.01$) and *esr1* ($p=0.02$) showed decreased expression (S2 Table), but did not meet the chosen threshold requirements (adjusted $p<0.05$) for downstream analysis. None of the F_1 DEGs were in matching with those in F_0 generation due to the chosen cut-off threshold.

Table 2. Significant canonical pathways associated with DEGs from F_1 high ARA livers proposed by IPA®.

Canonical Pathways	p-value	z-score [1]	Gene symbol [2]
EIF2 Signaling	3.98E-13	-2.887	RPL32,RPL11,RPL36A,RPS27,RPS8,RPS18,RPL29,EIF4G3,EIF2S1,XIAP,RPS28,RPS7,RPS26,SREBF1,RPL19,RPL21,RPL5,RPS25,RPS15A,RPS2,RPL36,RPS17,RPL18,RPL38
LXR/RXR Activation	5.13E-03	-2.000	APOB,C3,SREBF1,FASN,ACACA,SERPINA1,RXRA
Neuropathic Pain Signaling In Dorsal Horn Neurons	4.47E-02	-1.342	PLCD3,PLCE1,PRKCQ,PRKAG2,GRM6
Melatonin Signaling	3.80E-02	-1.000	PLCD3,PLCE1,PRKCQ,PRKAG2
PPARα/RXRα Activation	3.31E-03	0.447	PLCD3,PLCE1,GPD1,HELZ2,ACOX1,FASN,PRKAG2,RXRA,ADIPOR1
LPS/IL-1 Mediated Inhibition of RXR Function	3.89E-04	0.447	ALDH1L1,SCARB1,CYP3A4,SREBF1,ACOX1,SULT1A1,ALDH1A2,SULT1A3/SULT1A4,FABP7,GSTA1,RXRA,FMO5
Sperm Motility	2.82E-02	-0.447	PLCD3,PLCE1,PRKCQ,CACNA1H,PRKAG2,PDE4B
Regulation of eIF4 and p70S6K Signaling	2.63E-06		ITGB1,RPS27,RPS8,RPS18,EIF4G3,EIF2S1,RPS28,RPS7,RPS26,RPS25,RPS15A,RPS2,RPS17
mTOR Signaling	7.76E-06		RPS28,RPS7,DGKZ,PRKCQ,RPS26,RPS27,RPS18,RPS8,PRKAG2,EIF4G3,RPS25,RPS15A,RPS2,RPS17
Polyamine Regulation in Colon Cancer	3.02E-05		AZIN1,SAT2,PSME4,OAZ1,OAZ2
Unfolded protein response	2.45E-04		HSPA4,SREBF1,EDEM1,HSPA9,VCP,CANX
Dopamine Degradation	1.20E-03		ALDH1L1,COMT,SULT1A1,ALDH1A2,SULT1A3/SULT1A4
Cysteine Biosynthesis III (mammalia)	1.78E-03		CBS/CBSL,MAT1A,CTH,PRMT1
FXR/RXR Activation	1.91E-03		APOB,C3,SCARB1,SREBF1,FASN,SERPINA1,RXRA,VLDLR
Aryl Hydrocarbon Receptor Signaling	2.82E-03		ALDH1L1,TFDP1,ALDH1A2,GSTA1,RXRA,ESR1,PTGES3,AHR
Protein Ubiquitination Pathway	3.80E-03		B2M,HSPA4,UBE2D2,UBE4B,UBE2H,DNAJB11,DNAJC19,HSPA9,HSPD1,THOP1,XIAP
Superpathway of Methionine Degradation	4.47E-03		CBS/CBSL,MAT1A,GOT1,CTH,PRMT1
Histidine Degradation VI	5.37E-03		CYP46A1,UROC1,MICAL2
Caveolar-mediated Endocytosis Signaling	6.92E-03		ITGB1,B2M,COPG2,COPB2,COPB1
Cysteine Biosynthesis/Homocysteine Degradation	7.08E-03		CBS/CBSL,CTH
Citrulline Biosynthesis	8.51E-03		LOC102724788/PRODH,OAT,GLS2
Xenobiotic Metabolism Signaling	8.51E-03		ALDH1L1,PRKCQ,CYP3A4,SULT1A1,ALDH1A2,SULT1A3/SULT1A4,GSTA1,RXRA,FMO5,PTGES3,AHR
Aldosterone Signaling in Epithelial Cells	8.71E-03		PLCD3,HSPA4,PLCE1,PRKCQ,DNAJB11,DNAJC19,HSPA9,HSPD1
tRNA Charging	1.12E-02		LARS,CARS,TARS,VARS,QARS
GPCR-Mediated Nutrient Sensing in Enteroendocrine Cells	1.78E-02		PLCD3,PLCE1,PRKCQ,PRKAG2,TAS1R3
Putrescine Degradation III	1.95E-02		ALDH1L1,ALDH1A2,SAT2
Superpathway of Citrulline Metabolism	2.40E-02		LOC102724788/PRODH,OAT,GLS2

Prostanoid Biosynthesis	2.45E-02	PTGDS,PTGES3
PXR/RXR Activation	2.75E-02	CYP3A4,PRKAG2,GSTA1,RXRA
TR/RXR Activation	2.95E-02	SCARB1,SREBF1,FASN,ACACA,RXRA
Pregnenolone Biosynthesis	3.09E-02	CYP46A1,MICAL2
Neuroprotective Role of THOP1 in Alzheimer's Disease	3.89E-02	PRKAG2,THOP1,ACE
VDR/RXR Activation	4.07E-02	SERPINB1,YY1,PRKCQ,RXRA
RAR Activation	4.37E-02	PRKCQ,ALDH1A2,PRKAG2,RBP2,SMARCD1,RXRA,PRMT1
Endoplasmic Reticulum Stress Pathway	4.57E-02	EIF2S1,TAOK3
Glucose and Glucose-1-phosphate Degradation	5.01E-02	RGN,PGM2
Pentose Phosphate Pathway	5.01E-02	PGLS,RPIA
Phagosome Formation	5.01E-02	ITGB1,MRC1,PLCD3,PLCE1,PRKCQ

[1] IPA® predicts upregulation (positive z-score) or downregulation (negative z-score) of canonical pathways.
[2] Gene symbols are reported as human orthologue gene symbols.

Table 3. The top most significantly enriched lipid metabolism related biological functions associated with DEGs from F_1 high ARA livers proposed by IPA®.

Biological Functions	p-value	z-score [1]	Gene symbol [2]
Concentration of phospholipid	3.82E-04	1.969	ACACA,CBS/CBSL,CHKA,DGKZ,FASN,LYST,NPC2,PITPNB,PLPP2,RGN,SCARB1,SREBF1,VLDLR
Clearance of lipid	5.72E-03	-1.960	C3,CYP3A4,SCARB1,VLDLR
Oxidation of fatty acid	2.04E-03	1.622	ACACA,ACOX1,ADIPOR1,C3,CYP3A4,FASN,PDK4,PRKAG2,SLC25A17,SLCO2A1,SREBF1
Synthesis of lipid	3.27E-06	-1.375	ACACA,ACLY,AHR,AKR1B1,ALDH1A2,APOB,ATP1A1,BCO1,C3,CACNA1H,CD9,CERS5,CHKA,CREB3L3,CYP39A1,CYP3A4,CYP46A1,DAGLA,DGKZ,ELOVL4,ESR1,FASN,FDX1,GSTA1,IGFBP2,ITGB1,LEPR,NPC2,PARK7,PDK4,PLCE1,PRKAG2,PTGDS,PTGES3,RGN,RXRA,SCARB1,SERPINA1,SH3KBP1,SREBF1
Concentration of choline-phospholipid	6.15E-03	1.342	ACACA,CHKA,FASN,LYST,SREBF1
Transport of fatty acid	4.49E-03	1.257	ABCC6,FABP7,SCARB1,SLC13A3,SLC25A17,SLCO2A1
Concentration of acylglycerol	2.26E-06	1.145	ACACA,ACLY,ADIPOR1,AKR1B1,APOB,ATP2A2,C3,CBS/CBSL,CHKA,CREB3L3,CYP3A4,DAGLA,FASN,FMO5,HELZ2,LEPR,MGLL,PDK4,RGN,RXRA,SCARB1,SREBF1,STEAP4,VLDLR
Synthesis of terpenoid	4.41E-04	-1.131	ACLY,AHR,ALDH1A2,APOB,ATP1A1,BCO1,CACNA1H,CYP39A1,CYP46A1,ESR1,FDX1,GSTA1,IGFBP2,PRKAG2,SCARB1,SERPINA1,SREBF1
Incorporation of lipid	6.20E-03	-1.127	ACLY,C3,FASN,SCARB1
Concentration of progesterone	5.80E-03	-1.067	CBS/CBSL,COMT,ESR1,LEPR,SCARB1

[1] IPA® predicts upregulation (positive z-score) or downregulation (negative z-score) of canonical pathways.
[2] Gene symbols are reported as human orthologue gene symbols.

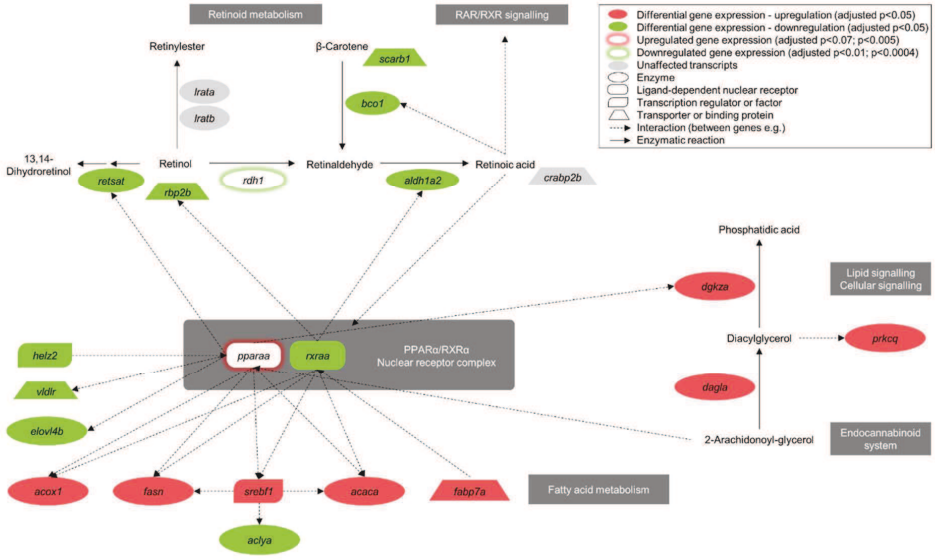

Fig 4. Differential expression of genes involved in retinoid processing and putative interactions with the PPARα/RXRα pathway and lipid signalling in male livers that are associated with parental high dietary ARA levels.

Filled shapes in the figure represent overlapping differentially expressed genes between RefSeq and Ensembl annotation. Shapes that are highlighted with glow underlie gene expression information exclusively from Ensembl annotation (S4 Table); a*caca* (acetyl-CoA carboxylase alpha), *aclya* (ATP citrate lyase a); *acox1* (acyl-CoA oxidase 1, palmitoyl; alias: peroxisomal acyl-CoA oxidase 1); *aldh1a2* (aldehyde dehydrogenase 1 family, member A2); *bco1* (beta-carotene oxygenase 1); *crabp2b* (cellular retinoic acid binding protein 2, b); *dagla* (diacylglycerol lipase, alpha); *dgkza* (diacylglycerol kinase, zeta a), *elovl4b* (ELOVL fatty acid elongase 4b); *fabp7a* (fatty acid binding protein 7, brain, a); *fasn* (fatty acid synthase); *helz2* (helicase with zinc finger 2, transcriptional coactivator); *lrata* (lecithin retinol acyltransferase a (phosphatidylcholine-retinol O-acyltransferase)); *lratb* (lecithin retinol acyltransferase b (phosphatidylcholine-retinol O-acyltransferase)); *pparaa* (peroxisome proliferator-activated receptor alpha a); *prkcq* (protein kinase C, theta); *rbp2b* (retinol binding protein 2b, cellular); *rdh1* (retinol dehydrogenase 1); *retsat* (retinol saturase (all-*trans*-retinol 13,14-reductase)); *rxraa* (retinoid X receptor, alpha a); *scarb1* (scavenger receptor class B, member 1); *srebf1* (sterol regulatory element binding transcription factor 1); *vldlr* (very low density lipoprotein receptor)

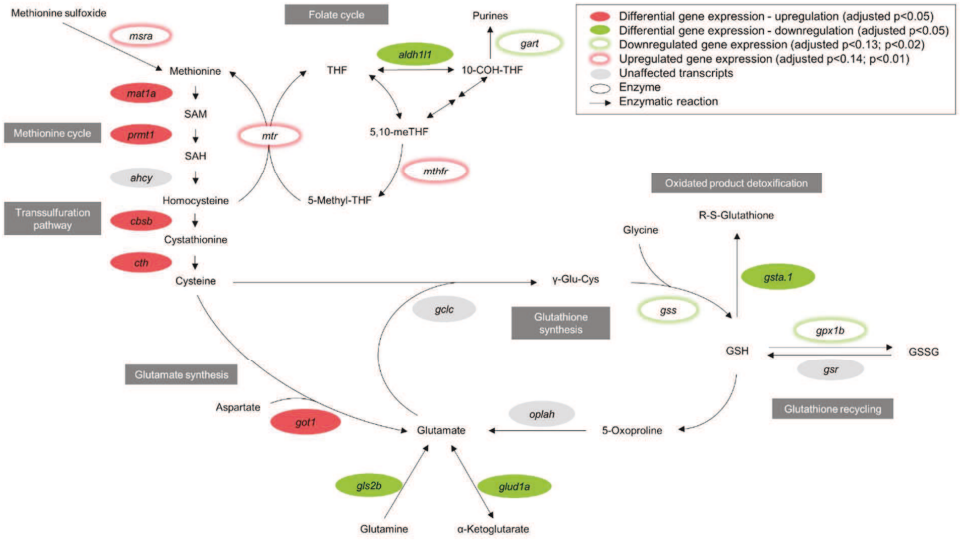

Fig 5. Parental high ARA levels are associated with differential expression of genes involved in methionine cycle, transsulfuration pathway, and glutamate and glutathione metabolism in male F_1 livers.

Filled shapes in the figure represent overlapping differentially expressed genes between RefSeq and Ensembl annotation. Shapes that are highlighted with glow underlie gene expression information exclusively from Ensembl annotation (S4 Table); *ahcy* (adenosylhomocysteinase); *aldh1l1* (aldehyde dehydrogenase 1 family, member L1); *cbsb* (cystathionine-beta-synthase b); *cth* (cystathionase (cystathionine gamma-lyase)); *gart* (phosphoribosylglycinamide formyltransferase); *gclc* (glutamate-cysteine ligase, catalytic subunit); *gls2b* (glutaminase 2b (liver, mitochondrial); *glud1a* (glutamate dehydrogenase 1a); *got1* (glutamic-oxaloacetic transaminase 1); *gpx1b* (glutathione peroxidase 1b); GSH (reduced glutathione); *gsr* (glutathione-disulfide reductase); *gss* (glutathione synthetase); GSSG (oxidized glutathione); *gsta.1* (glutathione S-transferase, alpha tandem duplicate 1); *mat1a* (methionine adenosyltransferase I alpha); *msra* (methionine sulfoxide reductase A); *mthfr* (methylenetetrahydrofolate reductase (NAD(P)H)); *mtr* (5-methyltetrahydrofolate-homocysteine methyltransferase); *oplah* (5-oxoprolinase (ATP-hydrolysing)); *prmt1* (protein arginine methyltransferase 1); R-S-glutathione (glutathione-S-conjugate); SAH (S-adenosyl-homocysteine); SAM (S-adenosyl-methionine); γ-Glu-Cys (gamma-glutamyl-cysteine); THF (tetrahydrofolate); 5-methyl-THF (5-methyltetrahydrofolate); 10-COH-THF (10-formyl-tetrahydrofolate); 5,10-meTHF (5,10-methylene-THF)

Gene expression correlation analysis of RNA-seq and qPCR results

Gene expression patterns of *acaca* and *map2k6* were investigated in both F_0 and F_1 livers by real-time qRT-PCR for validation purposes. Real-time qRT-PCR and RNA-seq derived gene expression for both genes correlated significantly in both generations (S5 File).

Discussion

In the present study we identified changes in hepatic gene expression patterns in the adult zebrafish progeny of parental fish given high dietary ARA levels. Despite the large mRNA expression differences observed in F_1 generation, surprisingly few DEGs were found in F_0 generation. The parental fish (F_0) that were given the high ARA or control diet for 17 days (44 DPF) showed major metabolic profile differences as investigated in our previous article [21]. Thus it is difficult to explain the low effect at gene expression level in the parental generation. Variation in gene expression is often invoked to explain metabolic differences [62]. In addition, for this study we found no overlap between the two generations, and even reducing the cut-off stringency for DEGs in F_0 generation gave very few genes overlapping between F_0 and F_1 generation. Furthermore, the differences between F_0 control and F_1 control patterns were larger than expected. One would expect that they cluster closer together as both were fed the control diet. Although many factors might apply, it is conceivable that a differently composed diet fed to previous generations of the present F_0 generation might have influenced F_0 transcriptomic patterns. The latter one would also explain the small differential gene expression between F_0 control and high ARA group. When comparing body weight in F_0 and F_1 generation, we previously reported a difference in F_0 juveniles, whereas this effect disappeared at later stages (91 DPF) [21]. Similar, there were no differences in body weight between the two groups of adult F_1 progeny.

Despite the weak coherence between transcriptomic and metabolic profile in F_0, there was a link between the parental metabolic data and the gene expression in the F_1 progeny. In the previous metabolomics study of the F_0 parental fish, we observed decreased levels of glutathione, glutamine and cysteine and increased levels of oxidized

metabolites of cysteine and methionine derivatives indicating an oxidized environment due to increased n-6 PUFA levels. Similarly in the present study, transcriptomic analysis of progeny livers (F_1 high ARA) implied an increased expression of genes involved in the methionine cycle, cysteine (transsulfuration) and glutamate synthesis (Fig 5). This can in turn involve alterations in pathways such as glutathione or folate-mediated 1-C metabolism by affecting redox homeostasis or methylation-dependent functions such as for epigenetic modifications. The findings described above indicate major compensatory mechanisms in the progeny most likely owing a modulation of the transcriptome by the oxidized and pro-inflammatory environment previously reported in the parental fish fed high ARA. Despite giving both progeny groups the control diet, we found major differences in hepatic transcriptomic profiles at adult stage.

The present results suggest that parental high dietary ARA affected RXR activated pathways in the progeny. 'PPARα/RXRα Activation' and 'LXR/RXR Activation' pathways were among the top affected pathways according to the IPA® analysis. Several genes belonging to retinol metabolic pathway were differentially expressed in adult progeny. Retinoids and their metabolites are potent activators controlling a range of essential physiological processes such as growth, limb patterning, eye vision, spermatogenesis and cell differentiation [63, 64]. Regulating their action is important for normal embryo development and epithelial differentiation, and disruption of signalling can have detrimental effects on the organism [65-68].

Retinoid metabolites act on lipid signalling pathways by activating RXRs which in turn form heterodimers with PPAR-lipid complexes (Fig 4). *Rxraa*, coding the RXRα protein, was shown to be downregulated in F_1 high ARA livers consistent with the observed downregulation of other genes involved in retinoid signalling. The PPAR complex controls transcription of target genes involved in lipid signalling and metabolism [69-71]. Several genes acting downstream of the nuclear receptor PPARα/RXRα complex were also found to be differently expressed in F_1 high ARA livers. For example *elovl4b*, *acox1*, *acaca* and *fasn* encode enzymes regulating fatty acid levels, particularly by influencing fatty acid biosynthesis, transport and peroxisomal *β*-oxidation. Modulating these pathways influence energy expenditure, membrane composition and fatty acid distribution. In addition, oxidation of fatty acids were among the top lipid metabolic

pathways enriched in the F_1 high ARA group. We reported previously that elongated ARA metabolites and dicarboxylic acid levels were increased in the parental high ARA group [21]. Those results suggested an increased *β*-oxidation, particularly peroxisomal *β*-oxidative degradation of long-chain fatty acids, which can be associated with the observed transcriptomic changes related to fatty acid metabolism in the progeny.

Furthermore, studies have also shown a link between lipid and folate metabolism where PPARα seem to be involved in the regulation of key enzymes along the choline oxidative pathway which is closely linked to the methionine cycle [72, 73]. In the present study IPA® reported the methionine degradation pathway as one of the significantly affected pathways in the progeny, despite no indicated direction of influence (z-score). It has been previously shown that the expression of genes regulating homocysteine synthesis from methionine were sensitive to either high dietary n-3 or n-6 PUFAs [74]. It is conceivable that our above described gene expression changes in fatty acid metabolism and the observed expression changes of genes involved in transsulfuration pathway and methionine cycle are linked.

Increased transcripts of estrogen receptor 1 and vitellogenin 5 were observed in male progeny livers associated with parental high dietary ARA levels. Interestingly, the same transcripts showed oppositely directed gene expression (regardless the chosen threshold) in the parental fish (F_0 high ARA) suggesting compensatory mechanisms being involved. Vitellogenin, a lipid transporting protein, is produced in the liver and transported to the ovary as an egg yolk protein, under the regulation of estrogens in female fish [75-77]. Dietary ARA has been suggested to affect oocyte maturation and especially steroidogenesis in fish, but knowledge on mechanisms and impact on the following generations is lacking [78-80]. Induction of vitellogenin in male fish is commonly used as a marker for endocrine disruption [28] and studies on zebrafish have shown that vitellogenin synthesis can be induced in male fish when exposed to an estrogen (steroid hormone) named 17*β*-estradiol [81, 82]. In addition, vitellogenin has also an immunological role facilitating the defence against virus and bacteria [83, 84]. The exact biological role of vitellogenin in male fish is not clear. In humans, actions of estrogens are mediated by estrogen receptors and their role has been linked to metabolic inflammation [85]. Estrogens can regulate various energy metabolism pathways and

disturbance by endocrine disruptors has been discussed in development of obesity [86]. However, our results from livers of male zebrafish progeny suggest that hepatic gene regulation related to steroidogenesis and estrogen signalling are sensitive to parental dietary high ARA intake.

We hypothesise that the observed DEGs in F_1 high ARA livers compared to the control group, were associated with parental diet, but the exact modulatory mechanisms are not known. One mechanism whereby maternal diet can impact the progeny's physiological status is the contribution to the nutrient reservoir in the developing oocyte [28, 87, 88]. Maternal transcripts present in the fertilized egg can steer gene expression in the developing embryo [89-91]. Epigenetic modifications of the genome in the gametes have also been identified as likely mechanisms through which environmental conditions, such as diet, can affect progeny transcriptomic patterns [26, 92]. Previous studies on zebrafish have shown that nutritional status of the parents such as vitamin B or vitamin E deficiency altered the transcriptome of their embryos [31, 32]. Dietary PUFAs have been shown to affect membrane composition, cell signalling, gene expression and metabolism of the developing oocyte and thereby being able to influence the development of the next generation [7, 36, 93, 94].

Zebrafish has become a favoured research tool to investigate both molecular biological processes and the importance of nutrition in developmental, health and disease outcomes in vertebrates [44, 49, 95, 96]. Due to genetic, anatomical and physiological similarities to other vertebrates, zebrafish can be a useful model to evaluate the influence of dietary profiles on gene expression that can persist throughout life and across multiple generations as shown for different teleost species before [31, 32, 45, 50, 97]. Here, we demonstrated that parental diet affect the hepatic transcriptomic profiles in adult progeny. At the transcriptional level, we found that parental high ARA had a greater effect in the progeny than in the parents who were directly exposed to the experimental diets. These results are surprising, and show that the nutritional priming from parental generation has substantial effect on the progeny transcriptional profile. However, other factors could have influenced the lack of differential expression in the parental generation such as previous dietary treatments and time of sampling in relation to feeding. More knowledge is needed on how parental dietary habits can shape the progeny's transcriptome and

thereby possibly alter metabolic pathways in the progeny. Whether these differences can be inherited to further generations is an area for further research.

Conclusions

Our work has shown that the parental diet modulated the transcription of a range of genes in the adult progeny connected to the fatty acid and retinoid metabolism, methionine cycle, transsulfuration pathway and estrogen signalling. We cannot distinguish if the effect at the transcriptional level is due to the nutritional composition of eggs (oocytes), maternal mRNA deposition or progeny transcriptome potential through programming of the gametes. Modulation of the transcriptome at early life stages can in turn affect metabolic pathways and their activity at later life stages. To our knowledge, the present study is the first one investigating liver transcriptome characteristics of adult zebrafish progeny from parents fed high ARA levels. Nevertheless, further study is required to understand deeper mechanisms on how those gene expression differences observed in adult progeny develop and if these effects can be transferred to future generations.

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Author Contributions

KKL, KHS and MM designed the study. ACA, KHS, KKL and MM performed the experiment. ACA, KHS, and KKL performed the analyses. PW and ACA analysed the data. ACA wrote the article with input from KKL, KHS, PW and MM.

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Supplementary information

S1 File. Composition of control and high ARA diet.

Table 4. Ash, lipid, protein and energy composition.

	Ash (g/100g ww) [1]	Lipid (g/100g ww) [1]	Protein (g/100g ww) [1]	Energy (J/g ww) [1]
Control diet	6.8	12.2	50	21100
High ARA diet	7.0	12.9	50	21400

[1] Data are expressed as mean of two technical replicates.

Table 5. Ingredients and nutrient composition.

Diet preparation has been previously presented: Adam AC, Lie KK, Moren M, Skjaerven KH. *High dietary arachidonic acid levels induce changes in complex lipids and immune-related eicosanoids and increase levels of oxidised metabolites in zebrafish (Danio rerio).* Br J Nutr. 2017 May 09:1-11.

Ingredients	Control (g/kg DM)	High ARA (g/kg DM)
Protein blend *	767·9	767·9
Agar †	1·0	1·0
Fish oil ‡	8·0	8·0
Rape seed oil §	48·0	20·0
Flax seed oil §	20·0	4·0
Cargill´s ARA-rich oil ‖	4·0	48·0
Dextrin †	46·17	46·17
Cellulose ¶	19·3	19·3
Lecithin **	20·0	20·0
Mineral mix ††	50·0	50·0
Vitamin mix ‡‡	10·0	10·0
Methionine §§	2·5	2·5
Cyanocobalamin (1 %) ‖‖	0·99998	0·99998
Folic acid (97 %) ‖‖	0·0111	0·0111
Pyridoxine hydrochloride ‖‖	0·0199	0·0199
Astaxanthin ¶¶	0·3	0·3
Sucrose †	1·0	1·0
Tocopherol mix ***	0·75	0·75

ARA, arachidonic acid.

* BioMar AS products: fish meal, 5 %; krill meal, 1 %; soya protein concentrate, 6·2 %; maize, 5 %; wheat, 7·5 %; wheat gluten, 13 %; pea protein, 49·8 %; field peas, 12·5 %.

† Dissolved in 200 ml heated Milli-Q water, Sigma Aldrich Norway AS.

‡ Cod liver oil; Møllers, Axellus AS.

§ Rømer Produkt.

‖ Donated by Cargill (40 % ARA, Alking Bioengineering).

¶ Sigma Aldrich.

** Alfa Aesar.

†† Merck; ingredients (g/kg of diet): $CaHPO_4$ x $2H_2O$, 30; $CoCl_2$ x $6H_2O$, 0·007; $CuSO_4$ x $5H_2O$, 0·02; K_2SO_4, 15; KI, 0·05; $MgSO_4$ $7H_2O$, 5; $MnSO_4$ x H_2O, 0·05; NaCl, 2·873; Se-yeast, 0·2; $ZnSO_4$ x $7H_2O$, 0·5; $FeSO_4$ x $7H_2O$, 0·6.

‡‡ Obtained from Vilomix Norway AS, Norway; without cyanocobalamin, folic acid and pyridoxine hydrochloride (vitamin B_6) because of the trial set up with two directions (mg/kg of diet): vitamin A, 20; vitamin D, 4; vitamin E (50 %, acetate), 200; vitamin K (50 %), 10; vitamin C (35 %, phosphate), 350; choline, 1000; ascorbic acid, 1000; thiamine hydrochloride, 15; riboflavin (80 %), 19; nicotinamide, 200; inositol, 400; calcium pantothenate, 60; biotin (2 %), 50; filler (protein blend), 6672.

§§ Sigma-Aldrich.

‖‖ Normin AS.

¶¶ Dissolved in the agar solution; provided as a gift from G.O. Johnsen AS.

*** Provided as a gift from BASF.

S2 File. *Danio rerio* primer sequences used for real-time qRT-PCR.

gene	accession no.	forward primer (5` - 3`)	reverse primer (5` - 3`)	amplicon size (bp)	PCR efficiency
eef1a1l1 *	NM_131263.1	AGACAACCCCAAGGCTCTCA	CTCATGTCACGCACAGCAAA	126	1.91
uba52 *	NM_001037113	CGAGCCTTCTCTCCGTCAGT	TTGTTGGTGTGTCCGCACTT	126	2.07
rpl13a **	NM_212784	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	148	1.85
acaca	XM_021476192.1	AACCATCACCGAGGAGGAGA	GTGGAGTCTTGCTCTGCCAT	157	2.00
map2k6	NM_001312870.1	GAGACCTGGACTCCAAAGCC	TTGTCCACCACTCCATACGC	115	1.99

* Olsvik PA, Williams TD, Tung HS, Mirbahai L, Sanden M, Skjaerven KH, et al. Impacts of TCDD and MeHg on DNA methylation in zebrafish (Danio rerio) across two generations. Comp Biochem Physiol C Toxicol Pharmacol. 2014 Sep;165:17-27.

** Tang R, Dodd A, Lai D, McNabb WC, Love DR. Validation of zebrafish (Danio rerio) reference genes for quantitative real-time RT-PCR normalization. Acta Biochim Biophys Sin (Shanghai). 2007 May;39(5):384-90.

S3 File. Gene counts of significant differentially expressed genes (adjusted $p<0.05$) in F_0 and F_1 zebrafish livers obtained from RNA-sequencing and read mapping to the RefSeq and Ensembl reference genome (GRCz10).

	RefSeq			Ensembl			Concordant genes
	Up	Down	Sum	Up	Down	Sum	Sum
F_0	27	12	39	19	8	27	20
F_1	267	315	582	290	315	605	458

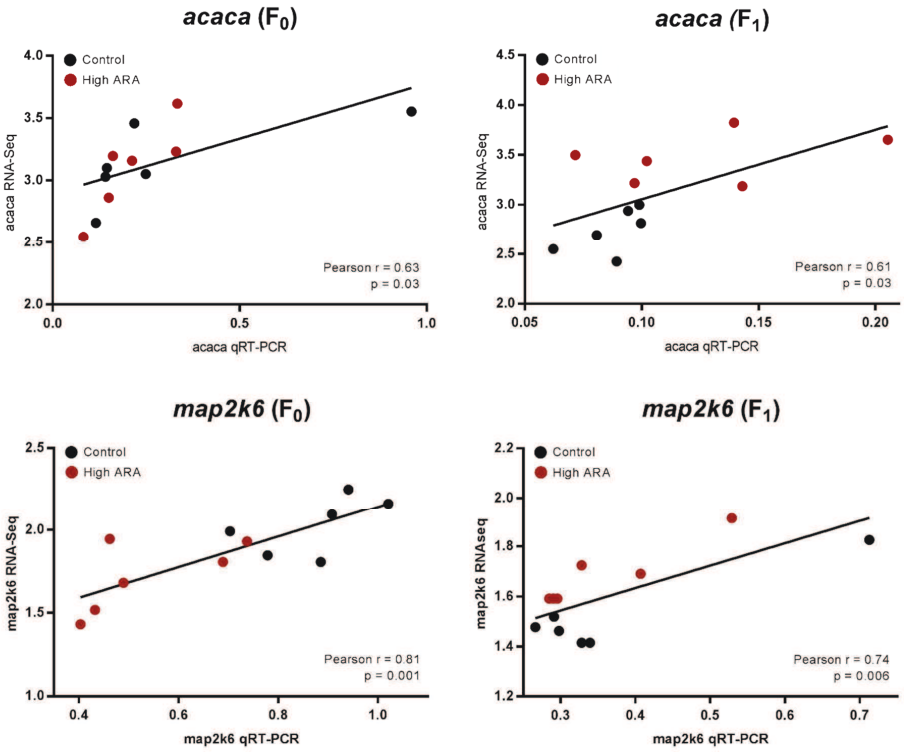
S4 File. Ingenuity® Pathway Analysis identified lipid metabolism associated biological functions represented by differentially expressed genes (adjusted p<0.05) from RNA-sequencing of F_1 high ARA compared to control livers.

Biological Functions	p-value	z-score [1]	Gene symbol [2]
concentration of phospholipid	3.82E-04	1.969	ACACA,CBS/CBSL,CHKA,DGKZ,FASN,LYST,NPC2,PITPNB,PLPP2,RGN,SCARB1,SREBF1,VLDLR
clearance of lipid	5.72E-03	-1.960	C3,CYP3A4,SCARB1,VLDLR
oxidation of fatty acid	2.04E-03	1.622	ACACA,ACOX1,ADIPOR1,C3,CYP3A4,FASN,PDK4,PRKAG2,SLC25A17,SLCO2A1,SREBF1
synthesis of lipid	3.27E-06	-1.375	ACACA,ACLY,AHR,AKR1B1,ALDH1A2,APOB,ATP1A1,BCO1,C3,CACNA1H,CD9,CERS5,CHKA,CREB3L3,CYP39A1,CYP3A4,CYP46A1,DAGLA,DGKZ,ELOVL4,ESR1,FASN,FDX1,GSTA1,IGFBP2,ITGB1,LEPR,NPC2,PARK7,PDK4,PLCE1,PRKAG2,PTGDS,PTGES3,RGN,RXRA,SCARB1,SERPINA1,SH3KBP1,SREBF1
concentration of choline-phospholipid	6.15E-03	1.342	ACACA,CHKA,FASN,LYST,SREBF1
transport of fatty acid	4.49E-03	1.257	ABCC6,FABP7,SCARB1,SLC13A3,SLC25A17,SLCO2A1
concentration of acylglycerol	2.26E-06	1.145	ACACA,ACLY,ADIPOR1,AKR1B1,APOB,ATP2A2,C3,CBS/CBSL,CHKA,CREB3L3,CYP3A4,DAGLA,FASN,FMO5,HELZ2,LEPR,MGLL,PDK4,RGN,RXRA,SCARB1,SREBF1,STEAP4,VLDLR
synthesis of terpenoid	4.41E-04	-1.131	ACLY,AHR,ALDH1A2,APOB,ATP1A1,BCO1,CACNA1H,CYP39A1,CYP46A1,ESR1,FDX1,GSTA1,IGFBP2,PRKAG2,SCARB1,SERPINA1,SREBF1
incorporation of lipid	6.20E-03	-1.127	ACLY,C3,FASN,SCARB1
concentration of progesterone	5.80E-03	-1.067	CBS/CBSL,COMT,ESR1,LEPR,SCARB1
concentration of long chain fatty acid	1.57E-03	1.067	ACLY,DAGLA,SCARB1,SREBF1
transport of steroid	2.60E-03	-1.026	ABCA12,APOB,CANX,CYP3A4,CYP46A1,NPC2,SCARB1,SLC25A1,SREBF1,VLDLR
export of cholesterol	9.21E-04	-0.998	ABCA12,APOB,CANX,CYP46A1,NPC2,SCARB1,SLC25A1,SREBF1,VLDLR
catabolism of lipid	2.61E-05	-0.931	AHR,APOB,COMT,CYP39A1,CYP3A4,CYP46A1,DAGLA,HEXA,MGLL,SCARB1,VLDLR
metabolism of membrane lipid derivative	2.61E-04	-0.919	ACLY,APOB,CD9,CERS5,CHKA,CYP39A1,CYP46A1,DGKZ,FASN,FDX1,HEXA,ITGB1,LEPR,LYST,NSDHL,RXRA,SCARB1,SERPINA1,SH3KBP1,SREBF1,VLDLR
accumulation of sphingolipid	1.72E-03	-0.857	ALDH1L1,CERS5,ELOVL4,HEXA,NPC2
conversion of fatty acid	2.72E-04	0.842	ACACA,ACLY,ACOX1,CYP3A4,FASN,SREBF1
exposure of lipid	1.58E-03	-0.811	CD9,CYP3A4,ITGB1,LGALS2,PRKCQ
exposure of phospholipid	9.63E-03	-0.811	CD9,ITGB1,LGALS2,PRKCQ
efflux of cholesterol	3.22E-03	-0.695	ABCA12,APOB,CANX,NPC2,SCARB1,SLC25A1,SREBF1,VLDLR
concentration of lipid	2.49E-06	0.672	ACACA,ACLY,ADIPOR1,AHR,AKR1B1,APOB,ATP1A1,ATP2A2,BCO1,C3,CBS/CBSL,CFD,CHKA,COMT,CREB3L3,CYP3A4,DAGLA,DGKZ,ESR1,FASN,FMO5,GNE,GSTA1,HELZ2,HMGN1,LEPR,LOC102724788/PRODH,LYST,MGLL,MRC1,NPC2,PDK4,PITPNB,PLPP2,PTGDS,PTGES3,RBP2,RGN,RXRA,SCARB1,SLCO2A1,SREBF1,STEAP4,VLDLR
metabolism of terpenoid	1.33E-08	-0.625	ACLY,AHR,ALDH1A2,APOB,BCO1,CACNA1H,COMT,CYP39A1,CYP3A4,CYP46A1,ESR1,FDX1,GSTA1,LEPR,NPC2,NSDHL,PLEKHA1,RBP2,RETSAT,RXRA,SCARB1,SERPINA1,SREBF1,SULT1A1,VLDLR
fatty acid metabolism	4.13E-06	-0.601	ABCA12,ABCC6,ACACA,ACLY,ACOX1,AKR1B1,APOB,CANX,CD9,CERS5,CHKA,CYP3A4,CYP46A1,ELOVL4,FABP7,FASN,FDX1,HBP1,LEPR,MGLL,NPC2,PARK7,PDK4,PRKAG2,PTGDS,PTGES3,RGN,RXRA,SCARB1,SLC13A3,SLC25A1,SLC25A17,SLCO2A1,SREBF1,VLDLR
steroid metabolism	3.81E-06	-0.581	ACLY,APOB,CACNA1H,COMT,CYP39A1,CYP3A4,CYP46A1,ESR1,FDX1,GSTA1,LEPR,NSDHL,PLEKHA1,RXRA,SCARB1,SERPINA1,SREBF1,SULT1A1,VLDLR
synthesis of steroid	1.24E-03	-0.542	ACLY,AHR,APOB,ATP1A1,CACNA1H,CYP39A1,CYP46A1,ESR1,FDX1,GSTA1,IGFBP2,PRKAG2,SCARB1,SERPINA1,SREBF1
accumulation of acylglycerol	4.35E-04	0.501	ACACA,ADIPOR1,APOB,MGLL,PDK4,SCARB1,SREBF1,TAS1R3,VLDLR
oxidation of lipid	2.58E-03	0.496	ACACA,ACOX1,ADIPOR1,ALDH1A2,C3,CYP3A4,FASN,PDK4,PRKAG2,SCARB1,SLC25A17,SLCO2A1,SREBF1
concentration of sterol	1.10E-03	0.409	AHR,APOB,ATP1A1,CBS/CBSL,CHKA,CYP3A4,ESR1,FMO5,LEPR,MGLL,NPC2,RGN,RXRA,SCARB1,SREBF1,STEAP4,VLDLR
synthesis of acylglycerol	1.83E-03	0.264	C3,CREB3L3,DAGLA,FASN,PLCE1,RGN,SCARB1,SREBF1
synthesis of fatty acid	6.58E-03	-0.258	ACACA,ACLY,AKR1B1,APOB,CYP3A4,ELOVL4,FASN,LEPR,NPC2,PARK7,PDK4,PTGDS,PTGES3,RGN,RXRA,SREBF1
accumulation of lipid	2.92E-06	-0.257	ACACA,ACOX1,ADIPOR1,AHR,ALDH1L1,APOB,BCO1,CERS5,COL14A1,ELOVL4,FABP7,FASN,HEXA,LEPR,MGLL,NPC2,PDK4,RETSAT,SCARB1,SREBF1,TAS1R3,VLDLR,YY1
concentration of cholesterol	1.45E-03	0.255	AHR,APOB,ATP1A1,CBS/CBSL,CYP3A4,ESR1,FMO5,LEPR,MGLL,NPC2,RGN,RXRA,SCARB1,SREBF1,STEAP4,VLDLR
metabolism of acylglycerol	2.15E-04	-0.237	APOB,C3,CREB3L3,DAGLA,DGKZ,FASN,MGLL,PLCE1,RGN,SCARB1,SREBF1
concentration of triacylglycerol	5.21E-06	0.228	ACACA,ACLY,ADIPOR1,AKR1B1,APOB,ATP2A2,C3,CBS/CBSL,CHKA,CYP3A4,FASN,FMO5,HELZ2,LEPR,MGLL,PDK4,RGN,RXRA,SCARB1,SREBF1,STEAP4,VLDLR
metabolism of cholesterol	1.04E-06	-0.226	ACLY,APOB,CYP39A1,CYP46A1,FDX1,LEPR,NSDHL,RXRA,SCARB1,SERPINA1,SREBF1,VLDLR

concentration of fatty acid	1.91E-04	0.137	ACACA,ACLY,AKR1B1,APOB,C3,CBS/CBSL,DAGLA,LEPR,LOC102724788/PRODH,MGLL,PDK4,PTGDS,PTGES3,RXRA,SCARB1,SLCO2A1,SREBF1,VLDLR
transport of lipid	4.70E-05	-0.099	ABCA12,ABCC6,APOB,CANX,CHKA,CYP3A4,CYP46A1,FABP7,HBP1,NPC2,SCARB1,SLC13A3,SLC25A1,SLC25A17,SLCO2A1,SREBF1,VLDLR
accumulation of triacylglycerol	1.30E-03	0.056	ACACA,ADIPOR1,APOB,PDK4,SCARB1,SREBF1,TAS1R3,VLDLR
flux of lipid	2.44E-03	0.005	ABCA12,APOB,CANX,NPC2,SCARB1,SLC25A1,SLCO2A1,SREBF1,VLDLR
catabolism of terpenoid	2.91E-06		AHR,COMT,CYP39A1,CYP3A4,CYP46A1,SCARB1,VLDLR
catabolism of steroid	1.17E-05		COMT,CYP39A1,CYP3A4,CYP46A1,SCARB1,VLDLR
catabolism of cholesterol	4.49E-05		CYP39A1,CYP46A1,SCARB1,VLDLR
conversion of malonyl-coenzyme A	2.67E-04		ACACA,FASN
metabolism of acetyl-coenzyme A	3.39E-04		ACACA,ACLY,FASN
metabolism of retinoid	6.76E-04		AHR,ALDH1A2,BCO1,CYP3A4,RBP2,RETSAT
synthesis of acyl-coenzyme A	6.80E-04		ACACA,ACLY,FASN,PDK4
synthesis of myristic acid	7.93E-04		FASN,SREBF1
concentration of malonyl-coenzyme A	7.93E-04		ACACA,ACLY,PDK4,SREBF1
distribution of cholesterol	8.57E-04		ATP1A1,CBS/CBSL,SCARB1
metabolism of estrogen	9.20E-04		COMT,CYP3A4,PLEKHA1,SULT1A1
conversion of cholesterol	1.10E-03		CYP3A4,CYP46A1,RXRA
homeostasis of lipid	1.27E-03		ABCA12,ACACA,APOB,C3,CYP3A4,GOT1,LYST,MAT1A,NPC2,SCARB1,SREBF1
conversion of acyl-coenzyme A	1.38E-03		ACACA,ACLY,FASN
synthesis of acetyl-coenzyme A	1.38E-03		ACLY,FASN,PDK4
synthesis of malonyl-coenzyme A	1.57E-03		ACACA,FASN
abnormal quantity of lipid	1.85E-03		ESR1,HELZ2,MRC1,NPC2,RBP2,RGN,SCARB1,SLCO2A1,STEAP4
accumulation of asialo GM2 ganglioside	2.59E-03		HEXA,NPC2
sulfation of 2-hydroxyestradiol	2.59E-03		SULT1A1,SULT1A3/SULT1A4
synthesis of palmitic acid	3.47E-03		ACACA,FASN,SREBF1
mobilization of acylglycerol	3.84E-03		APOB,DAGLA
sulfation of beta-estradiol	3.84E-03		SULT1A1,SULT1A3/SULT1A4
uptake of phospholipid	3.84E-03		PEBP1,SCARB1
catabolism of acylglycerol	4.03E-03		APOB,DAGLA,MGLL
quantity of monounsaturated fatty acids	4.65E-03		ACLY,SCARB1,SREBF1
synthesis of sterol	5.23E-03		ACLY,APOB,CYP46A1,PRKAG2,SERPINA1,SREBF1
concentration of 1,2-dipalmitoylphosphatidylcholine	5.31E-03		ACACA,FASN
conversion of acetyl-coenzyme A	5.31E-03		ACACA,ACLY
quantity of non-esterified fatty acid	6.82E-03		AKR1B1,CBS/CBSL,MGLL
accumulation of D-erythro-C16-ceramide	7.01E-03		ALDH1L1,CERS5
incorporation of glycosphingolipid	7.01E-03		ACLY,SCARB1
synthesis of cholesterol	7.74E-03		ACLY,APOB,CYP46A1,SERPINA1,SREBF1
abnormal quantity of cholesterol	8.55E-03		ESR1,SCARB1,STEAP4
secretion of cholesterol	8.55E-03		APOB,ESR1,SCARB1

[1] IPA® predicts upregulation (positive z-score) or downregulation (negative z-score) of biological functions.

[2] Gene symbols are reported as human orthologue gene symbols.



S5 File. Correlation between gene expression patterns obtained from real-time qRT-PCR and RNA-sequencing of *acaca* and *map2k6* in F_0 and F_1 zebrafish livers.
Line of best fit was applied to best present the relation between the gene expression patterns. Log-transformed raw count data from RNA-sequencing and mRNA levels expressed as mean normalized expression obtained from real-time qRT-PCR were used for Pearson correlation analysis and plotting using GraphPad. Pearson correlation coefficient (r) and p-values are indicated. Dots represent replicates from control (black) and high ARA group (dark red).

S1 Table. Differentially expressed genes in male F_0 high ARA livers compared to control livers using the RefSeq genome.

Please visit http://vedlegg.uib.no/?id=4782d82b2ac351fcd673fcf4ad466dc7 to access the file.

S2 Table. Differentially expressed genes in male F_0 high ARA livers compared to control livers using the Ensembl genome.

Please visit http://vedlegg.uib.no/?id=4782d82b2ac351fcd673fcf4ad466dc7 to access the file.

S3 Table. Differentially expressed genes in male F_1 high ARA livers compared to control livers using the RefSeq genome.

Please visit http://vedlegg.uib.no/?id=4782d82b2ac351fcd673fcf4ad466dc7 to access the file.

S4 Table. Differentially expressed genes in male F_1 high ARA livers compared to control livers using the Ensembl genome.

Please visit http://vedlegg.uib.no/?id=4782d82b2ac351fcd673fcf4ad466dc7 to access the file.

S5 Table. Concordant genes from the comparison of differentially expressed genes (adjusted $p<0.05$) in F_0 generation after mapping to the RefSeq (x) and Ensembl (y) reference genome (GRCz10).

Please visit http://vedlegg.uib.no/?id=4782d82b2ac351fcd673fcf4ad466dc7 to access the file.

S6 Table. Concordant genes from the comparison of differentially expressed genes (adjusted $p<0.05$) in F_1 generation after mapping to the RefSeq (x) and Ensembl (y) reference genome (GRCz10).

Please visit http://vedlegg.uib.no/?id=4782d82b2ac351fcd673fcf4ad466dc7 to access the file.

S7 Table. Concordant genes in F_1 generation uploaded into the Ingenuity® Pathway Analysis software suite using human orthologues.

Please visit http://vedlegg.uib.no/?id=4782d82b2ac351fcd673fcf4ad466dc7 to access the file.

Paper III

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