UNIVERSITY of STIRLING

THE CHSE-214 SALMON CELL LINE AS A MODEL TO STUDY MOLECULAR REGULATION OF LONG-CHAIN POLYUNSATURATED FATTY ACID BIOSYNTHESIS IN SALMONIDS

A THESIS SUBMITTED TO THE UNIVERSITY OF STIRLING

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

by

OLGA LILIANA RUBIO MEJIA

INSTITUTE OF AQUACULTURE, SCHOOL OF NATURAL SCIENCES,

UNIVERSITY OF STIRLING, STIRLING, SCOTLAND, UNITED KINGDOM

MARCH 2015

DECLARATION

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

Signature of candidate: _____

Olga Liliana Rubio Mejía

Signature of principal supervisor: _____

Prof. Douglas R. Tocher

Signature of additional supervisor: _____

Dr Michael J. Leaver

Date: _____

ACKNOWLEDGEMENTS

I am very pleased that my PhD has been a great story and I would like to thank in first instance one of the main characters, my principal supervisor. Thank you Douglas Tocher, for all the time invested in my academic formation. Your guidance, support and encouragement were the secret ingredients for making my PhD a wonderful experience. Thank you for being always available when I needed to talk to you, for taking very seriously your role as a supervisor and my role as a student, for all the feedback you gave me to improve my research work…you definitely went the extra mile! Without any doubt you are one of the best supervisors in the Institute (most probably the best, but don't tell the others…hahaha).

This work would not have been possible without the support of my superhero in the nutrition laboratory, thank you so much James Dick for finding solutions to all the mistakes (I mean, challenges) I made while I was learning. I am proud that I had contributed to improve your expertise, now you can really tell you are the "expert". I really enjoyed my time working with you and your excellent team. Thanks to Irene and Liz for making my time in the lab very enjoyable, you two were a great motivation for me to work in the lab with the nice atmosphere you have created, keep it up girls! I would also like to thank Fiona Muir for the help she offered me during the cell culture work, Matthew Sprague for his patience during the lipid class analyses, and in general to the entire staff of the Institute of Aquaculture.

I am deeply thankful with Monica Betancor and Christian De Santis for their supervision and assistance during the molecular analyses. Thank you guys for sharing your knowledge with me, you made this part of my study a lot easier.

One of the most enjoyable things in the Institute of Aquaculture is the opportunity to meet people and make friends from all over the world. I would like to thank my dear friends, Phuoc, Gustavo, Pedro, Wanna, Sharla, Chacha, Carina, Lislie, Claudia, Taslima, Aliya, Ying, Benedikt, Khalfan, Fani, Chris, Will, Trudie and Giacomo, for all the international dinners we shared, the laughs, parties and the beautiful moments we created together. You all made my PhD experience unforgettable.

I would like to thank my best friend and husband Giuseppe Paladini for being so tolerant, patient and for teaching me to enjoy every moment and to celebrate every success of my PhD, thanks for sharing your passion for the life and for taking me back on track when I lost my way. The care of the families Rubio, Mejía, Paladini and Giustiniani was fundamental to complete this stage of my life. Gracias infinitas a mis hermanas y a mis padres por todo su apoyo y motivación. Grazie mille anche alla mia nuova famiglia Italiana per il supporto e amore che mi date costantemente.

I am deeply thankful to my PhD Committee: Gabriel Mourente, Oscar Monroig and Herve Migaud for their comments and feedback which improved immensely the current study.

Finally, a huge thanks to Conacyt-Mexico (Consejo Nacional de Ciencia y Tecnología) for sponsoring this PhD programme and for allowing me to live all the experiences mentioned above and beyond.

ABSTRACT

The main source of omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA) in our diet is supplied by fish, and an ever-increasing proportion of these are being produced by aquaculture. The drive for the growing market demand and production from sustainable sources has led to the use of high-energy (fat) diets and, recently, to the replacement of fishmeal and fish oil with non-marine components, such as plant meals and vegetable oils that are devoid of n-3 LC-PUFA. Both changes impact greatly on lipid and fatty acid metabolism in fish, with health implications for the fish and the human consumer. This impact highlights the need to investigate the basic molecular mechanisms underlying the regulation of lipid and fatty acid metabolism in fish, specifically focussing on the pathways of lipid homeostasis and LC-PUFA synthesis. The aim of this study was to develop and utilise Chinook salmon embryo (CHSE-214) cell line as a model for Atlantic salmon, *Salmo salar* L., to enable an integrated approach to study the biochemical and molecular regulation of lipid metabolism in fish. In particular, α-linolenic acid (LNA, 18:3n-3) and linoleic acid (LOA, 18:2n-6), which are essential fatty acids abundantly found in vegetable oils, and are precursors of LC-PUFA, were supplemented in combination with other fatty acids, to explore the effect of these on total lipid content, lipid class, FA composition and gene expression of CHSE-214 cell line. Total lipid content was extracted, followed by determination of lipid class and fatty acid analyses. Gene expression analyses of transcription/nuclear factors and various target genes in Atlantic salmon, including those involved in pathways of LC-PUFA synthesis and fatty acid oxidation, were carried out. The results demonstrated that CHSE-214 cell line, under experimental conditions, is able to convert LNA to eicosapentaenoic acid (EPA, 20:5n-3), and LOA to arachidonic acid (ARA, 20:4n-6), but not LNA and/or EPA to docosahexaenoic acid (DHA, 22:6n-3), highlighting the activity of elongase and desaturase enzymes during the conversion process. Changes occurring on the fatty acid profile and also at molecular level were observed. Understanding the role that transcription factors play in the regulation of lipid biosynthesis in fish will allow endogenous LC-PUFA synthesis to be optimised. The results from this study could be used to improve the efficiency of alternative, sustainable diets in aquaculture, while maintaining the nutritional quality of farmed fish for the final consumer. CHSE-214 cell line can therefore be used as a model to study the molecular mechanisms involved in the LC-PUFA biosynthesis, particularly in the conversion of LNA to EPA, which can then be reproduced *in vivo*, saving time and money.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

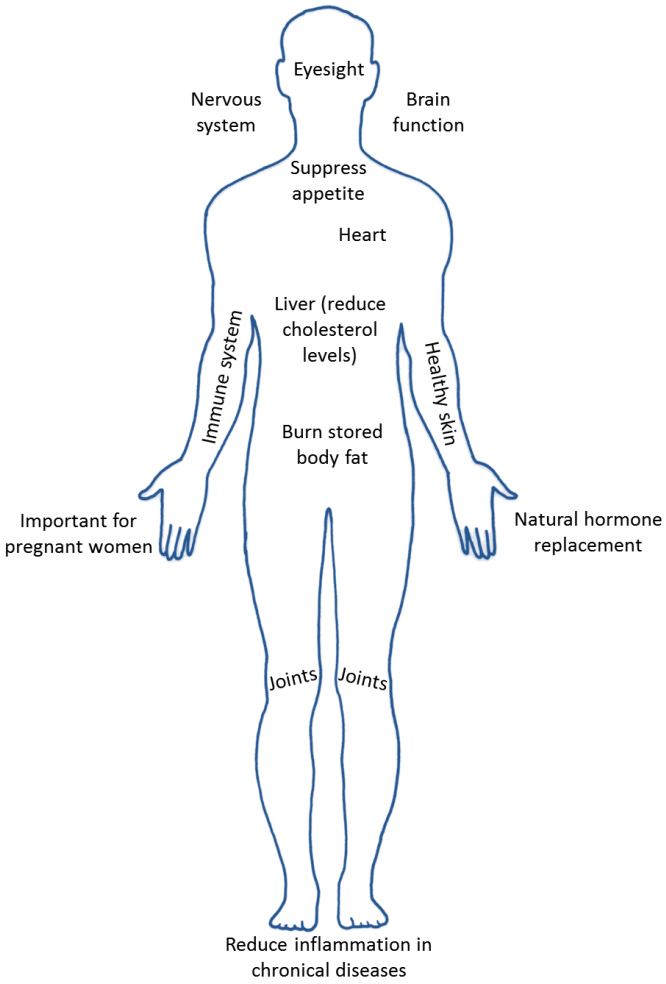
ACO - Acyl CoA oxidase
ARA - Arachidonic acid
AS - Atlantic salmon
β-ACT - β-actin
BHT - Butylated hydroxytoluene
CHOL - Cholesterol
CHSE - Chinook salmon embryo
CPT - Carnitine palmitoyl transferase
DHA - Docosahexaenoic acid
DPBS - Dulbecco's phosphate buffered saline
EDTA - Ethylenediaminetetraacetic acid
EFA - Essential fatty acid
ELOVL – Elongase of very long-chain fatty acids
EPA - Eicosapentaenoic acid
FA - Fatty acid
FABP - Fatty acid binding protein
FAD - Fatty acyl desaturase
FAS - Fatty acid synthase
FBS - Foetal bovine serum
FFA - Free fatty acid
FM - Fishmeal
FO - Fish oil
HPTLC - High-performance thin-layer chromatography
LC-PUFA - Long-chain polyunsaturated fatty acid
LNA - α-Linolenic acid
LOA - Linoleic acid
LXR - Liver X receptor
MTT - Methyl thiazolyl tetrazolium
MUFA - Monounsaturated fatty acid
OFN - Oxygen free nitrogen
PA/CL - Phosphatidic acid/cardiolipin
PBS - Phosphate buffer solution
PC - Phosphatidylcholine
PE - Phosphatidylethanolamine
PI - Phosphatidylinositol
PL - Phospholipids
PPAR - Peroxisome proliferator activated receptor
PS - Phosphatidylserine
PUFA - Polyunsaturated fatty acid
qPCR - Quantitative polymerase chain reaction
RXR - Retinoid X receptor
SFA - Saturated fatty acid
SHK - Salmon head kidney
SM - Sphingomyelin
SREBP - Sterol regulatory element binding protein
TAG - Triacylglycerol
TLC - Thin layer chromatography
UFA - Unsaturated fatty acid
VO - Vegetable oil

LIST OF TABLES

LIST OF FIGURES

Chapter 1

General introduction



Health benefits of n-3 LC-PUFA [original image]

"To eat is a necessity, but to eat intelligently is an art"

Francois de La Rochefoucauld

1.1. Lipids and fatty acids

Lipids, defined as biological substances that are hydrophobic in nature and in many cases soluble in organic solvents, such as chloroform, hydrocarbons or alcohols, represent a large group of chemically heterogeneous compounds, which can often contain long-chain fatty acids (FA) (Smith, 2000). According to their molecular structure, lipids can be subdivided into "simple" (yielding at most two types of products on hydrolysis) and "complex" groups (yielding three or more products on hydrolysis) (Fahy *et al.*, 2005). Moreover, lipids can be classified by their composition as: glycerolipids (containing glycerol), phospholipids (phosphoric acid), glycolipids (carbohydrate), sulpholipids (sulfur-containing group), sphingolipids (sphingosine or other long-chain base), and ether lipids (long-chain alkyl group combined as an ether). Based on their chemical functional backbone they can be categorised as polyketides, acylglycerols, sphingolipids, prenols and saccharolipids (Fahy *et al.*, 2005). Lipids that are found in animals, including fish, can also be classified in two groups according to their solubility: a) neutral, including triacylglycerols (TAG), wax esters, sterols, steryl esters, and free FA; and, b) polar lipids, including phosphoglycerides, sphingolipids, sulpholipids, and glycolipids (Sargent *et al.*, 2002).

1.1.1. Fatty acid nomenclature

As a result of the digestion and metabolism of lipids, smaller molecules called FA are produced. Gustone and Norris (1983) suggested the term "fatty acid" to describe any aliphatic acid, with a chain of ten or more carbon atoms, which occurs naturally in fats, oils and related compounds (lipids), and also for some other acids of closely related structure. In the biota, about 40 FA can be found; structural differences exist depending on their origin, with the more complex compositions occurring in animal oils in comparison with

vegetable oils (VO). FA present in fish oil (FO) can be saturated (all carbons saturated with hydrogen) or unsaturated containing double bonds that are usually in *cis* configuration, and are formed by straight chains with even numbers of carbon atoms in their molecules (between 12 and 24), ethylenic bonds, and a terminal carboxyl group (Turchini *et al.*, 2011).

Most of the FA are designated on the basis of their chain length, their degree of unsaturation (number of double bonds), and the position of their double bonds (Berg *et al.*, 2002). There are two accepted nomenclature systems used in lipid chemistry and aquaculture: the delta (Δ) configuration, and the shorthand systems (the omega (ω) / normal (n) -designation system), differing from each other in the way they describe the double bond position. The delta configuration uses numerical values to indicate the position of each double bond in relation to the carboxyl terminus of the chain. The shorthand system designates the location of the first double bond from the methyl end of FA, but both systems are similar in that they consist of two numbers separated by a colon. The number before the colon gives the number of carbons in the chain and the figure after the colon denotes the number of double bonds (Gurr *et al.*, 2002). The majority of FA also have a common name depending on their origins, and a few of them are just called by their Greek-Latin names, reflecting the number of carbon atoms and double bonds they contain (Table 1.1) (Sargent *et al.*, 2002).

1.1.2. Saturated, monounsaturated, polyunsaturated and long-chain polyunsaturated fatty acids

Depending on the saturation of the carbon chains, FA are classified as saturated (SFA) and unsaturated (UFA). SFA occur naturally in vertebrate species and are the simplest FA. These contain the maximum number of hydrogen atoms and no carbon double bonds, whereas UFA have C=C double bonds. The UFA can be further divided into

monounsaturated fatty acids (MUFA) with one double bond, where oleic acid (18:1n-9) is the most commonly found in the animal kingdom (Turchini *et al.*, 2011); and polyunsaturated fatty acids (PUFA) with two or more double bonds, both presenting Z (*cis*) configuration. Long-chain polyunsaturated fatty acids (LC-PUFA) are a group of PUFA defined by having chain lengths of ≥ C_{20} and with ≥ three double bonds (Gustone and Norris, 1983).

Table 1.1. The trivial names, n-designation, Δ-designation and systematic names of the fatty acids used in the current study

Trivial name	n-designation	Δ-designation	Systematic name
Saturated			
Palmitic	16:0	16:0	Hexadecanoic
Monounsaturated			
Oleic	18:1n-9	18:1Δ9	9-octadecenoic
Gadoleic	20:1n-9	20:1Δ9	11-eicosenoic
n-6 family			
Linoleic	18:2n-6	18:2Δ9,12	9,12-octadecadienoic
γ-Linolenic	18:3n-6	18:3Δ6,9,12	6,9,12-octadecatrienoic
Dihomo-γ-Linolenic	20:3n-6	20:3Δ8,11,14	8,11,14-eicostrienoic
Arachidonic	20:4n-6	20:4Δ5,8,11,14	5,8,11,14-eicosatetraenoic
n-3 family			
α-Linolenic	18:3n-3	18:3Δ9,12,15	9,12,15-octadecatrienoic
Timnodonic	20:5n-3	20:5Δ5,8,11,14,17	5,8,11,14,17-eicosapentaenoic
Clupanodonic	22:5n-3	22:5Δ7,10,13,16,19	7,10,13,16,19-docosapentaenoic
Cervonic	22:6n-3	22:5Δ4,7,10,13,16,19	4,7,10,13,16,19-docosahexaenoic

1.1.3. The n-3 and n-6 series

The n-3 series include α-linolenic acid (LNA, 18:3n-3) and its metabolic LC-PUFA products, *i.e.* eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3); while the n-6 series include linoleic acid (LOA, 18:2n-6) and its metabolic products, *e.g.* arachidonic acid (ARA, 20:4n-6). All of them play important roles in life and health, and PUFA are classified as essential because cannot be produced *de novo* by vertebrates. This means that they must be included in the diet in a proper balance to satisfy physiological requirements. Structurally, n-3 and n-6 series PUFA have generally chain lengths of C_{18}–C_{22} and with two to six double bonds. The main difference between them

lies on the position of the first bond in the carbon chain; n-6 FA have their first double bond between the 6th and the 7th carbon atoms, counted from the methyl (CH_3) terminal carbon atom, while the n-3 family between the 3rd and the 4th (Gustone and Norris, 1983). As both series have vital roles in metabolism, their inclusion in the diet is essential, but special attention must be paid in the ratio between them, as some of them, especially EPA and ARA, compete for the same enzyme systems including desaturases, elongases, cyclooxygenases and lipoxygenases (Okuyama *et al.*, 1997).

1.2. Roles of fatty acids in fish nutrition

In general terms, essential and non-essential FA are involved in several metabolic and physiological processes, such as source of metabolic energy for growth, reproduction, swimming, maintenance of the structure and function of cellular biomembranes, eicosanoid production, absorption and delivery of lipid-soluble nutrients (*e.g.* vitamins and carotenoid pigments), and lipid homeostasis (Tocher, 2003). This is the reason why particular consideration must be taken in current fish feeds regarding dietary oil inclusion.

1.2.1. Energy source

In all organisms, FA function as storage and generation of metabolic energy in form of ATP (adenosine triphosphate), via mitochondrial and peroxisomal β-oxidation, mainly used for heat production (heterothermic fish) and swimming (Sargent *et al.*, 1989). During reproduction FA support the energy requirements of both the parent and the future progeny. Energy requirement varies during embryogenesis and early larval development, depending on the species (Fraser *et al.*, 1988; Ostrowski and Divakaran, 1991; Rainuzzo *et al.*, 1992; Ronnestad *et al.*, 1994, 1995, 1998; Vazquez *et al.*, 1994; Finn *et al.*, 1995; Wiegand, 1996; Mourente and Vazquez, 1996; Mourente *et al.*, 1999). SFA and MUFA are

the main substrates for energy (Henderson, 1996), whereas PUFA are structural components of the cell membrane, and are precursors of biomolecules involved in biological processes such as homeostasis, cell signalling and immune and inflammatory responses (Simopoulos, 2008; Catal, 2009). All the aforementioned FA are introduced in fish feeds in the form of FO. Other lipid sources (*e.g.* VO) are also included providing SFA, MUFA and PUFA, but no LC-PUFA (Tacon and Metian, 2008).

Carnivorous fish have a very limited ability to digest and utilise carbohydrates, probably reflecting their low availability in the natural environment. As a result, dietary protein and lipid represent the major source of energy, with FA as the main source (Watanabe, 1982; Smith, 1989). When formulating diets in the aquaculture industry, protein and lipid are the predominant ingredients, followed by carbohydrates and low amounts of micronutrients including vitamins and minerals. For some species carbohydrates can also be included in significant amounts if their digestibility is improved by processing or cooking, and at level between 10% and 20% of total diet (Tocher, 2003). Protein is the most expensive basal component in a fish feed, which is why it is important to supply the minimum amount required, along with a balanced inclusion of other nutrients as a source of energy to guarantee optimal growth. In this sense, dietary lipids are the primary source to provide energy, having the highest protein-sparing effect due to the fact that lipids provide twice as much energy per unit mass compared to protein and carbohydrate (Sargent *et al.*, 1989; Hemre *et al.*, 1995; Bendiksen *et al.*, 2003). Protein-sparing effect has been defined as a process by which energy is obtained from non-protein sources, *i.e.* lipids and carbohydrates, allowing the protein to be conserved in muscle tissue rather than being used as energy source. With this understanding, high-energy diets, which are in fact high-oil fat diets, have become very popular in the aquaculture field. However, some studies of fish fed with these diets have reported alterations of lipid metabolism,

potentially compromising the health and welfare of the fish and, subsequently, the health of the human consumers. It has been reported that the use of high-energy diets can also change the taste of the final product (Sargent *et al.*, 2002). With most species, lipids can be included in the fish diet in a range of 10–20% of the dry weight of the diet, allowing the use of protein mainly for growth, and avoiding problems like body lipid deposition that can further be reflected in the oily texture of the fish, and could increase the predisposition to rancidity (Cowey and Sargent, 1979; Watanabe, 1982; Bell *et al.*, 1998). However, Atlantic salmon can tolerate higher lipid inclusion, Hemre and Sandnes (1999) reported greater growth in Atlantic salmon, *Salmo salar* L., fed diets containing 38–47% of lipids, compared with fish fed a diet containing 31%. Commercial diets for salmon are rich in oil containing 25–35% (Bell *et al.*, 2002).

1.2.2. Structural function (membranes)

Phospholipids, cholesterol and glycolipids are the major components of vertebrate cell and organelle membranes. These provide a semi-permeable barrier between the intra- and extracellular environments, regulating movements of metabolites and some nutrients into and out of the cell. If the cell membrane composition is altered, the membrane permeability barrier properties change too (Alberts *et al.*, 2002). Phosphoglycerides, a group of phospholipids, are the major constituents of cell membranes in fish, containing mainly 16:0, 18:1n-9, EPA, and DHA (Salem *et al.*, 2001; Sargent *et al.*, 2002). Their role is to maintain the structure and metabolic function of cellular membranes. Although n-3 PUFA are essential for the membrane function in neural tissue, they are also susceptible to the attack of oxygen and other organic radicals. The oxidative damage of n-3 PUFA present in the membrane can affect the cell membrane structure and fluidity, causing pathological effects on cells and tissues (Tocher, 1995, 2003).

1.2.3. Metabolic roles

Dietary lipids are the source for PUFA required for the synthesis of new cellular lipids to support growth and reproduction, and for turnover of existing lipids (Sargent *et al.*, 2002). Specific essential FA (EFA) requirements have to be determined for each fish species, as these vary specifically and quantitatively depending on the stage of the life cycle, the habitat (*i.e.* marine, freshwater), trophic level (*i.e.* herbivores, carnivores) and genetic back-up. A deficiency of EFA would affect biological processes, such as growth and reproduction, and in some cases different pathologies can appear, which may lead to death (Tocher, 2010). Another aspect to take into consideration when formulating fish diets is the competition in terms of desaturase and elongase enzymes that exists between the n-6 and n-3 PUFA, which in turns depends on their concentration and availability. The best results have been reported when EFA requirements and the optimal balance between n-3 and n-6 PUFA are determined in fish diets (Izquierdo, 1996).

1.2.3.1. Eicosanoids

The C_{20} PUFA (*i.e.* 20:3n-6, ARA and EPA) are precursors of biologically active compounds, collectively termed "eicosanoids", including prostaglandins, prostacyclins, thromboxanes, leukotrienes, and lipoxins (Henderson and Tocher, 1987; Sargent *et al.*, 1995a; Tocher, 1995). The eicosanoids are autocrines and have short half-lives. They are involved in the control and regulation of the immune response, inflammatory response, cardiovascular tone, renal function, neural function, blood clotting, timing and regulation of reproduction, and gene expression processes (De Pablo and Alvarez de Cienfuegos, 2000; Calder, 2001; Funk, 2001; Yaqoob, 2004). ARA and EPA are precursors of eicosanoids, while EPA and DHA are precursors of resolvins, maresins and protectins, the latter playing a role in resolving the inflammatory response and resuming homeostasis

(Serhan and Petasis, 2011; Weylandt *et al.*, 2012; Tocher, 2015). Eicosanoids are produced by all body tissues, and their synthesis is influenced by the availability of C_{20} PUFA, and most importantly the EPA:ARA ratio in cellular membranes, which in turn is largely determined by the dietary intake of n-3 and n-6 PUFA (Bell and Koppe, 2011). In mammals as in fish, ARA is the main precursor for the production of 2-series prostanoids, and 4-series leukotrienes. These have higher biological efficacy in comparison with 3-series prostaglandins, and 5-series leukotrienes, which are produced from EPA (Tocher and Sargent, 1987; Bell *et al.*, 1994, 1996a). Studies in fish suggested that EPA competes with ARA in the eicosanoid production, which in turn suppresses the production of ARA-derived eicosanoids, and increases the EPA-derived eicosanoids, which are less biological active (Bell *et al.*, 1994, 1996a). This knowledge must be taken into account when dietary FO is replaced by VO in fish diets, trying to avoid or to minimise the alteration of EPA:ARA ratio, especially because high incidences of cancer, cardiovascular diseases and inflammatory conditions have been reported in developed human populations. This has been associated with an increment of LOA in the diet compared with LNA, favouring the increment of ARA and eicosanoids (Okuyama *et al.*, 1997; Ruxton *et al.*, 2005). A normal physiological mechanism is that during stressful situations eicosanoid production increases, but an excessive production can trigger pathological conditions to the fish in first instance, and to the final consumer (Bell and Koppe, 2011).

1.2.3.2. Transcription factors involved in lipid metabolism in fish

There is a large number of genes involved in lipid homeostasis that are regulated at the transcriptional level, in order to maintain the balance between lipid uptake, transport, deposition, biosynthesis, and metabolism and catabolism (Jump and Clarke, 1999). Lipid-controlling transcription factors studied in fish include: sterol regulatory element-binding

protein (Srebp) 1 and 2, liver X receptor (Lxr), peroxisome proliferator-activated receptors (Ppar) α and β and retinoid X receptor (Rxr) (Cruz-Garcia *et al.*, 2009; Turchini *et al.*, 2011; Minghetti *et al.*, 2011; Carmona-Antoñanzas *et al.*, 2014).

1.3. Fatty acid metabolism

1.3.1. Digestion

From the literature it is known that the digestion process in fish is most likely the same as in mammals (Sargent *et al.*, 1989). However, it is always possible to find differences related to the anatomical body composition between fish species. The digestion process occurs in the intestinal lumen, with absorption in the intestinal epithelial of the enterocytes and subsequent transport of absorbed lipids through lymph or blood (Tocher, 2003).

Digestion of lipids involves some enzymes (lipases) that can be found mainly in the proximal part of the intestine and the pyloric caeca, if present, which also prolongs exposure time to digestive enzymes and provides a greater surface area for absorption. The main source of digestive lipase enzymes in fish is the pancreas or hepatopancreas and, in some species, intestinal cells can actively secrete lipolytic enzymes (Fänge and Grove, 1979). Some extrinsic and intrinsic factors can influence the apparent digestibility of dietary lipids, such as the temperature and/or the degree of lipid saturation (Leger, 1985). Hydrolytic enzymes include triacylglycerol lipases, phospholipases and bile salt-activated lipase, and there are also other less common enzymes, such as cholesteryl ester hydrolase (Sargent *et al.*, 2002).

1.3.2. Absorption and transportation

Free FA are the main product of lipid digestion in fish. In addition to these, acyl glycerols, 2-monoacylglycerols, diacylglycerols and glycerol are produced from the digestion of triacylglycerols; 1-acyl-lysoglycerophospholipid is produced from the digestion of phosphoglycerides, and cholesterol and long-chain alcohols from the hydrolysis of cholesteryl and wax esters, respectively. These products are solubilised or emulsified in bile salt micelles, which can pass through the intestinal mucosa by passive diffusion. This event can happen throughout the whole intestine, but in smaller quantities in distal portions. Inside the intestinal mucosal cells, free FA are re-esterified with glycerol, partial acylglycerols, and lysophospholipids to reform triacylglycerols and phosphoglycerides (Tocher, 2003). Lipids are exported from the intestine as chylomicrons and very low density lipoprotein, generally called "lipoproteins", synthesised in the intestine and liver, respectively, and in this form, they can be transported via lymph and blood to the sites of conversion, storage or energy utilisation (Tocher, 2003).

1.3.3. Biosynthesis (of FA and PUFA)

All organisms, including fish, are able to synthesise SFA, such as palmitic acid (16:0) and stearic acid (18:0), *de novo*, which are the main products of the fatty acid synthase (FAS) enzyme complex. These two FA can be desaturated by the action of stearoyl-CoA desaturase, a desaturase that introduces a double bond into 16:0 and 18:0 at the Δ9 position producing 16:1n-7 (palmitoleic acid) and 18:1n-9 (oleic acid), respectively. However, under normal feeding conditions, most of the SFA and MUFA are suggested to be obtained from the diet (Tocher, 2003). C_{18} PUFA, such as LOA and LNA, cannot be synthesised in vertebrates, and therefore, must be included in fish diets. In some species, these EFA can be further desaturated and elongated to form the LC-PUFA, such as ARA,

EPA and DHA (Figure 1.1), which appear in high concentrations in fish body tissue. These enzymatic reactions occur in the microsomal fraction of cells in various tissues, and although there is competition for the enzymes, the affinity of desaturases and elongases is generally higher for n-3 rather than for the n-6 series (Stubbs and Smith, 1984; Tocher *et al.*, 1989; Gregory *et al.*, 2011).

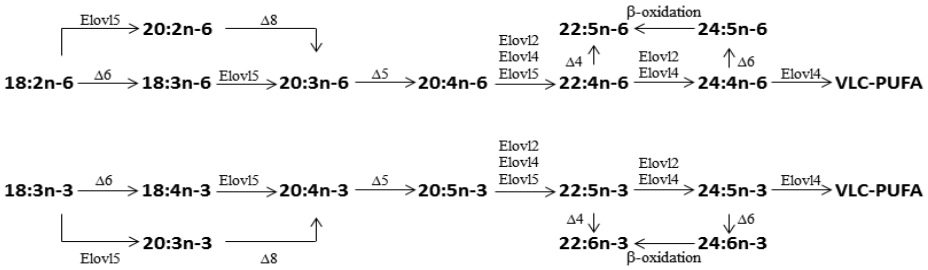


Figure 1.1. Schematic pathways of biosynthesis of long-chain polyunsaturated fatty acid in fish. Modified from Tocher (2015)

1.3.4. Essential fatty acids (EFA)

FA can be classed as essential if they are not synthesised by the organism, or non-essentials if they are. No vertebrate can synthesise PUFA from MUFA, and so PUFA in general are essential. Thus, EFA must be provided by the diet, whilst non-essential FA can be synthesised *de novo* by pathways from non-lipid sources that are similar in mammals (Henderson, 1996). SFA, MUFA and PUFA are found in most living organisms, but n-3 LC-PUFA are mainly in marine organisms, which is explained by the fact that 16:0 and 18:0 can be synthesised *de novo* by all the organisms (Sargent *et al.*, 2002). Fish species can desaturase (via the microsomal Δ9-desaturase) 18:0 to produce 18:1n-9, but the latter cannot be further converted to LOA and LNA, because of the lack of both Δ12 and Δ15 desaturases, which makes these last two PUFA essential (Tinoco, 1982; Stubbs and Smith, 1984; Holman, 1986). Given this, the FA to be considered in fish diets are ARA, and its

metabolic precursor LOA, together with EPA, DHA, and their metabolic precursor LNA (Sargent *et al.*, 2002).

1.3.5. Catabolism and other pathways

Excess dietary lipid is stored mainly in adipose tissue as TAG to cover future energy requirements, representing the most important source of FA for catabolism (Nelson and Cox, 2008). FA β-oxidation is a metabolic process that takes place mainly in the mitochondria (in all tissues), but also in the peroxisomes. It comprises four reactions: oxidation, hydration, second oxidation and thiolysis to produce acetyl-CoA (Gurr *et al.*, 2002). The main purpose of this process is to generate energy from FA by oxidising fatty-acyl-CoA at the β-carbon position, and sequentially removing two carbon units (Henderson, 1996). Once the acetyl-CoA is produced, it enters the citric acid (Krebs) cycle to produce $FADH_2$ and NADH. These last two molecules are further transferred to the electron transport chain, providing the energy for ATP production and oxidative phosphorylation. If fatty-acyl-CoA have more than 12 carbon atoms in their chain, they require to be linked to carnitine via carnitine palmitoyltransferase (CPT-I and CPT-II) and a translocase to enter the mitochondrial matrix. In peroxisomal β-oxidation two-carbon acyl-CoA and hydrogen peroxide are produced instead of $FADH_2$. As this system cannot oxidise long-chain fatty-acyl-CoA completely, its function is to shorten long and very-long FA chains, which can be later transferred to the mitochondria for oxidation via acyl-carnitines (Henderson, 1996). SFA and MUFA (16:0; 18:1n-9; 20:1n-9; and 22:1n-11) are reported as the preferred FA substrates for mitochondrial β-oxidation in fish, while PUFA and LC-PUFA (*i.e.* EPA and DHA) are more retained in tissues to cover other metabolic functions (Sargent *et al.*, 2002).

1.4. Fatty acids in fish nutrition

1.4.1. Fish and aquaculture

Over time, different activities have been developed in order to satisfy human food requirements. However, many of these activities were primarily focused on the quantity rather than the quality of the final food product. This trend has changed recently, with more importance being given to the nutritional composition of food and how it affects human health, prioritising those dietary components that are beneficial to humans. Highly relevant to these activities are fish and seafood represented by wild capture fisheries and aquaculture, which are both focused on the production of food with high nutritional content. In particular, it is important to highlight that the products derived from these activities represent the main source of so-called "omega-3" FA, the n-3 LC-PUFA, EPA and DHA, which are involved in mechanisms of prevention and modulation of cardiovascular diseases, autoimmune diseases, hypertension and neurodegenerative diseases, *e.g.* "Alzheimer" (Givens and Gibbs, 2006).

Traditionally fishmeal (FM) and FO were the main ingredients for fish feeds; however, the drive for increasing production and sustainable development has led to the use of high-energy (fat) diets. The main strategy has been the replacement of FM and FO with non-marine components, such as plant meals and VO that are devoid of n-3 LC-PUFA (Bell *et al.,* 1998). Both of these changes impact greatly on lipid and FA metabolism of the fish, and on health, not only of the fish, but also of the human consumer (Bell *et al.*, 2001, 2003a; Pratoomyot *et al.,* 2008; Betancor *et al.*, 2015).

As aquaculture has been growing faster in recent years, researchers from all over the world have become interested in this field with an objective in common: the provision of farmed fish with a high n-3 LC-PUFA content for human consumption, pushing the

progress of the farms, and paying more attention to the quality of the final product. It is well known that the most expensive input in farm production is the feed, and within the feed, the FM and FO represent the ingredients with the highest cost for fish manufacturers (FAO, 2014).

The growth of aquaculture production estimated for 2012 was higher than expected (2.1%), providing about 50% of fish for human consumption (FAO, 2014). Given that fish obtained from fisheries is decreasing and fish demand for human consumption is increasing, it is estimated that by 2030 aquaculture production will have to satisfy 62% of the total human demand for fish. Therefore, there are good opportunities for aquaculture activities if these are developed and practiced with responsibility, because these activities offer benefit not just for food security but also for economic growth (FAO, 2014). The prices for some captured species, such as Atlantic bluefin tuna *Thunnus thynnus* (L.), Atlantic herring *Clupea harengus* (L.), Atlantic mackerel *Scomber scombrus* (L.), and European squid *Loligo vulgaris* (Lamarck), have increased recently, while farmed fish prices, *e.g.* Atlantic salmon, have been going down as a result of its increasing global aquaculture production, as shown in Figure 1.2.

1.4.2. Atlantic salmon farming

The worldwide production of farmed Atlantic salmon reported by 2012 exceeded two million tonnes (FAO, 2014). Farmed Atlantic salmon constitutes more than the 90% of the farmed salmon market, and more than the 50% of the global salmon market, with the European Union and North America being the major consumers (FAO, 2014). Farmed Atlantic salmon can be sold as a fresh product (*e.g.* whole, chunked or filleted), processed (*e.g.* frozen, smoked) or non-processed, to supply value-added products into the market. As production has increased over the last 10–15 years, the availability of suitable sites for

farming has been decreasing, and farm prices have fallen sharply, restricting further growth in production in the Northern hemisphere. Chile is the most rapidly growing supplier now, which, according to FAO (2014), has low labour and material costs and can therefore effectively compete with traditional producing countries in distant markets.

The environmental effect of Atlantic salmon farming on wild fisheries has been controversial and questioned by many individuals and organisations. Since Atlantic salmon production has traditionally relied on supplies of FM and FO for feed production, the major ecologic areas of concern are represented by local nutrient pollution into water systems, waste feed/faeces, local chemical pollution, use of chemical treatments, escapees, disease spread, global environmental impact and issues of sustainability (FAO, 2014).

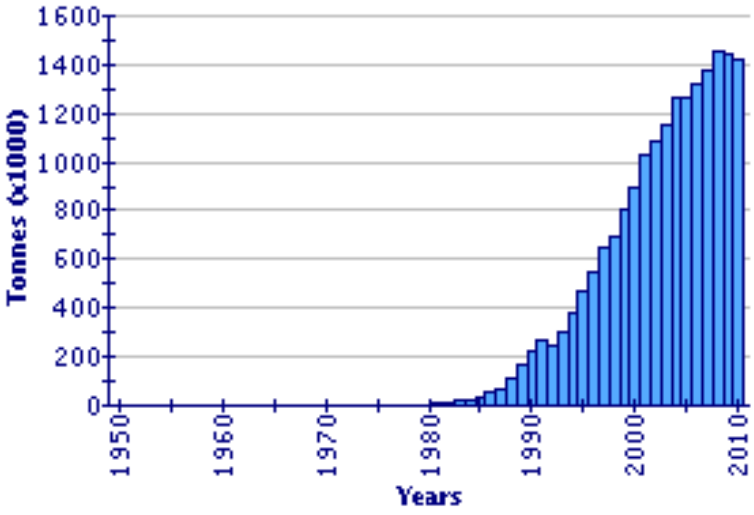


Figure 1.2. Global aquaculture production of Atlantic salmon, *Salmo salar* L., from 1950 to 2010 (from FAO Fishery Statistic, www.seafoodsource.com/newsarticledetail.aspx?id=16044).

1.4.3. Fish nutrition

As a response to the continued growth of the demand for seafood products and a decrease in the supply from natural sources, aquaculture production has become one of the biggest industries all over the world. In intensive aquaculture, nutrition plays a fundamental role, influencing the production cost, fish growth, health and waste production

(Gatlin, 2002). Balanced diet formulations and appropriate feeding practices are necessary to develop nutritious, cost-effective diets, and this goal can be reached by determining the nutritional requirements of fish in their different life stages.

1.4.4. Sustainability (FO substitution)

The FO composition depends primarily on the diets of the wild fish from which the oils are obtained, being predominantly influenced by the FA profiles of marine phytoplankton (the primary producers) and zooplankton, which contain high levels of n-3 LC-PUFA (*e.g.* EPA and DHA) (Jobling, 1993).

Peru, Chile, Thailand, China, USA, Japan, Denmark, Norway, Iceland, and South Africa, are the main global FO producing countries (FIN, 2008). In general FO can be extracted from the body of some so-called "oily" species, such as anchovy, sardine, menhaden, capelin, mackerel and herring and, secondly, from the liver of other fish species, particularly cod and halibut (FIN, 2008).

The price of FO is increasing as a consequence of the high demand and its finite and limited supply, having a negative impact on the cost of production of aquafeeds (FAO, 2014). It is therefore necessary to find new alternative dietary oil sources, in order to contribute to the expansion, development and sustainability of the world aquaculture industry. With the replacement of FO it is a fundamental aim not to compromise the growth of the fish, nor the quality of the final product (Hardy, 2010). As fish have traditionally been the primary and almost unique source of n-3 LC-PUFA in the human diet, strategies to replace dietary FO in aquaculture should be focused on the FA profile of the final product, trying to keep it as similar as possible to natural marine sources (Sargent *et al.*, 2002). The main barrier for FO replacement in this respect is its FA profile, which contains between 15-30% of n-3 LC-PUFA and low levels of n-6 LC-PUFA and LOA,

whereas VO contains neither n-3 LC-PUFA, nor ARA, low levels of LNA and a high amount of n-6 PUFA, mainly LOA (Hertrampf and Piedad-Pascual, 2000).

Many studies on salmon have been performed regarding to the substitution of FO with alternative oil from non-marine sources, such as fats from terrestrial animals (Henderson, 1996), and VO including palm oil (Bell *et al.*, 2002), rapeseed oil, olive oil (Torstensen *et al.*, 2004), sunflower oil, corn oil and soybean oil (Bell *et al.*, 1993; Rora *et al.*, 2005), all demonstrating that the use of alternative oil sources for salmonid production is a feasible solution (Bell *et al.*, 2001). The aim of the aquaculture research community working in this field is to find the adequate balance of EFA and FA for energy in aquafeeds in the replacement of FO with VO, taking into consideration several factors, such as the high growth and survival of the fish, their feed conversion efficiency, immune competence, disease resistance, and high-quality standards of fillet production in terms of n-3 LC-PUFA content (Peng *et al.*, 2008).

1.5. Cell culture

1.5.1. General

According to Lee (2010) tissue culture can be defined as the growth of tissue or cells separated from the organism. It is also known as a technique of keeping tissues alive and growing in an appropriate culture medium. Viable growing tissues, separated from the living animal, was possible using an appropriate culture medium, containing a mixture of nutrients (Lee, 2010). At the beginning of the 20th century tissue culture was first devised with the intention of studying the behaviour of animal cells, free of systemic variations that might affect *in vivo* (Harrison, 1907; Carrel, 1912). The term "cell culture" refers to a culture derived from dispersed cells taken from an original tissue, from a primary culture,

or from a cell line or cell strain by enzymatic, mechanical or chemical disaggregation. The L929 was the first cloned cell strain isolated from mouse L-cells in 1948 using capillary cloning (Sanford *et al.*, 1948). These procedures were facilitated when trypsin became more used for subcultures in the 1950s. In 1952 tissue culture became more widely used, with the introduction of antibiotics, and the development of defined media (Freshney, 2010). Nowadays, cell culture models represent an important biological tool to carry out investigation in research areas, including oncology, virology, physiology, toxicology, and genetics (Lakra *et al.*, 2011).

1.5.2. Fish cell culture

As the aquaculture industry has been growing, many cultures of fish cells of a wide variety of fish species, including Atlantic salmon, Arctic charr *Salvelinus alpinus* (L.), European whitefish *Coregonus lavaretus* (L.), common carp *Cyprinus carpio* (L.), goldfish *Carassius auratus* (L.), and zebrafish *Danio rerio* (Hamilton), have been developed, contributing to increase and/or improve fish production (Freshney, 2010). However, there are still several aquaculture species from which *in vitro* cell culture models are not yet developed.

Fish cells can be maintained *in vitro* as primary cell cultures, which are cells directly derived from the animal tissues, or cell lines, which are primary cells successfully sub-cultured weekly for few months, showing a stable phenotype (Adams, 1980). Cell lines can be derived from a number of fish organs and tissues, such as gonad, liver, pancreas, kidney, heart, spleen, skeletal muscle, and nervous tissue (Bols and Lee, 1991). Most fish cell lines are anchorage-dependent; they can attach and grow as a monolayer on the flask surface at pH 7.4, reaching 100% confluence within 5-7 days (Freshney and Freshney, 2002). In research areas, fish cell cultures are advantageous because of their low

cost, sensitivity, versatility and reproducibility (Visoottiviseth and Chanwanna, 2001), making possible and easier virology, cytogenetic, toxicology, oncology, immunology, temperature effects, and cell physiology studies (Tocher *et al.*, 1995).

1.5.3. Advantages and disadvantages of working with cell culture

The major advantages that cultured cells have over *in vivo* studies are that the living cells can be monitored under the microscope, they offer a homogeneous cell population of identical genetic make-up growing in a constant and/or controlled physico-chemical environment (*e.g.* pH, temperature, osmolality, dissolved gases), as well as they avoid ethical issues. Moreover, physiological conditions (*e.g.* control of hormones and nutrients, concentrations) can be easier controlled *in vitro* compared with *in vivo* experiments, as well as the microenvironment regulation of matrix, cell–cell interaction, and gaseous diffusion, all of these factors might interfere, or might be difficult to control in *in vivo* trials. In addition, cell cultures enable easier determination of cytology and immunostaining, validation and accreditation, origin, history, purity, and can be stored in liquid nitrogen. They also allow replication and variability, easier quantitation, reduced requirement of reagent, direct access to cells, lower cost, control of time ability to define dose, reduction of animal use, cytotoxicity and, moreover, screening of pharmaceutics, cosmetics, *etc.* (Freshney, 2010). However, *in vitro* experiments cannot fully replace *in vivo* studies, as they have some limitations which include necessary expertise, as well as infrastructure. Working with cell line also has a higher risk of microbial and cross-contaminations, and creates issues with containment and disposal of biohazards, quantity and cost capital equipment for scale-up, disposable plastics, genetic instability, markers not always expressed, *etc*. (Freshney, 2010), while *in vivo* experiments might represent more accurately a “snapshot” of the real situation.

1.5.4. State of the art of cell culture studies in fish lipid metabolism

Several successful trials have been carried out investigating lipid and FA metabolism using fish cell lines as model systems. These studies often involved supplementing fish cell lines with PUFA under controlled conditions. Some studies showed it was possible to demonstrate different PUFA requirements between freshwater, anadromous and marine fish species, as occurs in fish in their natural environment and that have been later proved correct with molecular biology procedures (Tocher and Dick, 1990; Tocher *et al.*, 1995; Ghioni *et al.*, 1999). In cell culture, foetal bovine serum (FBS) is added to the growth media and is the main PUFA source. According to Tocher and collaborators (1989), FBS is the serum of choice for fish cell culture due to the difficulties in obtaining fish serum and the risk of contaminating with accidentally introduced viruses. When working with cultured fish cell lines for investigating PUFA metabolism, it is imperative to consider the altered FA profile of fish cell lines, as they have lower levels of n-3 PUFA and LC-PUFA, and higher total n-6 PUFA, compared to the fish tissues they were derived from (Tocher *et al.*, 1989; Tocher and Dick, 1991). There are also some fish cell lines, *e.g.* turbot, *Scophthalmus maximus* (L.), fin cell line (TF) and the gilthead seabream, *Sparus aurata* L., fin cell line (SAF-1), with a limited ability to convert C_{18} to $C_{20,}$ because of their limited $C_{18\text{-}20}$ elongase or Δ5 fatty acyl desaturase (*fads2d5*) activities, respectively (Ghioni *et al.*, 1999). Although investigating LC-PUFA synthesis in cell cultures is less expensive and represents a quicker and more convenient alternative than fish feeding trials, there is no guarantee that the enzymes involved in the LC-PUFA metabolic pathway will be expressed in cultured cells the same way they are expressed in living fish (Tocher, 2003). According to Tocher *et al.* (1989), Tocher and Dick (1991) and Ghioni *et al.* (1999) fish cell lines are useful for investigating the LC-PUFA synthesis pathway, but they have to be subsequently compared with *in vivo* trials.

1.5.5. CHSE-214 cell line

In the current study, the main target species was Atlantic salmon, but there were no appropriate cell lines available. Originally this study was planned in the Atlantic salmon cell line, called AS, used in previous studies (Tocher, 1990; Tocher *et al.*, 1995; Ghioni *et al.*, 1999). However, this cell line was no longer available, and it was not sourced anymore. Other Atlantic salmon cell lines, such as SHK-1, have shown poor growth, and therefore are difficult to be used on a large scale (Minghetti *et al.*, 2011; Carmona-Antoñanzas *et al.*, 2014). As alternative, CHSE-214 cell line, derived from Chinook salmon (*Oncorhynchus tshawytscha* (Walbaum)) embryo, was chosen since it grows fast and it is easy to culture, which in turn enable the generation of a large numbers of cells in a short period of time.

CHSE-214 is an undifferentiated fibroblast-like cell line, widely used in viral diagnoses for fish, including infectious hematopoietic necrosis, viral haemorrhagic septicaemia, spring viraemia of carp, and infectious pancreatic necrosis. Morphologically the cell line CHSE-214 has a fibroblast-like appearance and is adherent to the plate. The method to grow the cell line has been developed and it is nowadays well known (Lannan *et al.*, 1984).

1.6. Gene expression studies

Over the last few years, the basic molecular mechanisms in the control and regulation of lipid and FA metabolism in fish have been investigated, focusing on the pathways of lipid homeostasis and LC-PUFA synthesis. Genes involved in the regulation of FA metabolism can be grouped as follows:

a) Genes involved in LC-PUFA biosynthesis: *fads2d5*, *fads2d6*, *elovl2*, *elovl5*

b) Transcription factors: *srebp1*, *srebp2*, *lxr*, *pparα*, *pparβ*, *rxr*

c) Genes involved in FA metabolism: *fas*, *cpt1*, *aco* (acyl-CoA oxidase), *fabp* (fatty acid binding protein)

1.6.1. Genes involved in LC-PUFA biosynthesis

Long-chain PUFA metabolism in fish has been extensively studied, focusing on the molecular components and regulation of the biosynthesis pathways (Zheng *et al.*, 2004; Morais *et al.*, 2009; Monroig *et al.*, 2009, 2010; Minghetti *et al.*, 2011; Carmona-Antoñanzas *et al.*, 2011; Martinez-Rubio *et al.*, 2013; Betancor *et al.*, 2014, 2015). Thus, cDNAs for fatty acyl desaturases and PUFA elongases (*elovl5*-like elongases) have been cloned from several freshwater and marine fish species (Monroig *et al.*, 2010). All the main components of the pathway, including *fads2d5*, *fads2d6* and *fads2d8*, *elovl2*, and *elovl5* variants a and b, have been cloned from Atlantic salmon (Hastings *et al.*, 2005; Zheng *et al.*, 2005, 2009a; Morais *et al.*, 2009; Monroig *et al.*, 2010, 2011a). The expression of desaturases, and in some circumstances, the *elovl5* is up-regulated in liver and intestine of Atlantic salmon fed with VO, correlated with increased LC-PUFA synthesis in these tissues (Zheng *et al.*, 2004, 2005). The molecular mechanisms involved in regulating desaturase and elongase expression in fish are currently being investigated. There are some studies, in which desaturase and elongase genes from Atlantic salmon and Atlantic cod have been sequenced including up to 4 Kb 5' upstream regulatory region (Tocher *et al.*, 2006; Zheng *et al.*, 2009b).

1.6.2. Transcription factors

SREBP 1 and 2, *LXR*, *PPAR* α and β, and *RXR* are well-known to be transcription factors. All of them play important roles in the gene regulation of LC-PUFA biosynthesis (Duplus *et al.*, 2000; Cruz-Garcia *et al.*, 2009; Turchini *et al.*, 2011; Minghetti *et al.*, 2011;

Carmona-Antoñanzas *et al.*, 2014). *SREBP* belong to the basic helix-loop-helix leucine zipper transcription factor family. Given that SREBP are synthesised as inactive precursors they need to be maturated to function as transcription factors (Desvergne *et al.*, 2006). In mammals, SREBP1 regulates FA/lipid biosynthetic genes, whereas SREBP2 regulates cholesterol biosynthetic genes (Horton *et al.*, 2003). There have been identified three *LXR*: *LXRs*, *LXRα* and *LXRβ*, these nuclear receptors are involved in the cholesterol and lipid metabolism. The *LXR* are transcription factors intimately involved with lipid metabolism, whose activity is modulated by sterols, inducing cholesterol catabolism and *de novo* FA biosynthesis in liver (Repa *et al.*, 2000; Schultz *et al.*, 2000; Cruz-Garcia *et al.*, 2009). Activated *LXR* also stimulates the expression of *SREBP1c* (Schultz *et al.*, 2000), which activates other genes involved in lipogenesis and triglyceride metabolism (Jung *et al.*, 2011). The tissue and nutritional expression of *lxr* of Atlantic salmon and rainbow trout, *Oncorhynchus mykiss* (Walbaum), have been cloned and characterised (Cruz-Garcia *et al.*, 2009). There have been identified three *PPAR* in mammals: *PPARα, PPARβ*, and *PPARγ*. The activation of *PPAR* occurs when these bind FA or their oxidised derivatives (Duplus *et al.*, 2000; Carmona-Antoñanzas *et al.*, 2014). *Ppar* regulate lipid and FA homeostasis in fish, particularly in relation to energy balance, FA oxidation and lipid deposition in tissues (Boukouvala *et al.*, 2004; Kennedy *et al.*, 2006; Leaver *et al.*, 2006; Diez *et al.*, 2007). Studies in Atlantic salmon failed to establish a clear link between *ppar* and expression of desaturase and elongase genes or regulation of LC-PUFA synthesis (Kennedy *et al.*, 2006; Leaver *et al.*, 2006; Villeneuve *et al.*, 2007). *RXR* play multiple roles in the metabolic systems, particularly when they form heterodimers with other nuclear receptors (Pérez *et al.*, 2012). They have been related to the regulation of cell growth, development, survival, cell differentiation and cell death (Dawson and Xia, 2012).

1.6.3. Genes involved in FA metabolism

Some of the genes involved in FA metabolism that have been studied in fish are: *fas*, *cpt1*, *fabp* and *aco*. Of these, *fas* is a key enzyme of lipogenesis; *cpt1* and *aco* are involved in mitochondrial and peroxisomal β-oxidation, respectively (McGarry and Brown, 1997) and *fabp* function as promoters of cellular uptake and transport of FA, and participate in the regulation of cell growth and gene expression (Haunerland and Spener, 2004).

1.7. Aims of the current study

The overarching aim of the present study was to develop and utilise an *in vitro* cell culture model to enable an integrated approach to study the biochemical and molecular regulation of lipid metabolism in fish. Specifically, the plan was to develop a cell culture model for salmon (using the CHSE-214 cell line), and a multi-well plate system to study the roles of various transcription factors in the control and regulation of lipid and FA metabolism, and in particular their potential role in the control of n-3 LC-PUFA synthesis. The study was a blend of cell culture assays, FA and lipid analyses, and gene expression (qPCR) methodologies, designed to investigate the molecular regulation of lipid and FA metabolism. In particular, the initial objective was to determine the effects of dietary (supplemental) FA on cellular lipid content, lipid class, and FA composition of total lipids. In addition, a further objective was to relate the effects on lipid and FA composition on the expression of genes, including the main lipid transcription/nuclear factors (*srebp1*, *srebp2*, *pparα*, *pparβ*, *lxr*, and *rxr*), and some of their key target genes, including those involved in pathways of LC-PUFA synthesis (*fads2d5*, *fads2d6*, *elovl2*, and *elovl5*), and FA oxidation (*fas*, *cpt1*, *aco*, and *fabp*), in Atlantic salmon. The specific objectives were designed in order to achieve the overall

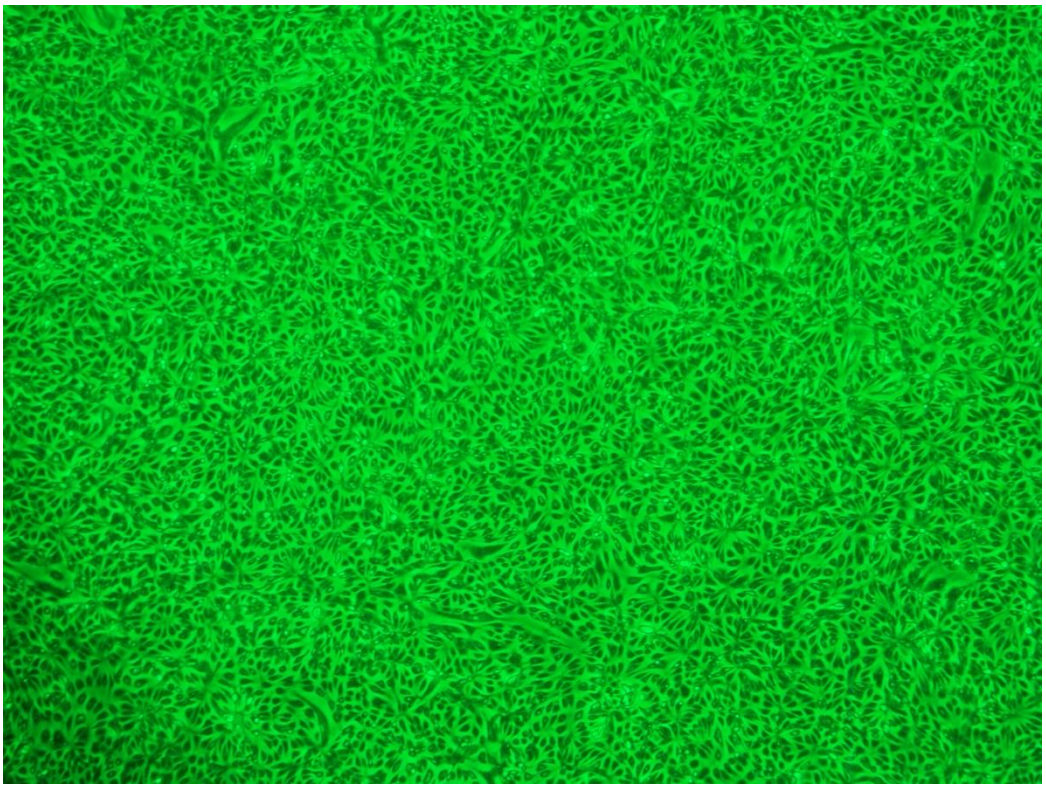
aim by developing the basic science of transcriptional control of lipid metabolism in fish to a position where it is possible to understand the roles of the transcription factors/nuclear receptor, and some of the mechanisms involved in the regulation of lipid and FA metabolism in fish, including how nutritional factors interact and potentially modulate this mechanism.

In summary, the aims of the present study were:

a) To develop and characterise a cell culture model utilising the CHSE-214 cell line to investigate the genetic regulation and control of lipid and FA metabolism in salmonids.
b) To develop appropriate qPCR gene expression assays (primers design) for a wide range of genes related to lipid and FA metabolism.
c) To use the above developed tools to elucidate the roles of key transcription factors/nuclear receptors in the control and regulation of LC-PUFA synthesis.
d) To use the above tools to elucidate the roles of key transcription factors in the control and regulation of FA oxidation, and metabolism.
e) To use the data obtained to generate a model for the integrated control of lipid and FA metabolism in fish.

Chapter 2

Materials and methods



Light micrograph of CHSE-214 cell line [original image]

"Cell culture is a little like gardening. You sit and you look at cells, and then you see something and say, 'You know, that doesn't look right'"

Siddhartha Mukherjee

2.1. CHSE-214 cell culture

2.1.1. Brief description of CHSE-214 cell line

Chinook salmon embryo 214 (CHSE-214) cells were obtained from the Laboratory of Disease and Virology, Institute of Aquaculture, School of Natural Sciences, University of Stirling, Stirling (UK). The established cell line was originally derived from Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum) embryo following enzymatic treatment of the tissue to release the cells using trypsin (Fryer *et al.*, 1965). This monolayer culture has fibroblast-like morphology and has been used in many studies spanning several fields including biomedical research (Amaro and Sánchez, 1997), toxicology (Davoren *et al.*, 2005), virology (Lorenzen *et al.*, 1999; Jensen *et al.*, 2002; McLoughlin and Graham, 2007; Jørgensen *et al.*, 2007; Herath *et al.*, 2009), and bacteriology (Smith *et al.*, 2010).

2.1.2. Routine culture of CHSE-214

Most of the work involving fish cell culture was carried out in the Laboratory of Disease and Virology (UoS) under a vertical laminar airflow hoods (Class II Biohazard Safety Cabinet). The cabinet surface was cleaned and sterilised using 70% ethanol prior to and after the work.

2.1.2.1. Preparation of the media and basic growth conditions

For routine culture to maintain stocks and provide experimental material, cells were grown in 75 cm^2 tissue culture flasks (Sarstedt AG & Co. Laboratories, Nümbrecht, Germany) in 20 ml of Leibovitz's L-15 medium with GlutaMAX™-1 (Gibco®, Life Technologies Ltd., Paisley, UK) supplemented with 200 µM L-glutamine and 10% foetal bovine serum (FBS) at 20ºC. For experiments, cells were also grown in L-15 medium with

200 µM L-glutamine and 5% FBS (to retain normal growth rate) at 20ºC. For lipid and fatty acid analyses 75 cm^2 tissue culture flasks were used in 10 ml. All experiments were performed on cultures at around 80% confluence to ensure that growth continued. In order to provide around 80% cell confluence within 24 h, the seeding density used was $10^4/cm^2$. Subsequently, the experimental settings were applied. All media were prepared under sterile conditions and were kept at room temperature (20ºC) and supplemented weekly with 200 µM L-glutamine.

2.1.2.2. Cell harvesting

CHSE-214 cells were harvested from the 75 cm^2 tissue culture flasks for subsequent procedures, including stock production, preparation of experimental flasks/plates and cell counting. The medium was removed from flasks by aspiration or decanting and the cell monolayers were washed twice with 10 ml Dulbecco's phosphate buffered saline (DPBS). The excess DPBS was removed by pipetting, and cells were harvested by adding 2 ml of 0.05% trypsin/ 0.02% ethylenediaminetetraacetic acid (T/EDTA), with incubation for 2–5 min at 20ºC. When the monolayer became opaque, excess T/EDTA was removed by pipetting and the cells were dislodged by gently tapping the flasks with the hand. Cells were re-suspended in the appropriate volume of the particular medium required depending upon use and gently mixed with the aid of a pipette in order to produce an homogeneous cell suspension for further procedures (*e.g.* cell passaging, cell counting, cell freezing).

2.1.2.3. Cell passaging

Cell passaging (*i.e.* subculturing or cell splitting) is the method whereby portions of cells (aliquots) from a parent culture are transferred into a new flask with fresh medium.

Flasks for routine culture and provision of stock cells were harvested when 100% confluent approximately every week, as described in section 2.1.2.2. Once the cells were detached from the flask they were re-suspended in 3 ml of fresh medium. Three fresh 75 cm^2 flasks were prepared by adding 19 ml of fresh medium to each flask and 1 ml of the homogeneous cell suspension was added to each flask. The cell suspension was gently mixed and the flasks incubated at 20ºC. On the following day, cells were observed under the inverted microscope (Olympus IMT-2) to check on growth and to ensure they reached around 80% confluence if they were to be used in experiments.

2.1.2.4. Cell counting using a haemocytometer

Samples of cell suspension were incubated with trypan blue solution, which is a selective or vital stain used to identify living and dead cells. Dead cells take up the dye and are coloured blue, while viable cells remain colourless due to their membrane permeability and active exclusion of the dye from the cell. This staining method, also known as "dye exclusion method", was used to count the number of viable cells in a suspension using a standard Neubauer chamber haemocytometer (Depth 0.1 mm or 1/400 mm^2). Cells were harvested as described in section 2.1.2.2 and, once the cells detached from the flask, 5 ml of fresh medium were added to re-suspend the cells followed by gentle pipetting to ensure homogeneity. The cell suspension was transferred into a fresh Bijou sample container, from which 0.1 ml was aliquoted and mixed with 0.1 ml of 0.5% trypan blue dye. This mixture was then allowed to stand for 1 min. The haemocytometer coverslip 20×26×0.4 mm (Fisher Scientific, Loughborough, UK) was pressed onto the slide, ensuring there was a good adherence and both chambers of the haemocytometer filled by capillary action with the stained cell suspension using a P100 Gilson pipette. The haemocytometer was placed on the microscope stage, the ruled pattern of the counting chamber was located under a low

power objective, and cells were counted within 1 mm^2 of the central portion of the ruled pattern. This square is divided into 25 smaller squares, each sub-divided in turn into 16 further squares marked by a single line. Only the viable cells were counted.

Calculation:
Depth of fluid in the counting chamber = 0.1 mm
The volume of the central 1 mm^2 was $1\times1\times0.1=0.1$ mm^3 or 0.0001 cm^3 (or 10^{-4} cm^3)
As 1 cm^3 is equivalent to 1 ml, cell concentration per ml will be:
Average count per 1 mm^2 × dilution factor × 10^4

2.1.2.5. Cell freezing and storage

Cells were harvested as described in section 2.1.2.2. Once the cells were detached from the flask, 3 ml of growth medium were added and cells re-suspended by gently pipetting up and down. The mixed cell suspension was collected in a universal container and a viable cell count was performed as described above (section 2.1.2.4). Each cryovial was labelled with the following information: cell line, passage number, freezing date and number of cells. Volume was calculated and adjusted using freezing medium (growth media containg 10% FBS + 10% dimethyl sulphoxide, DMSO) to produce a solution of 3×10^6 cells ml^{-1} per cryovial. All cryovials were placed in the -20ºC freezer for 1 h before being transferred to the -70ºC freezer for a further 2 h. Finally, the cryovials were removed from the polystyrene box, placed in marked cans, and quickly immersed in liquid nitrogen (-196ºC) to avoid any cell thawing.

2.2. Incubation of CHSE-214 cells with fatty acids

2.2.1. Preparation of fatty acid-bovine serum albumin complexes

Cells were incubated with fatty acids (FA) that were added as bovine serum albumin (BSA) complexes (Spector and Hoak, 1969; Tocher *et al.*, 1989). The FA were purchased

from a commercial provider (Sigma-Aldrich® Company Ltd., Dorset, UK) at a purity of 99%. First, 25 mg (25-30 µl) of liquid FA were added to a 10 ml Quickfit tube and dissolved in 200 µl of chloroform/methanol (C/M, 2:1, by volume) containing 0.01% of butylated hydroxytoluene (BHT) as antioxidant. In the same tube, 7 ml of isohexane were added and the FA suspension mixed thoroughly before transferring the solution into a foil-covered 50 ml conical flask containing 800 mg of diatomaceous earth (Celite®, Sigma-Aldrich® Company Ltd., Dorset, UK). The whole mixture was swirled to mix thoroughly and the solvent was then evaporated under a stream of oxygen-free nitrogen (OFN), using the N-Evap evaporator (Organomation Associates Inc., Berlin, MA, USA). A solution of 16.5 mg ml^{-1} of FA-free BSA in sterile DPBS was prepared and 25 ml were added to the dry Celite/FA mixture. The mixture was gassed with nitrogen and closed with a glass stopper, then incubated for 30 min at 20ºC with moderately vigorous stirring using a magnetic flea and a stirring plate. The solution was filtered through filter paper (Whatman No.1) into a 30 ml quickfit tube, then sterilised using a Minisart (Sartorius Stedim Biotech, Goettingen, Germany) high-flow sterile syringe filter of 0.2 µm pore size and aliquoted into sterile, foil-covered Bijous (5 ml) in the vertical laminar flow hood. FA concentration was measured by aliquoting 100 µl of the FA/BSA preparation with 100 µl of the standard FA 17:0 at a known concentration of 1 mg ml^{-1}, followed by lipid extraction, transmethylation, and gas chromatography (GC) analysis according to Christie (2003) (see section 2.3.3).

2.2.2. Incubation of CHSE-214 with fatty acids

For experiments investigating the effects of supplemental FA on lipid and FA compositions of CHSE-214 cells, the cells were routinely subcultured at a split ratio of 1:2 into 75 cm^2 tissue culture flasks in a volume of 10 ml of L-15 medium containing 5% FBS

and 200 µM of L-glutamine. One day after subculture, when cells in the flasks were approximately 80% confluent, FA were added as complexes directly to flasks in triplicate. The FA used during this study were: 16:0, 18:1n-9, 20:1n-9, 18:2n-6, 18:3n-6, 20:3n-6, 20:4n-6, 18:3n-3: 20:5n-3, 22:5n-3 and 22:6n-3. The final concentration of FA was dependent upon the particular experiment and will be indicated in each case. Cells were harvested after 5 d incubation with the FA substrates at 20ºC. After the incubation time the medium was decanted and the monolayer carefully washed twice with 2 x 5 ml portions of DPBS. An amount of 0.5 ml T/EDTA solution was added to each flask to dissociate the cells. A Corning® cell scraper (Sigma-Aldrich® Company Ltd., Dorset, UK) was used in order to maximise the recovery of cells. A total of 5 ml of DPBS were added to each flask to re-suspend the detached cells and the suspensions transferred into labelled 10 ml glass conical tubes and then centrifuged at 400 g for 5 min (Jouan C312, France) to pellet the cells. The supernatant was removed and the cell pellet washed by adding 5 ml of DPBS, the cell pellet was re-suspended and tubes centrifuged at 400 g for 5 min. The supernatant was removed and the pellet was allowed to drain (carefully and quickly) on tissue paper prior to the lipid extraction.

2.3. Lipid and fatty acid analyses

2.3.1. Lipid extraction

Total lipids were extracted from cells according to the methods of *Folch et al.* (1957). To prevent lipid oxidation, most of the solvents contained 0.01% BHT as antioxidant and all solutions were chilled in an ice box. All procedures involving organic solvents were performed in glassware. Once the cell pellets were drained on the tissue paper, 5 ml of ice-cold chloroform/methanol (C/M, 2:1, by volume) containing 0.01%

BHT were added using a glass pipette and the cell pellet completely disrupted by pipetting and mixed on a vortex mixer. After leaving on ice for 10 min to ensure complete lipid extraction, 1 ml of ice-cold 0.88% potassium chloride (KCl) was added and the solution mixed thoroughly before the tubes were centrifuged at 400 g for 5 min. After centrifugation, samples were separated into three distinct layers: upper aqueous, intermediate white protein layer, and lower organic layer. The lower organic layer was transferred into a clean 10 ml glass test tube, and the solvent evaporated under a stream of OFN. Total lipid extracts were re-suspended in 100 µl of C/M (2:1, by vol.), mixed, and transferred into previously weighed and labelled small glass bottles. The solvent was then evaporated under a stream of OFN, and the extracts dried in a vacuum desiccator for 4 h. To quantify the extracted lipid, the bottles were re-weighed and the final total lipid extracts were re-suspended at a concentration of 10 mg ml^{-1} in C/M (2:1, by vol.) containing 0.01% BHT. Samples were stored at -20ºC prior to lipid class and FA analyses.

2.3.2. Determination of lipid class composition

Lipid class composition analysis was performed by high-performance thin-layer chromatography (HPTLC) and quantitative densitometry essentially according to Henderson and Tocher (1992). Initial separation of lipid classes was performed using 10 × 10 cm × 0.25 mm HPTLC plates (Merck, Darmstadt, Germany). Six 3 mm origins were marked with pencil at a distance of 1.2 cm between them. Plates were cleaned by fully developing in C/M (2:1, by volume), allowing the solvent to evaporate by air drying in the fume cupboard, followed by activation at 110ºC for 15 min. Routinely, 3 samples of 20 µg total lipid (*i.e.* 2 µl of 10 mg ml^{-1}), two lipid class standards (one neutral and one polar classes), and one blank were applied to each 3 mm origin using a Microliter™ glass syringe (Hamilton®, Bonaduz, Switzerland). Plates were developed to 5.5 cm in the first

solvent, using methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by volume), to separate polar lipid classes. The plate was removed from the tank, excess solvent evaporated by air drying. Once the plates were dried, they were fully developed in the same direction in the second solvent containing isohexane/diethyl ether/acetic acid (85:15:1, by volume) to separate neutral lipid classes. Plates were air dried briefly to evaporate excess solvent, and then sprayed with 3% cupric acetate in 8% phosphoric acid staining solution. After excess solution was drained, the plates were charred at 160ºC for 15 min in an oven. After cooling, the plates were scanned in a densitometer (Camag 3 TLC Scanner, Muttenz, Switzerland) equipped with winCATS software to quantify the different lipid classes (Henderson and Tocher, 1992).

2.3.3. Determination of fatty acid composition

The FA profile of cellular lipid was determined by analysis of fatty acid methyl esters (FAME) by gas chromatography (Christie, 2003). The FAME were prepared by transmethylation. To this end, 100 µl (at a concentration of 10 mg μl^{-1}) of total lipid extract were aliquoted into a quick-fit test tube and the solvent evaporated under a stream of OFN. One ml of toluene and 2.5 ml of 1% sulphuric acid (H_2SO_4) in methanol were added to each lipid sample. Samples tubes were gassed with OFN, sealed with glass stoppers and a small piece of paper tissue and incubated overnight in a hot block at 50ºC. After cooling, FAME were extracted by adding 2 ml of 2% potassium bicarbonate ($KHCO_3$), followed by 5 ml of isohexane/diethyl ether (1:1, by volume) containing 0.01% BHT. Tubes were vortexed and centrifuged at 400 g for 5 min to separate the two phases. The upper layer was collected and transferred into a clean test tube. Further 5 ml isohexane/diethyl ether (1:1, by volume without BHT) were added to the remaining lower layer and the extraction procedure repeated to ensure maximum recovery of FAME. After mixing and centrifuging

at 400 g for 5 min, the upper layer was collected and combined with the first upper layer. The combined upper layers were placed under a stream of OFN to evaporate the solvent and the dry FAME extract re-suspended in 100 µl of isohexane containing 0.01% BHT. The FAME extracts were purified by thin-layer chromatography (TLC) on 20 × 20 cm × 0.25 mm plates (Merck, Darmstadt, Germany). The plates were marked by pencil with four origins of 2.5 cm length, with 2 cm between them, starting at 2 cm from the side and 1.5 cm from the bottom of the plates. A neutral lipid class standard mixture containing FAME was used at each side of the plate for indicating the position of the FAME in the samples. The location of the FAME was revealed by spraying the standard lanes at the sides of plates with 1% iodine in chloroform. The position of FAME in the experimental samples was marked with pencil, and the silica containing the FAME was scraped off and transferred into fresh test tubes. Five ml isohexane/diethyl ether (1:1, by volume), plus 1 ml isohexane/diethyl ether (1:1, by volume) containing BHT were added to the tubes. Tubes were mixed and centrifuged at 400 g for 2 min. The supernatant was carefully transferred into a clean test tube by pipetting and the solvent evaporated under a stream of OFN. Dry purified FAME samples were re-suspended in isohexane containing 0.01% BHT at a concentration of 1 mg FAME μl^{-1}. FAME were quantified by gas chromatography using a Fisons GC 8160 gas chromatograph (Fisons Ltd, Crawley, UK) equipped with on-column injector, flame ionisation detector and fitted with a ZB Wax column (30 m × 0.32 mm), using hydrogen as a carrier gas at a flow rate of 2.0 ml/min. The temperature was programmed initially from 50 ºC to 180 ºC and then to 225 ºC, at rates of 40ºC/min and 2 ºC/min, respectively, and then held at 225ºC for 5 min. Individual FAME were identified by comparing the profile of the samples with known standards. FAME were quantified and data collected with Chrom-card™ ver. 1.19 (Thermoquest Italia S.p.A, Italy) for Microsoft® Windows®.

2.4. Statistical analysis

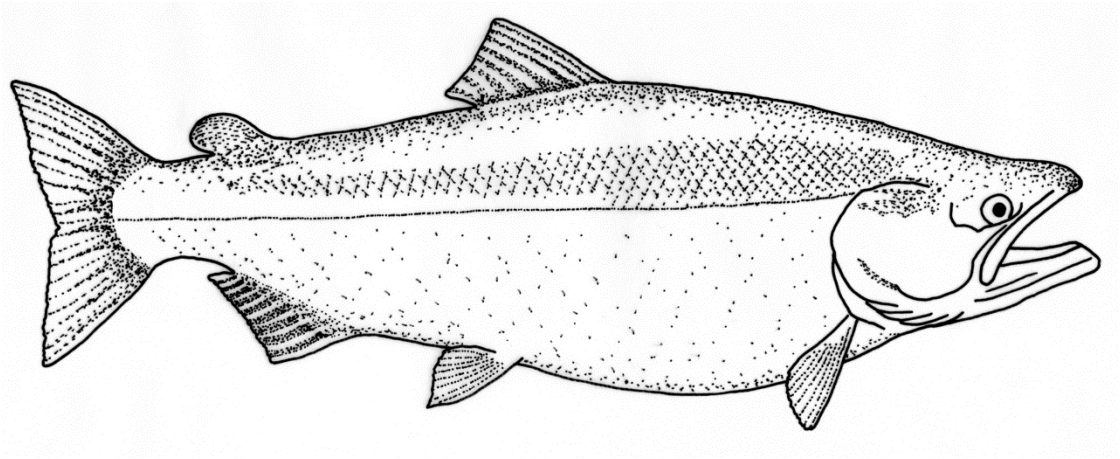
The data are presented as means ± standard deviation (SD) with n = 3. Results of fatty acid analysis and lipid class composition were analysed by regression analysis at the 95% significance level, using the statistics software IBM SPSS Statistics for Microsoft Windows®, ver. 19.0 (Armonk, NY: IBM Corp.). Arcsine square root transformations were applied to the variables expressed in percentage in order to normalise the data according to Sokal and Rohlf (1998). The conversion of the precursors and intermediary metabolites in LC-PUFA (*i.e.* LOA → ARA; LNA → EPA and DHA), was represented graphically using Microsoft® Excel®, in Chapters 3, 4 and 5. The effect of the FA supplemented (variable 1), their concentrations (variable 2), and their interaction was determined by two-way analysis of variance (ANOVA) ($p < 0.05$).

2.5. Materials

Tissue culture flasks and plastic pipettes were obtained from Sarstedt AG & Co. Laboratories, Nümbrecht, Germany. All solvents were HPLC grade and were obtained from Fisher Scientific UK, Loughborough. Compressed gases were obtained from British Oxygen Company (BOC, Glasgow, UK).

Chapter 3

Effects of fatty acid supplementation on the lipid and fatty acid compositions of the CHSE-214 cell line



Line drawing of Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum) [original image]

"If you focus on results, you will never change. If you focus on change, you will get results"

Jack Dixon

3.1. Introduction

Atlantic salmon (*Salmo salar* L.), one of the main finfish species cultured intensively in the world, has the ability to store large quantities of n-3 long-chain-polyunsaturated fatty acids (LC-PUFA) in its flesh (Miller *et al.*, 2008). In the natural environment salmon are predominantly carnivorous, having access to high levels of n-3 LC-PUFA, principally eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids, and n-6 LC-PUFA in lower level, mainly arachidonic acid (ARA, 20:4n-6) (Sargent *et al.*, 2002). For this reason Atlantic salmon in intensive culture has been fed diets containing fish oil (FO) and fishmeal, which are obtained primarily from marine wild-caught sources. However, the over-dependence on FO and fishmeal for the formulation and manufacture of salmon aquafeeds, is one of the major challenges that has to be addressed for the sustainable development of the aquaculture industry (Naylor *et al.*, 2000; Tacon and Metian, 2008). As an alternative to overcome this situation, it has been proposed that FO could be replaced by vegetable oil (VO), due to its availability, sustainability and the lower production costs in comparison with FO (Turchini *et al.*, 2009). The main difference between FO and VO in terms of their fatty acid (FA) profile is that FO is rich in n-3 LC-PUFA while VO completely lacks LC-PUFA, but can be abundant in linoleic (LOA, 18:2n-6) and α-linolenic (LNA, 18:3n-3) acids. The replacement of dietary FO by VO is not a major problem for freshwater and herbivorous fish, as these have the necessary enzymes to convert LNA into EPA and DHA and, LOA into ARA (Tocher, 2003). Marine carnivorous fish, on the other hand, present a low conversion of both acids, *i.e.* LOA into ARA, and LNA into EPA and DHA (Sargent *et al.*, 1997; Turchini *et al.*, 2009). The inclusion of these FA in fish feeds is important since phospholipids in fish tissues are rich in n-3 LC-PUFA (*i.e.* EPA and DHA) with ARA in lower concentration (Sargent *et al.*, 1989, 2002); ARA also represents the main precursor

for eicosanoid production (Henderson and Tocher, 1987; Tocher, 1995). The FA profile of Atlantic salmon is affected when FO is replaced with VO, resulting in low n-3 LC-PUFA levels (Torstensen *et al.*, 2000; Bell *et al.*, 2002; Bransden *et al.*, 2003; Carter *et al.*, 2003; Turchini *et al.*, 2009). Therefore**,** the study of FA metabolism in fish has a vital role, as a change in FA composition of the fish diet may affect the health and welfare of the fish, which may also lose their beneficial properties for the human consumer.

Lipids are the main energy source for salmonids, therefore, they must be included in the diet at the appropriate inclusion level to cover essential FA requirements, avoiding nutritional diseases and/or pathological issues such as: poor feed efficiency, vacuolated pyloric caeca tissue and reduced growth (Bell *et al.*, 1991; Tacon, 1996; Seierstad *et al.*, 2005). Heart lesions, thinning of ventricular walls, and arteriosclerosis changes in Atlantic salmon fed diets with 100% of VO have been also reported (Bell *et al.*, 1991; Seierstad *et al.*, 2005). It has been shown that dietary FO can be replaced up to 75% without compromising the growth, health and/or welfare of Atlantic salmon if n-3 LC-PUFA requirements are covered (Polvi and Ackman, 1992; Rosenlund *et al.*, 2001; Torstensen *et al.*, 2005).

Some of the most researched VO used in the aquafeed industry for Atlantic salmon are listed in Table 3.1, where their corresponding FA profiles (%) are also presented, with particular focus on the FA supplemented during the experiments described in this Chapter. Fatty acids such as 16:0 (palmitic acid), 18:1n-9 (oleic acid), LOA, and LNA are present in high levels in many of the VO listed. It can be observed that n-3 LC- PUFA such as EPA, 22:5n-3, and DHA are reported only in FO. The monounsaturated (MUFA) 20:1n-9 (eicosenoic) is rare in the VO mentioned. Regarding n-6 FA, 18:3n-6 is present only in echium oil.

Table 3.1. Incidence (%) of FA added to the CHSE-214 in vegetable or plant oils that can be used to replace fish oil in aquafeed for Atlantic salmon

	Oil sources							
Fatty acid	Palm	Rapeseed	Linseed	Sunflower-seed	Olive	Soya	Echium	Fish
16:0	46.7	4.7	6.3	6.4	10.8	9.7	7.5	16.4
18:1n-9	33.8	58.3	18.3	25.3	75.4	22.5	17.2	21.1
20:1n-9	0.0	1.1	0.0	0.0	0.0	0.0	0.8	3.8
18:2n-6	11.4	21.6	15.7	60.7	6.8	55.2	19.5	3.4
18:3n-6	0.0	0.0	0.0	0.0	0.0	0.0	9.8	0.0
20:3n-6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20:4n-6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0
18:3n-3	0.0	7.3	53.3	0.0	0.0	6.4	28.1	0.6
20:5n-3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	12.7
22:5n-3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7
22:6n-3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.3

Modified from Miller, M.R; Nichols, P.D; Carter, C.G. (2008)

Cell culture represents a biological tool, which is widely used in the research field (Lakra *et al.*, 2011). The RTG-2 cell line was the first permanent fish cell line, and was produced from gonad of rainbow trout (*Oncorhynchus mykiss* Walbaum) reported by Wolf and Quimby (1962). Since then, fish cell culture has represented a major biological tool for research in fish (Lakra *et al.*, 2011; Lee *et al.*, 2013). In the past, cell culture was mainly used for virology studies (Wolf, 1988), but nowadays they are frequently used in a wider range of areas such as immunology (Bols *et al.*, 2001), toxicology (Bahich and Borenfreund, 1991), ecotoxicology (Schirmer, 2006), endocrinology (Bols and Lee, 1991), temperature effect studies (Tocher and Sargent, 1990), and FA metabolism(Tocher *et al.*, 1989, 1992, 1995, 1996; Tocher and Sargent, 1990; Tocher and Dick, 1999; Ghioni *et al.*, 1999; Minghetti *et al.*, 2011; Gregory *et al.*, 2011; Viegas *et al.*, 2012). The use of cell culture has become more popular since they are relatively easy to manipulate and maintain, and because the results can be more reproducible than those obtained from fish studies (Wolf and Quimby, 1976). Lakra *et al*. (2011) reported that approximately 283 finfish cell lines around the world have been established so far. The methodology required for the culture of fish and mammalian cell lines are generally similar, but fish cell lines have a

wider range of optimal incubation temperatures and, in the case of cell lines derived from marine fish, the osmolality of the medium has to be adjusted (Lakra *et al.*, 2011). Cell culture has been an important *in vitro* tool to study fish lipid metabolism (Bailey and Dunbar, 1973; Spector *et al.*, 1981). However, there have been some issues regarding the FA profile of the cell lines used. Although aquatic organisms and more specifically marine organisms are dominated by the n-3 LC-PUFA series (Sargent, 1976), the FA profile of the cells in culture is dependent upon the culture medium and therefore is generally different from the tissue that they were originally derived from. This has been associated mainly to the culture of the fish cell lines using foetal bovine serum (FBS), as a result, the FA composition of the cell line reflects the FA profile of FBS, which is rich in n-6, and deficient in n-3 LC-PUFA (Tocher *et al.*, 1988). Another factor may be the incubation temperature, as in the case of the marine fish cell lines, which are routinely cultured at a higher temperature (22ºC approximately) compared to the natural environment where the fish were obtained from (5–15ºC), having effects on lipid and FA metabolism, and therefore, altering the FA composition of the fish cell lines to some extent (Tocher and Sargent, 1990).

The aim of the studies in Chapter 3 is to determine the effects of supplementing individual FA, and FA concentration on total lipid content, lipid class composition and FA composition of CHSE-214 cell line. The data produced will provide information to enable the enzymatic capabilities of the CHSE-214 cell line to be investigated. Particular attention was paid to the activity of the LC-PUFA biosynthesis pathway. The supplementation of FA in increasing concentrations also provided data to enable the ideal concentration of FA supplementation required to alter FA composition, without negatively affecting cell growth and/or altering lipid metabolism towards a storage mode. Increased lipid deposition and storage would be reflected in the cellular deposition of lipid droplets and in altered lipid

class compositions, which would show an increment of triacylglycerol (TAG). The information obtained in this Chapter was necessary to determine the concentration of FA supplementation required to carry out the FA competition experiments in subsequent Chapters.

3.2. Materials and Methods

3.2.1. Cell line and routine culture procedures

The specifications of the CHSE-214 cell line, the preparation of the media and the routine culture procedures of the cells are provided in Chapter 2, sections 2.1.1 and 2.1.2.

3.2.2. Individual FA supplementation experiments

FA were supplemented to the cell line as complexes. These were prepared following the method described by Spector and Hoak (1969). Full details of the procedure are provided in Chapter 2, section 2.2.1. Table 3.2 lists the FA supplemented, their concentrations in the BSA complex, and the amount (in μl) added to the flasks to obtain final planned FA concentrations. Palmitic acid (16:0) and oleic acid (18:1n-9) were supplemented because they are the most abundantly found SFA and MUFA, respectively, in VO used for the formulation of aquafeeds. The 20:1n-9 was supplemented since it is a MUFA mainly found in the FO. LOA was supplemented because it is the precursor of ARA, and because was necessary to observe if the cell line could convert it, as well as other n-6 intermediaries, *i.e.* 18:3n-6, 20:3n-6. LNA was supplemented as this is the precursor of the n-3 LC-PUFA and therefore it was important to confirm the CHSE-214 cell line was able to metabolise it. Finally EPA, DPA and DHA were supplemented as

these are EFA and are only present in FO. Graded concentrations were supplemented in order to analyse the data using regression analysis.

Table 3.2. Initial concentration of FA in the BSA complexes used as substrates for CHSE-214 cell line and amount added to the cells to obtain concentrations of 20, 50 and 100 μM

Fatty acid complex	Concentration (mM)	Volume (μl) for 20 μM	Volume (μl) for 50 μM	Volume (μl) for 100 μM
16:0 (Palmitic)	1.56	128	320	640
18:1n-9 (Oleic)	1.82	110	275	550
20:1n-9 (Eicosenoic)	1.12	180	450	900
18:2n-6 (Linoleic)	2.04	98	245	490
18:3n-6 (γ-Linolenic)	2.52	80	200	400
20:3n-6 (Dihomo-γ-linolenic)	1.76	115	285	570
20:4n-6 (Arachidonic)	2.09	96	240	480
18:3n-3 (α-Linolenic)	2.00	100	250	500
20:5n-3 (Eicosapentaenoic)	1.83	110	275	550
22:5n-3 (Docosapentaenoic)	1.46	137	342	685
22:6n-3 (Docosahexaenoic)	1.49	134	335	670

3.2.3. Supplementation of cultures with FA complexes

The protocol used for the supplementation of FA to the cell line has been described in Chapter 2, section 2.2.2. The concentrations of FA supplemented to the cells were 20, 50 and 100 μM for lipid incorporation assays, and 0, 20, 40, 60, 80 and 100 μM for toxicity assays. For lipid analyses, experimental control flasks did not receive any treatment whereas for toxicity analyses, experimental control wells were incubated with the BSA solution without FA. After the incubation period, cells were harvested and washed as previously described in Chapter 2, sections 2.1.2.2 and 2.2.2 for further lipid analyses.

3.2.4. Lipid analyses

Cellular lipids were extracted according to Folch *et al.* (1957) as described in Chapter 2, section 2.3.1. Lipid class analyses were carried out by one-dimensional double development high-performance thin-layer chromatography, as described in Chapter 2,

section 2.3.2 (Henderson and Tocher, 1992). Fatty acid methyl esters (FAME) were prepared by acid-catalysed transmethylation according to Christie (2003). Full details of the procedure are provided in Chapter 2, section 2.3.3.

3.2.5. Cell proliferation and toxicity analysis (MTT cell proliferation assay)

The MTT cell assay was used to determine the effects of fatty acid supplementation on cell proliferation and also to determine any toxic effects of different concentrations of FA added to the cell cultures. The MTT (thiazolyl blue tetrazolium bromide, Sigma-Aldrich® Company Ltd., Dorset, UK) is a vital staining dye, which is used to determine cell growth and toxic effects by counting viable cells. Cells were harvested from the 75 cm^2 tissue culture flasks as described in Chapter 2, section 2.1.2.2, and counted as previously detailed in section 2.1.2.4. Homogeneous cell suspensions were distributed in six-well plates at a density of 1.6×10^6 cells per well in 3 ml of L-15 media supplemented with 5% FBS. Twenty-four hours after seeding, FA were added in triplicate to the wells at concentrations previously specified. The plates were then incubated for 5 d at 20ºC. On the fifth day, MTT powder was weighted and dissolved in Milli-Q water at a concentration of 5 mg ml^{-1} to make the stock solution that was filter-sterilised prior to use by syringe filtration (0.2 µm pore size). The sterile MTT solution was added to the growth medium that was used for fatty acid supplementation experiments (*i.e.* 5% FBS and 200 µM L-glutamine) at a final concentration of 0.5 mg MTT ml^{-1} medium. Cells in the six-well plates were washed twice with DPBS (1 ml per well per wash) and 2.5 ml of media containing MTT added to each well and the plates incubated for 4 h at 20ºC. The MTT/medium was removed from each well by pipetting, and plates with the lid open were air dried and kept in darkness for 40 min at 20ºC in the incubator. When the plates were completely dry, 1 ml of DMSO solution was added to each well to dissolve the crystals of

MTT. Plates were wrapped in aluminium foil and incubated for further 30 min, gently shaking every 10 min. Four replicates of 200 µL MTT/DMSO solution per sample were transferred from each well in the six-well plates to individual wells in a 96-well plate. The absorbance of the MTT blue dye was determined in a spectrophotometer at 570 nm in a Multiskan® plate reader (Multiskan® EX, MTX Lab Systems, Inc., U.S.A.) using Genesis Lite software (MTX Lab Systems, Inc., U.S.A.). The output obtained was a txt file extension and this was converted into a Microsoft® Excel® file in order to organise the data and to represent them graphically.

3.2.6. Statistical analysis

For all assays, the experimental conditions were applied in triplicate, with each flask considered a replicate in the supplementation studies for effects on lipid and FA composition, and each well a replicate in the cell proliferation/toxicity analyses. Results are presented as means ± 1 standard deviation (SD). Detailed statistical analyses are provided in Chapter 2, section 2.4.

3.3. Results

3.3.1. Lipid content, lipid class and FA composition of CHSE-214 cells grown in 10% FBS (baseline)

As a starting point, the lipid content, lipid class and FA composition of three 75 cm^2 flasks of CHSE-214 cells growing under conditions to provide experimental material were determined. The lipid content and lipid class composition of the CHSE-214 grown in 20 ml of L-15 media with 10% of FBS are shown in Table 3.3. In average 444.7 µg of lipids were extracted from the CHSE-214 growing in 75 cm^2 tissue culture flasks. The

percentage of total polar lipids was almost the double of the percentage of the total neutral lipids. The most abundant polar lipids were: PE > PC > PI, while the major component of the total neutral lipids was cholesterol.

Table 3.3. Lipid content and lipid class composition of CHSE-214 cells growing in L-15 media with 10% of FBS

Lipid	CHSE-214 in 10% of FBS
Lipid content (µg)	444.7 ± 15.2
Class composition (%)	
PC	16.0 ± 2.1
PE	21.7 ± 0.1
PS	8.1 ± 0.5
PI	11.9 ± 2.3
PA/CL	1.8 ± 0.1
SM	5.1 ± 0.2
Total polar	64.6 ± 1.0
Total neutral	35.4 ± 1.0
TAG	12.3 ± 0.2
CHOL	16.8 ± 0.9
FFA	6.4 ± 0.2

Footnotes: Results are expressed as mean ± 1 SD (n=3). Lipid class composition is given as a percentage of the total lipid content. *Abbreviations:* PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids.

The FA profile of the CHSE-214 cell line grown under routine culture conditions in medium containing 10% FBS for provision of experimental material is shown in Table 3.4. The major FA groups were present in the rank order MUFA > SFA > PUFA. The most abundant SFA in cell total lipid were 16:0 and 18:0, accounting for 15.6% and 10.5% of total fatty acids (TFA), respectively. Regarding MUFA, 18:1n-9 was the most abundant at 39.7% of TFA. Total n-6 PUFA accounted for almost 12% of TFA with individual n-6 PUFA in the rank order LOA > 20:3n-6 > ARA. The n-3 LC-PUFA were found in lower proportions, with 2.8% of DHA, 1% of 22:5n-3, and 0.6% EPA. The PUFA composition was characterised by the presence of n-9 PUFA, specifically 18:2n-9 at 2.4%, and 20:2n-9 at 1.7%.

Table 3.4. Fatty acid composition (%) of total lipid of CHSE-214 cells grown in routine culture conditions for the provision of stock

Fatty acid	Percentage
14:0	1.8 ± 0.1
15:0	0.7 ± 0.1
16:0	15.6 ± 0.5
17:0	0.4 ± 0.1
18:0	10.5 ± 1.6
22:0	0.7 ± 0.1
Total saturated	**29.8 ± 1.4**
16:1n-9	3.2 ± 0.2
16:1n-7	2.7 ± 0.3
18:1n-9	39.7 ± 1.0
18:1n-7	3.1 ± 0.1
24:1n-9	1.1 ± 0.1
Total monounsaturated	**49.9 ± 0.8**
18:2n-6	5.5 ± 0.3
18:3n-6	0.5 ± 0.1
20:2n-6 (contains 20:3n-9)	0.5 ± 0.1
20:3n-6	2.8 ± 0.3
20:4n-6	2.4 ± 0.4
Total n-6 PUFA	**11.8 ± 0.9**
20:5n-3	0.6 ± 0.1
22:5n-3	1.0 ± 0.1
22:6n-3	2.8 ± 0.3
Total n-3 PUFA	**4.5 ± 0.3**
18:2n-9	2.4 ± 0.4
20:2n-9	1.7 ± 0.1
Total n-9 PUFA	**4.1 ± 0.4**
Total PUFA	**20.4 ± 1.4**

Footnotes: Results are expressed as mean ± 1 standard deviation (SD) (n=3) Fatty acid composition is given as a percentage of the total fatty acid content. *Abbreviations:* PUFA = polyunsaturated fatty acid.

3.3.2. Effect of supplemental SFA (16:0) and MUFA (18:1n-9, 20:1n-9) fatty acids on lipid content, lipid class and fatty acid compositions of CHSE-214 cells

3.3.2.1. Supplementation with 16:0

The lipid content and lipid class composition of the CHSE-214 cell line incubated with increasing concentrations of 16:0 are presented in Table 3.5. Cell lipid content data did not show a clear trend with graded supplementation of 16:0 (R^2 = 0.000; p = 0.953). Furthermore, there were no clear trends in the lipid class composition data with graded

supplementation of 16:0, apart from the increasing TAG, which increased with the supplementation of 16:0 at 20 µM to 100 µM; however, results were not statistically significant (R^2 = 0.001; p = 0.943).

Table 3.5. Lipid content and lipid class composition of CHSE-214 cell line incubated with 16:0

Lipid	Control	20 µM	50 µM	100 µM	R^2	P-value
LC (µg)	423.3 ± 81.4	506.7 ± 107.9	430.0 ± 108.2	443.3 ± 140.1	0.000	0.953
CC (%)						
PC	23.7 ± 1.6	23.9 ± 1.1	28.1± 1.7	25.4 ± 0.4	0.046	0.504
PE	14.4 ± 1.8	20.1 ± 1.2	19.4 ± 0.7	15.1 ± 1.7	0.566	0.005
PS	4.9 ± 0.1	6.2 ± 0.8	7.6 ± 0.6	4.8 ± 0.3	0.005	0.824
PI	10.3 ± 0.1	4.0 ± 1.2	4.1 ± 0.9	3.6 ± 0.7	0.002	0.878
PA/CL	1.5 ± 0.2	1.5 ± 0.1	1.3 ± 0.2	2.0 ± 0.2	0.440	0.019
SM	5.2 ± 0.5	4.8 ± 0.2	4.3 ± 0.8	6.2 ± 0.4	0.094	0.331
TP	60.0 ± 2.0	60.5 ± 1.2	64.8 ± 0.4	57.1 ± 1.2	0.064	0.426
TN	40.0 ± 2.0	39.5 ± 1.2	35.2 ± 0.4	42.9 ± 1.2	0.064	0.426
TAG	4.7 ± 0.8	2.3 ± 1.1	3.3 ± 1.6	3.8 ± 1.1	0.001	0.943
CHOL	35.3 ± 1.3	37.2 ± 2.3	31.9 ± 2.0	39.1 ± 0.3	0.071	0.402
FFA	ND	ND	ND	ND	-	-

Footnotes: Results are expressed as mean ± 1 SD (n=3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA profile of CHSE-214 cells incubated with increasing concentrations of 16:0 is shown in Table 3.6. Saturated fatty acids (SFA) accounted for about 20–35% of TFA and, as supplemental 16:0 increased graded increase of total SFA (R^2 = 0.782; p = 0.036) and 16:0 (R^2 = 0.872; p = 0.042) was observed. However, the proportions of MUFA (R^2 = 0.323; p = 0.054), n-6 (R^2 = 0.011; p = 0.749), n-3 (R^2 = 0.626; p = 0.002), n-9 (R^2 = 0.624; p = 0.002) and total PUFA (R^2 = 0.700; p = 0.001) all decreased. Almost all individual FA showed generally graded decreasing trends, apart from 18:0, which showed increasing proportions at 20 µM and 50 µM and (R^2 = 0.230; p = 0.115) and 16:1n-7 which increased by almost two-fold at 20 µM, almost three-fold at 50 µM and over six-fold at 100 µM (R^2 = 0.851; p = 0.000). A similar trend to a lesser extent was observed in the

percentages reported for 16:1n-9 (R^2 = 0.719; p = 0.000) and 18:1n-7 (R^2 = 0.912; p = 0.000).

Table 3.6. Fatty acid composition (%) of CHSE-214 incubated with 20, 50 and 100 µM of 16:0

Fatty acid	Control	20 µM	50 µM	100 µM	R^2	P-value
14:0	1.8 ± 0.2	1.8 ± 0.4	1.6 ± 0.4	1.4 ± 0.3	0.065	0.424
15:0	0.3 ± 0.0	0.6 ± 0.2	0.4 ± 0.1	0.9 ± 0.1	0.150	0.213
16:0	12.6 ± 1.3	16.7 ± 2.3	18.4 ± 2.1	26.6 ± 1.0	0.872	0.042
17:0	ND	1.0 ± 0.2	1.1 ± 0.2	1.2 ± 0.1	0.194	0.152
18:0	6.8 ± 0.4	8.9 ± 1.3	10.2 ± 1.5	5.0 ± 0.5	0.230	0.115
22:0	0.4 ± 0.2	0.6 ± 0.1	0.3 ± 0.1	0.7	0.344	0.045
Σ SFA	**21.9 ± 1.4**	**29.5 ± 1.7**	**32.0 ± 1.3**	**35.8 ± 0.8**	**0.782**	**0.036**
16:1n-9	4.1 ± 0.1	5.0 ± 0.4	5.7 ± 0.3	5.9 ± 1.0	0.719	0.000
16:1n-7	3.2 ± 0.6	5.5 ± 0.4	8.6 ± 1.0	21.8 ± 0.6	0.851	0.000
18:1n-9	44.9 ± 3.1	37.3 ± 1.7	33.7 ± 0.6	20.0 ± 1.5	0.366	0.037
18:1n-7	2.4 ± 0.9	3.8 ± 0.4	3.2 ± 0.3	1.7 ± 0.2	0.912	0.000
24:1n-9	1.2 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.9 ± 0.3	0.015	0.702
Σ MUFA	**55.9 ± 1.2**	**52.5 ± 1.3**	**52.1 ± 1.2**	**51.3 ± 1.0**	**0.323**	**0.054**
18:2n-6	2.9 ± 0.1	3.1 ± 0.1	2.4 ± 0.1	2.9 ± 0.3	0.172	0.180
18:3n-6	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.041	0.529
20:2n-6*	1.2 ± 0.1	0.9 ± 0.3	0.7 ± 0.1	0.6 ± 0.1	0.615	0.003
20:3n-6	1.2 ± 0.3	0.8 ± 0.2	1.3 ± 0.2	1.3 ± 0.2	0.164	0.191
20:4n-6	1.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	0.9 ± 0.1	0.150	0.213
Σ n-6 PUFA	**7.2 ± 0.4**	**5.7 ± 0.5**	**5.3 ± 0.4**	**6.2 ± 0.5**	**0.011**	**0.749**
20:5n-3	0.5 ± 0.1	0.6 ± 0.1	1.0 ± 0.1	0.5 ± 0.1	0.196	0.149
22:5n-3	1.0 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.260	0.090
22:6n-3	1.6 ± 0.3	1.3 ± 0.2	1.2 ± 0.3	1.1 ± 0.1	0.666	0.001
Σ n-3 PUFA	**3.2 ± 0.1**	**2.5 ± 0.2**	**2.7 ± 0.4**	**2.3 ± 0.2**	**0.626**	**0.002**
18:2n-9	6.4 ± 0.5	5.0 ± 0.9	4.7 ± 0.2	3.3 ± 0.2	0.293	0.069
20:2n-9	5.4 ± 0.6	4.7 ± 0.5	3.1 ± 0.1	1.2 ± 0.1	0.776	0.000
Σ n-9 PUFA	**11.8 ± 0.3**	**9.7 ± 0.2**	**7.8 ± 0.3**	**4.5 ± 0.2**	**0.624**	**0.002**
Σ PUFA	**19.6 ± 3.5**	**18.0 ± 1.9**	**15.9 ± 0.9**	**12.9 ± 0.2**	**0.700**	**0.001**

Footnotes: Results are expressed as mean ± 1 SD (n=3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; ND = not detected; *contains 20:3n-9.

3.3.2.2. Supplementation with 18:1n-9

In Table 3.7 the lipid content and the lipid class compositions of CHSE-214 cells incubated with 18:1n-9 are presented. No clear trend was observed in the cell total lipid with the graded supplementation of 18:1n-9 (R^2 = 0.265; p = 0.087). The proportions of total polar lipids were in the range of 49.9–59.2%. The percentages of PC decreased (R^2 =

0.818; p = 0.000) and PE increased with the graded supplementation of 18:1n-9 (R^2 = 0.551; p = 0.006). Total neutral lipids increased with supplementation of 20 µM and 50 µM 18:1n-9, and TAG showed graded increments, particularly at 50 µM by almost two-fold, and over three-fold at 100 µM (R^2 = 0.869; p = 0.000).

Table 3.7. Lipid content and lipid class composition of CHSE-214 incubated with 18:1n-9

Lipid	Control	20 µM	50 µM	100 µM	R^2	P-value
LC (µg)	426.6 ± 41.6	565.4 ± 98.5	515.9 ± 51.3	590.3 ± 15.3	0.265	0.087
CC (%)						
PC	22.7 ± 1.6	16.4 ± 0.4	12.2 ± 1.9	11.6 ± 2.2	0.818	0.000
PE	14.2 ± 1.8	16.0 ± 0.9	16.2 ± 0.7	24.8 ± 1.3	0.551	0.006
PS	4.6 ± 1.2	4.9 ± 0.5	3.9 ± 0.7	3.6 ± 0.4	0.255	0.094
PI	10.4 ± 2.0	11.3 ± 0.7	11.0 ± 0.6	8.2 ± 0.3	0.260	0.090
PA/CL	1.6 ± 0.2	1.6 ± 0.3	1.5 ± 0.3	1.6 ± 0.2	0.003	0.877
SM	5.7 ± 0.3	5.8 ± 0.4	5.1 ± 0.6	4.6 ± 0.5	0.507	0.009
TP	59.2 ± 3.0	56.0 ± 1.9	49.9 ± 1.1	54.4 ± 3.6	0.287	0.072
TN	40.8 ± 3.0	44.0 ± 1.9	50.1 ± 1.1	45.6 ± 3.6	0.287	0.072
TAG	4.1 ± 0.5	3.9 ± 0.5	7.5 ± 0.8	15.1 ± 1.3	0.869	0.000
CHOL	36.7 ± 2.6	40.1 ± 1.9	42.6 ± 1.3	30.5 ± 2.3	0.151	0.212
FFA	ND	ND	ND	ND	-	-

Footnotes: Results are expressed as mean ± 1 SD (n=3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA composition of the CHSE-214 cell line incubated with increasing concentrations of 18:1n-9 is presented in Table 3.8. With the exception of the control (unsupplemented) cells, the proportions of the major FA groups were in the rank order MUFA > PUFA > SFA. Proportions of total SFA and, particularly, 16:0 and 18:0 decreased with the graded supplementation of 18:1n-9 (R^2 = 0.675; p = 0.001 and R^2 = 0.814; p = 0.000, respectively), while total MUFA proportionally increased (R^2 = 0.814; p = 0.000). The 18:1n-9 was incorporated with the graded supplementation of the FA itself (R^2 = 0.740; p = 0.000). No major metabolites were observed, apart from 16:1n-9 (R^2 = 0.960; p = 0.000) and, at levels lower than 0.5%, 20:1n-9, 22:1n-9 and 24:1n-9.

Supplementing 18:1n-9 at 100 µM significantly decreased SFA, n-6 PUFA, n-9 PUFA and total PUFA.

Table 3.8. Fatty acid composition (%) of CHSE-214 incubated with 20, 50 and 100 µM of 18:1n-9

Fatty acid	Control	20 µM	50 µM	100 µM	R^2	P-value
14:0	1.5 ± 0.1	1.6 ± 0.1	1.7 ± 0.2	1.3 ± 0.1	0.174	0.177
15:0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.107	0.299
16:0	12.3 ± 0.6	12.0 ± 0.1	11.3 ± 0.5	7.8 ± 0.5	0.675	0.001
18:0	6.9 ± 0.5	6.7 ± 0.3	6.0 ± 0.1	4.3 ± 0.1	0.814	0.000
22:0	0.4 ± 0.2	0.6 ± 0.2	0.3 ± 0.0	0.2 ± 0.0	0.107	0.245
Σ SFA	**21.4± 1.2**	**21.2 ± 0.5**	**19.6 ± 0.5**	**13.9 ± 0.8**	**0.784**	**0.000**
16:1n-9	4.2 ± 0.2	4.8 ± 0.1	5.6 ± 0.1	6.0 ± 0.1	0.960	0.000
16:1n-7	3.1 ± 0.1	2.5 ± 0.1	2.2 ± 0.2	1.4 ± 0.1	0.827	0.000
18:1n-9	46.0 ± 1.2	45.4 ± 0.9	47.5 ± 0.7	58.1 ± 1.3	0.740	0.000
18:1n-7	1.9 ± 0.2	1.9 ± 0.1	1.7 ± 0.1	0.9 ± 0.7	0.393	0.029
20:1n-9	ND	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	-	-
20:1n-7	ND	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	-	-
22:1n-9	ND	0.3 ± 0.0	0.4 ± 0.1	0.2 ± 0.0	-	-
24:1n-9	1.7 ± 0.2	2.1 ± 0.4	1.9 ± 0.6	1.4 ± 0.3	0.097	0.324
Σ MUFA	**56.9 ± 1.1**	**57.5 ± 0.6**	**59.9 ± 0.1**	**68.5 ± 1.3**	**0.814**	**0.000**
18:2n-6	2.0 ± 0.1	1.8 ±0.1	1.9 ± 0.2	1.5 ± 0.1	0.541	0.006
18:3n-6	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.709	0.001
20:2n-6*	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	0.7 ± 0.0	0.722	0.000
20:3n-6	2.4 ± 0.1	2.0 ± 0.5	1.7 ± 0.6	0.9 ± 0.2	0.727	0.000
20:4n-6	0.7 ± 0.1	0.9 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	0.407	0.026
Σ PUFA	**6.8 ± 0.1**	**6.3 ± 0.7**	**6.1 ± 0.8**	**4.3 ± 0.1**	**0.692**	**0.001**
20:5n-3	0.2 ± 0.1	0.5 ± 0.1	0.7 ± 0.2	1.0 ± 0.3	0.249	0.099
22:5n-3	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.1	0.5 ± 0.1	0.499	0.010
22:6n-3	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.2	0.9 ± 0.1	0.642	0.002
Σ n-3 PUFA	**2.3 ± 0.1**	**3.5 ± 0.3**	**2.6 ± 0.1**	**2.5 ± 0.4**	**0.002**	**0.896**
18:2n-9	6.2 ± 0.2	6.6 ± 0.2	6.3 ± 0.2	6.4 ± 0.2	0.010	0.757
20:2n-9	6.0 ± 0.1	5.6 ± 0.1	5.0 ± 0.1	3.9 ± 0.1	0.968	0.000
22:2n-9	0.4 ± 0	0.5 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.248	0.100
Σ n-9 PUFA	**12.6 ± 0.3**	**12.6 ± 0.0**	**11.9 ± 0.3**	**10.8 ± 0.2**	**0.933**	**0.000**
Total PUFA	**21.7± 0.4**	**22.4 ± 1.0**	**20.6 ± 0.6**	**17.6 ± 0.5**	**0.773**	**0.000**

Footnotes: Results are expressed as mean ± 1 SD (n=3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; ND = not detected; *contains 20:3n-9.

3.3.2.3. Supplementation with 20:1n-9

The lipid content and the lipid class composition of CHSE-214 incubated with increasing concentrations of 20:1n-9 are presented in Table 3.9. No clear trend was observed in the cellular total lipid content with the graded supplementation of 20:1n-9 (R^2

= 0.051; p = 0.481). Proportions of total polar lipid (R^2 = 0.436; p = 0.020), PC (R^2 = 0.626; p = 0.002), PE (R^2 = 0.953; p = 0.000), PS (R^2 = 0.800; p = 0.000) and PI (R^2 = 0.774; p = 0.000) all decreased with the graded supplementation of 20:1n-9, except for PA/CL (R^2 = 0.905; p = 0.000) and SM (R^2 = 0.061; p = 0.439). The proportions of total neutral lipid classes increased (R^2 = 0.436; p = 0.020), as a result of the increment of TAG with the graded supplementation of 20:1n-9 (R^2 = 0.855; p = 0.000).

Table 3.9. Lipid content and lipid class composition of CHSE-214 incubated with 20:1n-9

Lipid	Control	20 µM	50 µM	100 µM	R^2	P-value
LC (µg)	470.0 ± 34.6	613.3 ± 140.1	546.7 ± 98.1	560.0 ± 121.7	0.051	0.481
CC (%)						
PC	23.9 ± 1.1	19.0 ± 0.2	18.6 ± 1.1	18.3 ± 0.9	0.626	0.002
PE	14.1 ± 0.3	11.8 ± 0.5	8.9 ± 0.3	8.8 ± 0.2	0.953	0.000
PS	4.6 ± 0.6	3.9 ± 0.1	3.8 ± 0.2	2.8 ± 0.1	0.800	0.000
PI	10.4 ± 0.7	9.1 ± 0.4	8.6 ± 0.1	6.4 ± 0.1	0.774	0.000
PA/CL	2.6 ± 0.3	3.3 ± 0.6	5.6 ± 0.2	4.5 ± 0.9	0.905	0.000
SM	4.3 ± 0.5	4.2 ± 0.1	4.4 ± 0.2	4.1 ± 0.2	0.061	0.439
TP	59.9 ± 0.9	54.0 ± 1.5	49.9 ± 1.0	44.9 ± 1.2	0.436	0.020
TN	40.1 ± 0.9	46.0 ± 1.5	50.1 ± 1.0	55.1 ± 1.2	0.436	0.020
TAG	4.4 ± 0.8	4.2 ± 0.9	11.0 ± 0.6	24.2 ± 1.1	0.855	0.000
CHOL	35.7 ± 1.7	44.5± 2.3	39.1 ± 1.2	30.9 ± 0.1	0.201	0.144
FFA	ND	ND	ND	ND	-	-

Footnotes: Results are expressed as mean ± 1 SD (n=3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA composition of CHSE-214 cells incubated with 20:1n-9 is presented in Table 3.10. The proportions of the major FA groups were as follows: MUFA > SFA > PUFA in all four treatments. Percentages of total SFA (R^2 = 0.566; p = 0.005), n-9 PUFA (R^2 = 0.875; p = 0.000) and total PUFA (R^2 = 0.944; p = 0.000) all decreased with the graded supplementation of 20:1n-9. There were observed graded increasing proportions of 20:1n-9 in the cells as concentration of supplementation increased (R^2 = 0.991; p = 0.000).

In terms of metabolism, some elongation to 22:1n-9 was observed (R^2 = 0.804; p = 0.000) but no further metabolites were detected. No metabolism to n-9 PUFA was observed.

Table 3.10. Fatty acid composition of CHSE-214 incubated with 20, 50 and 100 µM of 20:1n-9

Fatty acid	Control	20 µM	50 µM	100 µM	R^2	P-value
14:0	1.6 ± 0.2	2.1 ± 0.5	2.0 ± 0.1	1.1 ± 0.3	0.167	0.187
15:0	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.304	0.063
16:0	13.4 ± 0.4	16.9 ± 0.7	14.7 ± 0.9	9.6 ± 0.3	0.527	0.008
17:0	ND	4.1 ± 0.6	1.2 ± 0.6	3.6 ± 0.2	0.421	0.022
18:0	6.1 ± 0.4	7.0 ± 0.1	5.0 ± 0.2	3.1 ± 0.4	0.971	0.000
Σ SFA	**21.3 ± 0.3**	**30.4 ± 1.4**	**23.2 ± 1.6**	**17.7 ± 0.8**	**0.566**	**0.005**
16:1n-9	4.7 ± 0.5	4.3 ± 0.3	4.6 ± 0.3	3.5 ± 0.5	0.008	0.785
16:1n-7	2.8 ± 0.2	3.4 ± 0.8	2.6 ± 0.1	1.7 ± 0.1	0.469	0.014
18:1n-9	45.9 ± 1.5	39.1 ± 1.4	36.7 ± 1.0	28.5 ± 1.2	0.956	0.000
18:1n-7	2.3 ± 0.3	1.9 ± 0.1	1.5 ± 0.1	1.9 ± 0.2	0.224	0.120
20:1n-9	ND	7.6 ± 0.5	20.7 ± 0.8	38.4 ± 0.6	0.991	0.000
22:1n-9	ND	0.9 ± 0.1	0.8 ± 0.2	2.8 ± 1.3	0.804	0.000
24:1n-9	1.1 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	0.490	0.011
Σ MUFA	**56.8 ± 1.6**	**57.6 ± 1.0**	**67.4 ± 1.4**	**77.2 ± 1.1**	**0.904**	**0.000**
18:2n-6	2.4 ± 0.2	1.6 ± 0.2	1.3 ± 0.1	1.1 ± 0.2	0.377	0.034
18:3n-6	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.042	0.522
20:2n-6*	0.7 ± 0.0	0.7 ± 0.	0.8 ± 0.2	0.2 ± 0.0	0.043	0.517
20:4n-6	1.5 ± 0.4	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.651	0.002
Σ n-6 PUFA	**6.8 ± 0.8**	**3.1 ± 0.2**	**3.6 ± 0.6**	**2.0 ± 0.3**	**0.742**	**0.000**
20:5n-3	0.5 ± 0.0	0.5 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	0.000	0.974
22:6n-3	1.5 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.7 ± 0.3	0.232	0.113
Σ n-3 PUFA	**2.4 ± 0.2**	**0.8 ± 0.1**	**0.4 ± 0.1**	**1.8 ± 0.3**	**0.237**	**0.108**
18:2n-9	6.6 ± 0.1	4.0 ± 0.3	3.1 ± 0.1	ND	-	-
20:2n-9	6.1 ± 0.6	4.1 ± 0.3	3.2 ± 0.1	1.9 ± 0.2	0.938	0.000
Σ n-9 PUFA	**12.7 ± 0.5**	**8.2 ± 0.6**	**6.3 ± 0.2**	**1.9 ± 0.2**	**0.875**	**0.000**
Σ PUFA	**21.9 ± 1.4**	**12.0 ± 0.5**	**10.4 ± 0.7**	**5.2 ± 0.4**	**0.944**	**0.000**

Footnotes: Results are expressed as mean ± 1 SD (n=3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* SFA = saturated fatty acid; MUFA = saturated fatty acid; PUFA = polyunsaturated fatty acid; ND = not detected; *contains 20:3n-9.

3.3.3. Effect of supplemental n-6 PUFA (LOA, 18:3n-6, 20:3n-6 and ARA) on lipid content, class and fatty acid compositions of CHSE-214 cells

3.3.3.1. Supplementation with LOA

In Table 3.11 the lipid content and the lipid class composition of CHSE-214 cells incubated with increasing concentrations of LOA are presented. No clear trend was

observed in total lipid content with the graded supplementation of LOA (R^2 = 0.148; p = 0.218). Apart from TAG, which increased with the graded supplementation of LOA (R^2 = 0.855; p = 0.000), no clear trends were observed in most of the lipid classes.

Table 3.11. Lipid content and lipid class composition of CHSE-214 incubated with 20, 50 and 100 µM LOA

Lipid	Control	20 µM	50 µM	100 µM	R^2	P-value
LC (µg)	406.7 ± 5.8	373.3 ± 30.6	440.0 ± 70.0	433.3 ± 30.6	0.148	0.218
CC (%)						
PC	23.0 ± 0.6	22.2± 0.6	22.6 ± 0.5	25.8 ± 1.1	0.410	0.025
PE	15.6 ± 0.2	17.4 ± 0.4	17.2 ± 0.3	19.0 ± 0.2	0.832	0.000
PS	4.2 ± 0.9	3.8 ± 0.1	3.8 ± 0.1	4.1 ± 0.3	0.101	0.755
PI	10.8 ± 0.7	9.8 ± 0.3	8.0 ± 0.2	8.0 ± 0.1	0.839	0.000
PA/CL	2.1 ± 0.3	1.2 ± 0.3	0.7 ± 0.1	0.4 ± 0.1	0.764	0.000
SM	4.2 ± 0.6	2.5 ± 0.4	2.6 ± 0.2	3.8 ± 0.5	0.027	0.613
TP	59.9 ± 1.5	56.9 ± 0.7	54.9 ± 0.6	61.1± 0.8	0.009	0.764
TN	40.1 ± 1.5	43.1 ± 0.7	45.1 ± 0.6	40.0 ± 0.8	0.009	0.764
TAG	4.1 ± 0.2	4.6 ± 0.4	10.0 ± 0.5	10.8 ± 1.4	0.855	0.000
CHOL	36.0 ± 1.6	38.5 ± 1.4	35.1 ± 0.8	28.1 ± 1.9	0.557	0.005
FFA	ND	ND	ND	ND	-	-

Footnotes: Results are expressed as mean ± 1 SD (n=3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LOA = linoleic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA composition of CHSE-214 cells incubated with increasing concentrations of LOA is presented in Table 3.12. Clearly, the relative levels of PUFA increased with increasing LOA supplementation (R^2 = 0.964; p = 0.000). Therefore, the proportions of the major FA groups in control (unsupplemented) cells was as follows: MUFA > SFA > PUFA; with 20 µM: MUFA > PUFA > SFA; and with 50 µM and 100 µM: PUFA > MUFA > SFA. Graded increased cellular LOA with increased supplementation of LOA was observed (R^2 = 0.938; p = 0.000).

Table 3.12. Fatty acid composition (%) of CHSE-214 incubated with 20, 50 and 100 µM of LOA

Fatty acid	Control	20 µM	50 µM	100 µM	R^2	P-value
14:0	1.7 ± 0.3	1.1 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	0.602	0.003
15:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.152	0.210
16:0	12.7 ± 0.1	12.1 ± 0.3	11.8 ± 0.6	10.2 ± 0.7	0.708	0.001
17:0	ND	0.3 ± 0.1	0.4 ± 0.0	0.2 ± 0.0	0.208	0.101
18:0	7.0 ± 0.2	8.7 ± 0.1	9.6 ± 0.3	8.4 ± 0.5	0.368	0.036
22:0	ND	0.4 ± 0.1	0.5 ± 0.0	0.1 ± 0.0	0.368	0.067
24:0	ND	ND	0.5 ± 0.0	0.5 ± 0.0	-	-
Σ SFA	**21.6 ± 0.1**	**22.8 ± 0.6**	**24.1 ± 0.5**	**20.7 ± 1.2**	**0.010**	**0.761**
16:1n-9	4.3 ± 0.2	3.5 ± 0.0	2.6 ± 0.1	2.3 ± 0.2	0.939	0.000
16:1n-7	2.6 ± 0.2	1.6 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	0.901	0.000
18:1n-9	47.5 ± 0.8	35.2 ± 0.6	24.2 ± 0.3	20.2 ± 1.9	0.960	0.000
18:1n-7	1.7 ± 0.2	1.8 ± 0.2	1.7 ± 0.4	1.7 ± 0.1	0.000	0.991
22:1n-9	0.2 ± 0.0	ND	0.1 ± 0.0	0.2 ± 0.0	-	-
24:1n-9	1.2 ± 0.3	ND	ND	ND	-	-
Σ MUFA	**57.5 ± 0.7**	**42.1 ± 0.5**	**29.8 ± 0.3**	**25.5 ± 2.2**	**0.948**	**0.000**
16:2n-6	ND	0.5 ± 0.0	1.0 ± 0.0	1.4 ± 0.2	0.884	0.000
18:2n-6	2.0 ± 0.1	11.4 ± 0.4	19.6 ± 0.7	25.4 ± 3.0	0.938	0.000
18:3n-6	0.3 ± 0.1	2.5 ± 0.1	5.3 ± 0.2	7.2 ± 1.0	0.953	0.000
20:2n-6*	1.0 ± 0.1	0.8 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.738	0.000
20:3n-6	1.3 ± 0.1	4.5 ± 0.1	6.5 ± 0.2	6.8 ± 0.2	0.844	0.000
20:4n-6	1.2 ± 0.1	1.8 ± 0.0	2.6 ± 0.2	2.6 ± 0.1	0.888	0.000
22:2n-6	ND	0.5 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.328	0.051
22:3n-6	ND	0.3 ± 0.0	1.0 ± 0.0	1.3 ± 0.1	0.944	0.000
22:4n-6	ND	0.6 ± 0.0	0.2 ± 0.0	ND	-	-
24:2n-6	ND	0.4 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.398	0.028
Σ n-6 PUFA	**5.8 ± 0.1**	**23.3 ± 0.6**	**37.5 ± 1.0**	**45.8 ± 4.2**	**0.942**	**0.000**
20:5n-3	0.5 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.508	0.009
22:5n-3	0.7 ± 0.1	ND	ND	0.4 ± 0.0	-	-
22:6n-3	0.9 ± 0.2	1.3 ± 0.0	1.1 ± 0.0	1.1 ± 0.1	0.051	0.084
Σ n-3 PUFA	**2.1 ± 0.4**	**1.7 ± 0.0**	**1.5 ± 0.0**	**1.8 ± 0.1**	**0.320**	**0.055**
16:2n-9	ND	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.501	0.010
18:2n-9	6.7 ± 0.2	5.1 ± 0.2	3.5 ± 0.1	3.0 ± 0.3	0.943	0.000
20:2n-9	5.8 ± 0.2	3.3 ± 0.0	2.0 ± 0.1	1.6 ± 0.2	0.929	0.000
22:2n-9	0.5 ± 0.1	0.5 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.863	0.000
22:3n-9	ND	ND	0.6 ± 0.0	0.4 ± 0.0	-	-
24:2n-9	ND	0.7 ± 0.0	0.6 ± 0.0	0.5 ± 0.1	0.481	0.012
Σ n-9 PUFA	**13.0 ± 0.2**	**10.1 ± 0.3**	**7.3 ± 0.3**	**6.0 ± 0.7**	**0.952**	**0.000**
Σ PUFA	**20.9 ± 0.7**	**35.1 ± 0.6**	**46.1 ± 0.7**	**53.8 ± 3.4**	**0.964**	**0.000**

Footnotes: Results are expressed as mean ± SD (n=3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LOA = linoleic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; ND = not detected; *contains 20:3n-9.

There was some apparent desaturation of LOA as there were also increased percentages of 18:3n-6 with LOA supplementation ($R^2 = 0.953$; $p = 0.000$). In addition the proportions of other metabolites, such as 20:3n-6 ($R^2 = 0.844$; $p = 0.000$), ARA (R^2 =

0.888; $p = 0.000$) and, to a lesser extent, C22 n-6 PUFA and 24:2n-6, were also increased, suggesting further metabolism of LOA. In consequence, the apparent enzymatic activities of the LC-PUFA biosynthesis pathway on LOA, led to increased percentage of all n-6 PUFA ($R^2 = 0.942$; $p = 0.000$), and decreased the percentages of MUFA ($R^2 = 0.948$; $p = 0.000$) and n-3 PUFA ($R^2 = 0.320$; $p = 0.055$), but the percentages of total SFA were not altered ($R^2 = 0.010$; $p = 0.761$).

3.3.3.2. Supplementation with 18:3n-6

In Table 3.13 the lipid content and the lipid class composition of CHSE-214 incubated with increasing concentrations of 18:3n-6 are presented. Total cell lipid content showed a trend of increased levels with the graded supplementation of 18:3n-6; however results were not statistically significant ($R^2 = 0.245$; $p = 0.102$). No clear trends associated with supplementation of 18:3n-6 were observed in the lipid class composition data.

Table 3.13. Lipid content and lipid class composition of CHSE-214 incubated with 18:3n-6

Lipid	Control	20 µM	50 µM	100 µM	R^2	P-value
LC (µg)	426.7 ± 80.8	440.0 ± 87.2	456.7 ± 30.6	516.7 ± 55.1	0.245	0.102
CC (%)						
PC	23.6 ± 1.0	18.5 ± 2.5	18.2 ± 0.9	19.7 ± 1.3	0.099	0.318
PE	14.3 ± 1.4	15.5 ± 1.1	17.2 ± 0.8	15.8 ± 0.7	0.264	0.087
PS	4.0 ± 2.1	5.3 ± 0.6	5.1 ± 0.3	4.6 ± 0.3	0.256	0.093
PI	9.2 ± 4.6	10.7 ± 0.6	10.8 ± 0.4	9.7 ± 0.2	0.056	0.458
PA/CL	2.5 ± 0.7	3.0 ± 1.0	2.7 ± 0.5	2.1 ± 0.2	0.023	0.641
SM	5.0 ± 0.2	3.5 ± 1.3	4.2 ± 0.7	3.9 ± 0.8	0.126	0.257
TP	58.6 ± 8.5	56.5 ± 3.6	58.2 ± 2.4	55.8 ± 0.8	0.037	0.549
TN	41.4 ± 8.5	43.5 ± 3.6	41.8 ± 2.4	44.2 ± 0.8	0.037	0.549
TAG	4.7 ± 0.5	2.8 ± 0.6	1.9 ± 0.7	2.5 ± 0.4	0.326	0.053
CHOL	36.7 ± 7.6	40.7 ± 2.5	39.9 ± 1.8	41.1 ± 1.3	0.285	0.074
FFA	ND	ND	ND	0.6 ±0.2	-	-

Footnotes: Results are expressed as mean ± 1 SD (n=3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA compositions of CHSE-214 cells incubated with 18:3n-6 are presented in Table 3.14. As with LOA supplementation, increasing levels of supplemented LOA resulted in graded increments in cellular PUFA content ($R^2 = 0.970$; $p = 0.000$).

Table 3.14. Fatty acid composition (%) of CHSE-214 incubated with 20, 50 and 100µM of 18:3n-6

Fatty acid	Control	20 µM	50 µM	100 µM	R^2	P-value
14:0	1.4 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	0.633	0.002
15:0	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.9 ± 0.1	0.538	0.065
16:0	13.5 ± 1.8	12.8 ± 0.6	13.8 ± 1.0	9.8 ± 0.8	0.390	0.030
17:0	ND	2.8 ± 0.2	1.8 ± 0.2	2.5 ± 0.3	-	-
18:0	7.8 ± 0.9	9.6 ± 0.6	11.2 ± 0.9	7.5 ± 0.9	0.001	0.936
22:0	0.4 ± 0.2	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.071	0.401
Σ SFA	**23.5 ± 2.9**	**27.0 ± 1.4**	**28.6 ± 2.2**	**22.2 ± 1.6**	**0.006**	**0.809**
16:1n-9	4.7 ± 0.1	3.2 ± 0.0	2.5 ± 0.0	2.0 ± 0.0	0.955	0.000
16:1n-7	2.5 ± 0.2	1.6 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	0.832	0.000
18:1n-9	46.1 ± 2.8	35.5 ± 0.3	26.5 ± 1.1	20.0 ± 0.7	0.980	0.000
18:1n-7	1.9 ± 0.1	1.6 ± 0.3	1.8 ± 0.2	1.5 ± 0.1	0.302	0.064
20:1n-11	ND	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	-	-
20:1n-9	ND	0.2 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	-	-
22:1n-11	ND	0.7 ± 0.1	0.5 ± 0.1	0.2 ± 0.0	-	-
22:1n-9	ND	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	-	-
24:1n-9	0.9 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	0.871	0.000
Σ MUFA	**56.1 ± 2.6**	**44.0 ± 0.2**	**34.0 ± 1.3**	**25.8 ± 1.0**	**0.983**	**0.000**
16:3n-6	ND	0.5 ± 0.0	1.1 ± 0.1	0.4 ± 0.1	-	-
18:2n-6	2.2 ± 0.6	1.6 ± 0.1	1.4 ± 0.1	1.5 ± 0.2	0.492	0.011
18:3n-6	0.3 ± 0.0	6.2 ± 0.4	13.0 ± 0.9	28.8 ± 2.4	0.986	0.000
20:2n-6*	1.0 ± 0.1	0.6 ± 0.0	0.5 ± 0.1	0.3 ± 0.0	0.916	0.000
20:3n-6	0.9 ± 0.1	5.8 ± 0.7	8.6 ± 0.6	10.0 ± 0.4	0.865	0.000
20:4n-6	1.1 ± 0.1	2.0 ± 0.3	2.7 ± 0.5	1.5 ± 0.2	0.142	0.228
22:4n-6	ND	0.8 ± 0.1	1.1 ± 0.1	1.5 ± 0.1	0.820	0.000
22:5n-6	ND	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.546	0.006
Σ n-6 PUFA	**5.6 ± 0.6**	**18.0 ± 1.4**	**28.9 ± 2.2**	**44.4 ± 2.5**	**0.985**	**0.000**
20:5n-3	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	1.1 ± 0.1	0.615	0.003
22:5n-3	0.7 ± 0.2	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.144	0.223
22:6n-3	1.7 ± 0.4	1.1 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	0.635	0.002
Σ n-3 PUFA	**3.0 ± 0.5**	**2.3 ± 0.3**	**2.2 ± 0.1**	**2.5 ± 0.2**	**0.164**	**0.192**
18:2n-9	6.7 ± 0.4	5.2 ± 0.1	3.8 ± 0.2	2.9 ± 0.1	0.979	0.000
20:2n-9	5.0 ± 0.9	3.0 ± 0.1	1.8 ± 0.2	1.5 ± 0.1	0.903	0.000
22:2n-9	ND	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	-	-
22:3n-9	ND	0.3 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	-	-
Σ n-9 PUFA	**11.8 ± 1.2**	**8.8 ± 0.2**	**6.3 ± 0.3**	**5.0 ± 0.3**	**0.955**	**0.000**
Σ PUFA	**20.4 ± 0.3**	**29.1 ± 1.6**	**37.4 ± 2.6**	**51.9 ± 2.4**	**0.970**	**0.000**

Footnotes: Results are expressed as mean ± 1 SD (n=3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; ND = not detected; *contains 20:3n-9.

Therefore, in the control (unsupplemented cells) treatment, the proportions of the FA groups were in the following rank order: MUFA > SFA > PUFA; in cells incubated with 20 µM 18:3n-6 MUFA > PUFA > SFA; and, in cells incubated with 50 µM and 100 µM 18:3n-6 the rank order was: PUFA > MUFA > SFA. There was a graded incorporation of 18:3n-6 itself (R^2 = 0.986; p = 0.000), as well as some elongation to 20:3n-6 (R^2 = 0.865; p = 0.000), and to a lesser extent some desaturation to ARA observed with supplementation at 20 µM and 50 µM, but results were not statistically significant (R^2 = 0.142; p = 0.228). Small percentages of C22 n-6 PUFA were also detected. The graded supplementation of 18:3n-6 reduced the percentages of MUFA (R^2 = 0.983; p = 0.000) and n-9 PUFA (R^2 = 0.955; p = 0.000), while the percentages of total PUFA (R^2 = 0.970; p = 0.000) and n-6 PUFA (R^2 = 0.985; p = 0.000) increased, with no alteration of total SFA (R^2 = 0.006; p = 0.809) and n-3 PUFA levels (R^2 = 0.164; p = 0.192).

3.3.3.3. Supplementation with 20:3n-6

The total lipid content and the lipid class composition of CHSE-214 cells incubated with increasing concentrations of 20:3n-6 are presented in Table 3.15. The cellular lipid content did not show a clear trend with the graded supplementation of 20:3n-6 (R^2 = 0.000; p = 0.955). Regarding class composition, TAG increased with the graded supplementation of 20:3n-6 (R^2 = 0.919; p = 0.000), which increased by four-fold at 50 µM and over six-fold at 100 µM, this increment was balanced by decreased polar class lipids.

Table 3.15. Lipid content and lipid class composition of CHSE-214 incubated with 20:3n-6

Lipid	Control	20 µM	50 µM	100 µM	R^2	P-value
LC (µg)	426.7 ± 90.7	369.9 ± 66.6	401.6 ± 95.4	274.5 ± 83.3	0.000	0.955
CC (%)						
PC	22.5 ± 0.6	21.9 ± 0.5	19.8 ± 1.3	24.8 ± 0.9	0.728	0.000
PE	14.6 ± 0.3	13.5 ± 0.3	12.4 ± 0.3	10.3 ± 0.7	0.912	0.000
PS	5.9 ± 0.5	6.0 ± 0.6	4.3 ± 0.1	3.0 ± 0.4	0.075	0.389
PI	9.9 ± 0.7	8.0 ± 0.6	4.5 ± 0.1	3.4 ± 0.9	0.813	0.000
PA/CL	2.8 ± 0.4	2.8 ± 0.8	1.9 ± 0.1	2.2 ± 0.2	0.929	0.000
SM	4.2 ± 0.1	4.6 ± 0.3	2.5 ± 0.1	2.3 ± 0.5	0.309	0.061
TP	59.9 ± 1.4	56.8 ± 2.0	45.4 ± 1.5	46.0 ± 2.9	0.803	0.000
TN	40.1 ± 1.4	43.2 ± 2.0	54.6± 1.5	54.0 ± 2.9	0.803	0.000
TAG	4.2 ± 0.5	5.1 ± 0.5	17.0 ± 1.9	25.8 ± 2.1	0.919	0.000
CHOL	35.9 ± 1.0	38.1 ± 2.6	37.6 ± 1.1	28.2 ± 0.9	0.407	0.026
FFA	ND	ND	ND	ND	-	-

Footnotes: Results are expressed as mean ± 1 SD (n=3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA compositions of CHSE-214 cells incubated with increasing concentrations of 20:3n-6 are presented in Table 3.16. Graded increased proportions of 20:3n-6 were observed with increased supplementation ($R^2 = 0.961$; $p = 0.000$). In addition, the data suggested some metabolism of 20:3n-6, including apparent retro-conversion of 20:3n-6 to 18:3n-6 ($R^2 = 0.972$; $p = 0.000$) and, to a lesser extent, 16:3n-6, and desaturation of 20:3n-6 to ARA ($R^2 = 0.870$; $p = 0.000$). Some C22 n-6 PUFA were also detected but only in low percentages. Supplementation of 20:3n-6 increased the proportions of total n-6 PUFA ($R^2 = 0.963$; $p = 0.000$), without affecting the total SFA ($R^2 = 0.035$; $p = 0.563$), but lowered the percentages of total MUFA ($R^2 = 0.971$; $p = 0.000$) and n-9 PUFA ($R^2 = 0.940$; $p = 0.000$).

Table 3.16. Fatty acid composition (%) of CHSE-214 incubated with 20, 50 and 100µM of 20:3n-6

Fatty acid	Control	20 µM	50 µM	100 µM	R^2	P-value
14:0	1.8 ± 0.3	1.1 ± 0.0	1.5 ± 0.2	0.9 ± 0.1	0.167	0.188
15:0	0.3 ± 0.0	0.3 ± 0.1	0.5 ± 0.0	0.3 ± 0.1	0.084	0.362
16:0	13.7± 0.6	17.0 ± 2.3	19.2 ± 1.2	9.5 ± 0.5	0.144	0.224
17:0	ND	0.6 ± 0.1	1.2 ± 0.2	1.5 ± 0.4	-	-
18:0	6.5 ± 0.2	8.5 ± 0.5	11.1 ± 0.5	5.1 ± 0.0	0.006	0.811
22:0	0.2 ± 0.1	0.6 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.000	0.987
Σ SFA	**22.5 ± 0.8**	**28.0 ± 2.6**	**33.8 ± 1.0**	**17.7 ± 0.8**	**0.035**	**0.563**
16:1n-9	5.1 ± 0.4	3.4 ± 0.5	2.8 ± 0.3	2.2 ± 0.3	0.861	0.000
16:1n-7	3.3 ± 0.2	2.5 ± 0.3	1.9 ± 0.2	1.7 ± 0.1	0.891	0.000
18:1n-9	45.5 ± 0.6	31.1 ± 1.6	24.5 ± 1.2	16.6 ± 1.2	0.969	0.000
18:1n-7	2.2 ± 0.3	2.2 ± 0.1	2.3 ± 0.1	1.5 ± 0.1	0.442	0.018
20:1n-9	ND	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	-	-
22:1n-9	ND	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	-	-
24:1n-9	1.0 ± 0.3	0.8 ± 0.3	0.8 ± 0.3	0.9 ± 0.2	0.046	0.504
Σ MUFA	**57.1 ± 0.2**	**40.5 ± 1.5**	**32.8 ± 0.8**	**23.3 ± 1.3**	**0.971**	**0.000**
16:3n-6	ND	2.6 ± 0.4	1.1 ± 0.0	2.1 ± 0.1	0.442	0.018
18:2n-6	1.8 ± 0.1	1.6 ± 0.3	1.3 ± 0.1	1.0 ± 0.0	0.810	0.000
18:3n-6	0.4 ± 0.1	1.2 ± 0.1	2.8 ± 0.3	5.9 ± 0.5	0.972	0.000
20:2n-6*	1.2 ± 0.1	0.8 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.863	0.000
20:3n-6	1.0 ± 0.1	10.4 ± 0.7	14.8 ± 0.9	33.7 ± 0.3	0.961	0.000
20:4n-6	1.0 ± 0.2	3.3 ± 0.2	3.4 ± 0.6	6.2 ± 0.7	0.870	0.000
22:3n-6	ND	0.7 ± 0.1	1.9 ± 0.3	2.1 ± 0.2	0.889	0.000
22:4n-6	ND	0.4 ± 0.0	0.5 ± 0.1	0.6 ± 0.1	0.795	0.000
Σ n-6 PUFA	**5.4 ± 0.4**	**18.3 ± 1.1**	**25.0 ± 1.0**	**50.0 ± 1.5**	**0.963**	**0.000**
20:5n-3	0.4 ± 0.2	0.5 ± 0.1	0.6 ± 0.0	0.4 ± 0.1	0.042	0.525
22:5n-3	0.8 ± 0.1	0.5 ± 0.1	0.4 ± 0.0	0.7 ± 0.1	0.153	0.209
22:6n-3	1.0 ± 0.1	0.9 ± 0.1	1.4 ± 0.2	2.1 ± 0.5	0.691	0.001
Σ n-3 PUFA	**2.2 ± 0.2**	**1.9 ± 0.1**	**2.4 ± 0.2**	**3.2 ± 0.6**	**0.464**	**0.015**
18:2n-9	6.1 ± 0.4	4.5 ± 0.5	2.6 ± 0.0	2.3 ± 0.2	0.918	0.000
20:2n-9	6.7 ± 0.2	3.1 ± 0.9	1.7 ± 0.1	1.4 ± 0.1	0.858	0.000
22:2n-9	ND	0.4 ± 0.1	0.3 ± 0.0	ND	-	-
22:3n-9	ND	0.6 ± 0.1	0.3 ± 0.0	ND	-	-
Σ n-9 PUFA	**12.8 ± 0.4**	**8.0 ± 0.9**	**4.6 ± 0.2**	**3.7 ± 0.2**	**0.940**	**0.000**
Σ PUFA	**20.4 ± 0.7**	**28.2 ± 1.4**	**32.0 ± 1.1**	**56.9 ± 1.6**	**0.874**	**0.000**

Footnotes: Results are expressed as mean ± 1 SD (n=3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; ND = not detected; *contains 20:3n-9.

3.3.3.4. Supplementation with ARA

The lipid content and the lipid class composition of CHSE-214 cells incubated with increasing concentrations of ARA are presented in Table 3.17. The cell total lipid content showed a trend to increase with graded supplementation of ARA from 20 µM to 50 µM and from 50 µM to 100 µM; however, results were not statistically significant ($R^2 = 0.053$;

p = 0.471). The lipid class composition showed some clear trends with increasing concentration of supplemented ARA, with graded increments of total neutral lipids (R^2 = 0.573; *p* = 0.004) and, particularly, TAG (R^2 = 0.779; *p* = 0.000), which was increased over two-fold at 20 µM, and by almost three-fold at 50 µM and 100 µM.

Table 3.17. Lipid content and lipid class composition of CHSE-214 incubated with ARA

Lipid	Control	20 µM	50 µM	100 µM	R^2	P-value
LC (µg)	423.3 ± 46.2	296.0 ± 90.0	320.0 ± 65.6	373.3 ± 49.3	0.053	0.471
CC (%)						
PC	24.5 ± 1.2	21.7 ± 0.7	28.7 ± 1.2	28.5 ± 0.9	0.748	0.000
PE	14.8 ± 0.8	17.0 ± 1.9	11.3 ± 0.5	10.4 ± 0.2	0.592	0.003
PS	4.0 ± 0.4	3.8 ± 0.9	2.2 ± 0.4	2.6 ± 0.3	0.479	0.013
PI	8.6 ± 0.7	7.6 ± 0.7	5.3 ± 0.6	5.7 ± 0.8	0.554	0.005
PA/CL	2.8 ± 0.0	2.5 ± 0.2	2.4 ± 0.6	2.3 ± 0.2	0.703	0.001
SM	5.4 ± 0.3	3.9 ± 0.4	2.3 ± 0.4	2.6 ± 0.1	0.245	0.102
TP	60.1 ± 2.3	56.4 ± 4.6	52.1 ± 3.3	52.1 ± 0.5	0.573	0.004
TN	39.9 ± 2.3	43.6 ± 4.6	47.9 ± 3.3	47.9 ± 0.5	0.573	0.004
TAG	4.2 ± 0.9	9.5 ± 0.7	12.1 ± 4.1	14.2 ± 0.8	0.779	0.000
CHOL	35.7 ± 1.7	34.1 ± 3.9	33.7 ± 1.3	32.0 ± 1.1	0.201	0.144
FFA	ND	ND	2.0 ± 0.8	1.7 ± 0.1	-	-

Footnotes: Results are expressed as mean ± 1 SD (n=3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* ARA = arachidonic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA composition of CHSE-214 cells incubated with increasing concentrations of ARA is presented in Table 3.18. There were graded increased proportions of cellular ARA with increased supplementation of this FA (R^2 = 0.989; *p* = 0.000). Some apparent elongation to 22:4n-6 was observed (R^2 = 0.818; *p* = 0.000), but no desaturation to 22:5n-6 was apparent. In addition, an increment of LOA (R^2 = 0.827; *p* = 0.000), particularly at 20 µM ARA was observed. The deposition of ARA, and the enzymatic activities of the LC-PUFA biosynthesis pathway on ARA led to increased percentages of total n-6 PUFA (R^2 = 0.997; *p* = 0.000), and decreased the proportions of total MUFA (R^2 = 0.995; *p* = 0.000)

and n-9 PUFA (R^2 = 0.732; p = 0.000), but the percentages of total SFA (R^2 = 0.007; p = 0.800) and n-3 PUFA (R^2 = 0.086; p = 0.356) were not altered.

Table 3.18. Fatty acid composition (%) of CHSE-214 incubated with 20, 50 and 100 µM of ARA

Fatty acid	Control	20 µM	50 µM	100 µM	R^2	P-value
14:0	1.4 ± 0.1	1.5 ± 0.2	1.8 ± 0.0	1.1 ± 0.1	0.103	0.310
15:0	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.7 ± 0.0	0.806	0.000
16:0	11.7 ± 0.6	18.7 ± 0.3	14.7 ± 0.2	11.9 ± 0.8	0.013	0.720
17:0	0.6 ± 0.1	0.9 ± 0.2	0.8 ± 0.2	0.8 ± 0.1	0.256	0.093
18:0	7.0 ± 0.1	10.5 ± 0.4	9.5 ± 0.3	6.2 ± 0.3	0.052	0.476
20:0	ND	0.4 ± 0.1	0.6 ± 0.0	0.6 ± 0.1	-	-
22:0	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.357	0.040
Σ SFA	**21.3 ± 0.7**	**32.7 ± 0.5**	**28.2 ± 0.2**	**21.5 ± 0.9**	**0.007**	**0.800**
16:1n-9	4.2 ± 0.1	2.9 ± 0.3	1.9 ± 0.2	1.7 ± 0.1	0.893	0.000
16:1n-7	4.0 ± 0.4	2.9 ± 0.1	2.9 ± 0.1	1.7 ± 0.0	0.873	0.000
18:1n-9	45.1 ± 0.6	27.9 ± 0.5	22.9 ± 0.1	14.9 ± 0.3	0.952	0.000
18:1n-7	1.8 ± 0.2	3.2 ± 0.5	2.7 ± 0.0	2.0 ± 0.3	0.001	0.932
20:1n-9	0.2 ± 0.1	0.6 ± 0.2	0.2 ± 0.0	0.4 ± 0.0	0.044	0.511
20:1n-7	0.2 ± 0.1	3.0 ± 0.3	2.3 ± 0.1	1.3 ± 0.0	0.200	0.145
22:1n-11	ND	1.7 ± 0.1	1.1 ± 0.0	0.9 ± 0.0	-	-
22:1n-9	0.1 0.0	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.059	0.448
24:1n-9	1.6 ± 0.2	1.1 ± 0.1	0.6 ± 0.0	0.5 ± 0.0	0.929	0.000
Σ MUFA	**57.2 ± 0.6**	**43.9 ± 0.5**	**34.6 ± 0.3**	**23.8 ± 0.6**	**0.995**	**0.000**
18:2n-6	2.0 ± 0.1	4.9 ± 0.2	3.3 ± 0.3	1.5 ± 0.1	0.094	0.332
18:3n-6	0.4 ± 0.1	0.4 ± 0.1	0.6 ± 0.0	1.2 ± 0.1	0.827	0.000
20:2n-6*	1.3 ± 0.0	0.6 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.767	0.000
20:3n-6	1.3 ± 0.1	1.9 ± 0.4	1.6 ± 0.2	2.5 ± 0.2	0.589	0.004
20:4n-6	0.7 ± 0.1	6.3 ± 0.4	16.4 ± 0.4	37.2 ± 1.9	0.989	0.000
22:4n-6	ND	2.0 ± 0.2	8.4 ± 0.2	6.6 ± 0.2	0.818	0.000
Σ n-6 PUFA	**5.8 ± 0.0**	**16.2 ± 0.9**	**30.7 ± 0.4**	**49.4 ± 1.9**	**0.997**	**0.000**
20:5n-3	0.8 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.102	0.312
22:5n-3	0.6 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.0 ± 0.2	0.253	0.095
22:6n-3	1.3 ± 0.1	1.6 ± 0.2	1.8 ± 0.2	1.6 ± 0.2	0.223	0.121
Σ n-3 PUFA	**2.8 ± 0.3**	**4.0 ± 0.2**	**4.0 ± 0.1**	**3.3 ± 0.6**	**0.086**	0.356
18:2n-9	6.6 ± 0.3	3.3 ± 0.2	2.5 ± 0.3	2.1 ± 0.1	0.842	0.000
20:2n-9	6.4 ± 0.2	ND	ND	ND	-	-
Σ n-9 PUFA	**13.0 ± 0.5**	**3.3 ± 0.2**	**2.5 ± 0.3**	**2.1 ± 0.1**	**0.732**	**0.000**
Σ PUFA	**21.6 ± 0.1**	**23.4 ± 1.0**	**37.1 ± 0.3**	**54.8 ± 1.4**	**0.913**	**0.000**

Footnotes: Results are expressed as mean ± 1 SD (n=3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* ARA = arachidonic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; ND = not detected; *contains 20:3n-9.

3.3.4. Effect of supplemental n-3 PUFA (LNA, EPA, 22:5n-3 and DHA) on lipid content, class and fatty acid compositions of CHSE-214 cells

3.3.4.1. Supplementation with LNA

The total cell lipid content and lipid class composition of CHSE-214 cells incubated with LNA are presented in Table 3.19. The total lipid content of the cells showed a graded increase with supplementation of LNA up to 50 µM, but with supplementation at 100 µM the apparent lipid content reduced to less than half (R^2 = 0.012; p = 0.734). The lipid class data was variable, but there was a clear trend with increasing supplementation of LNA, with a graded increment of total neutral lipids (R^2 = 0.887; p = 0.000), and particularly TAG (R^2 = 0.825; p = 0.000), which increased over two-fold at 50 µM, and over three-fold at 100 µM.

Table 3.19. Lipid content and lipid class composition of CHSE-214 incubated with LNA

Lipid	Control	20µM	50µM	100µM	R^2	P-value
LC (µg)	416.7 ± 25.2	438.9 ± 5.8	680.9 ± 55.1	299.4 ± 30.6	0.012	0.734
CC (%)						
PC	24.6 ± 1.0	22.4 ± 1.0	26.1 ± 0.9	25.1 ± 0.7	0.129	0.252
PE	14.1 ± 3.3	19.3 ± 0.2	16.8 ± 3.5	17.0 ± 0.3	0.068	0.414
PS	6.2 ± 0.2	4.6 ± 0.6	4.5 ± 0.4	3.0 ± 0.7	0.781	0.000
PI	10.4 ± 0.3	7.1 ± 0.5	6.2 ± 0.5	4.8 ± 0.9	0.880	0.000
PA/CL	2.4 ± 1.0	3.3 ± 0.2	2.5 ± 0.3	5.7 ± 0.2	0.502	0.010
SM	6.2 ± 0.7	5.6 ± 0.9	3.6 ± 0.7	1.7 ± 0.2	0.873	0.000
TP	64.0 ± 0.4	62.3 ± 1.4	59.8 ± 1.1	57.3 ± 1.2	0.887	0.000
TN	36.0 ± 0.4	37.7 ± 1.4	40.2 ± 1.1	42.7 ± 1.2	0.887	0.000
TAG	4.4 ± 0.4	4.3 ± 0.5	9.3 ± 0.8	15.8 ± 3.6	0.825	0.000
CHOL	31.6 ± 0.8	33.4 ± 1.9	30.9 ± 1.3	26.9 ± 2.5	0.448	0.017
FFA	ND	ND	ND	ND	-	-

Footnotes: Results are expressed as mean ± 1 SD (n=3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LNA = α-linolenic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA composition of CHSE-214 cells incubated with LNA is presented in Table 3.20. The graded supplementation of this FA increased the proportions of LNA itself in cells (R^2 = 0.972; p = 0.000), along with its elongation product, 18:4n-3 (R^2 = 0.971; p = 0.000). Other n-3 PUFA metabolites were also detected such as 20:4n-3 (R^2 = 0.579; p = 0.004), which increased at concentration up to 50 µM supplementation, and EPA, whose concentration only increased up to 20 µM supplementation (R^2 = 0.458; p = 0.016). The effects of increased total n-3 PUFA (R^2 = 0.970; p = 0.000) were balanced by decreased total MUFA (R^2 = 0.952; p = 0.000), n-6 PUFA (R^2 = 0.420; p = 0.023) and n-9 PUFA (R^2 = 0.871; p = 0.000), but did not affect the percentages of total SFA (R^2 = 0.076; p = 0.385).

Table 3.20. Fatty acid composition (%) of CHSE-214 incubated with 20, 50 and 100 µM of LNA

Fatty acid	Control	20 µM	50 µM	100 µM	R^2	P-value
14:0	1.8 ± 0.8	1.5 ± 0.3	1.8 ± 0.3	2.0 ± 0.8	0.061	0.438
15:0	0.3 ± 0.1	0.2 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.091	0.340
16:0	12.8 ± 1.0	14.9 ± 1.8	15.4 ± 0.6	10.6 ± 1.0	0.081	0.370
18:0	6.9 ± 0.1	11.0 ± 2.2	13.5 ± 0.6	10.0 ± 0.6	0.334	0.049
22:0	0.4 ± 0.0	0.8 ± 0.2	1.1 ± 0.3	0.7 ± 0.1	0.271	0.083
Σ SFA	**22.2 ± 1.8**	**28.4 ± 4.1**	**32.2 ± 0.8**	**23.6 ± 1.6**	**0.076**	**0.385**
16:1n-9	4.1 ± 0.2	3.2 ± 0.3	2.9 ± 0.4	2.9 ± 0.6	0.515	0.009
16:1n-7	3.2 ± 0.5	2.2 ± 0.1	1.7 ± 0.1	1.7 ± 0.3	0.728	0.000
18:1n-9	45.0 ± 1.1	35.4 ± 0.5	27.5 ± 4.1	22.8 ± 1.0	0.946	0.000
18:1n-7	2.5 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.9 ± 0.2	0.144	0.224
24:1n-9	1.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	0.536	0.007
Σ MUFA	**56.0 ± 0.7**	**44.4 ± 0.2**	**35.9 ± 3.9**	**31.3 ± 0.9**	**0.952**	**0.000**
18:2n-6	2.9 ± 0.2	2.1 ± 0.3	1.9 ± 0.2	2.0 ± 0.2	0.551	0.006
18:3n-6	0.4 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.020	0.659
20:2n-6*	1.2 ± 0.1	0.7± 0.0	0.6 ± 0.1	0.7 ± 0.4	0.328	0.052
20:3n-6	1.2 ± 0.1	1.1 ± 0.2	0.9 ± 0.2	1.1 ± 0.0	0.110	0.291
20:4n-6	1.6 ± 0.1	1.3 ± 0.6	1.0 ± 0.2	1.2 ± 0.2	0.205	0.140
22:4n-6	ND	0.4 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.001	0.939
Σ n-6 PUFA	**7.3 ± 0.5**	**5.8 ± 1.0**	**4.8 ± 0.6**	**5.5 ± 0.8**	**0.420**	**0.023**
18:3n-3	ND	3.8 ± 0.2	8.2 ± 1.2	17.9 ± 2.7	0.972	0.000
18:4n-3	ND	2.9 ± 0.1	6.0 ± 0.9	10.6 ± 0.1	0.971	0.000
20:3n-3	ND	ND	0.2 ± 0.0	0.5 ± 0.1	-	-
20:4n-3	ND	1.9 ± 0.2	3.3 ± 0.2	2.0 ± 0.7	0.579	0.004
20:5n-3	0.5 ± 0.0	4.0 ± 0.7	3.8 ± 0.4	3.2 ± 0.2	0.458	0.016
22:5n-3	0.9 ± 0.1	0.9 ± 0.3	0.8 ± 0.1	0.7 ± 0.1	0.227	0.117
22:6n-3	1.6 ± 0.1	1.3 ± 0.4	0.9 ± 0.1	1.0 ± 0.0	0.502	0.010
Σ n-3 PUFA	**3.0 ± 0.4**	**14.8 ± 1.9**	**23.1 ± 2.9**	**36.0 ± 1.8**	**0.970**	**0.000**
18:2n-9	6.1 ± 0.1	4.1 ± 0.4	2.8 ± 0.0	2.5 ± 0.1	0.901	0.000
20:2n-9	5.4 ± 0.3	2.5 ± 0.6	1.2 ± 0.1	1.0 ± 0.9	0.751	0.000
Σ n-9 PUFA	**11.5 ± 0.3**	**6.6 ± 1.1**	**4.0 ± 0.1**	**3.5 ± 0.8**	**0.871**	**0.000**
Σ PUFA	**21.8 ± 1.2**	**27.2 ± 3.9**	**31.9 ± 3.5**	**45.0 ± 2.4**	**0.865**	**0.000**

Footnotes: Results are expressed as mean ± 1 SD (n=3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LNA = α- linolenic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; ND = not detected; *contains 20:3n-9.

3.3.4.2. Supplementation with EPA

In Table 3.21 the cell lipid content and lipid class composition of CHSE-214 cells incubated with increasing concentrations of EPA are presented. There was no clear trend observed in the cell total lipid content with the graded supplementation of EPA (R^2 =

0.075; p = 0.389). It was particularly noteworthy that supplementation with EPA at 20 µM decreased the proportion of cell TAG by almost four-fold, as this was the first FA showing this TAG-lowering effect. However, TAG increased by almost two-fold at 50 µM and over three-fold at 100 µM supplementation of EPA (R^2 = 0.502; p = 0.010). Except for PC, which increased with the graded supplementation of EPA (R^2 = 0.869; p = 0.000), the remaining lipid class composition data showed no clear trends with increasing concentration of supplemented EPA.

Table 3.21. Lipid content and lipid class composition of CHSE-214 incubated with EPA

Lipid	Control	20 µM	50 µM	100 µM	R^2	P-value
LC (µg)	406.7 ± 64.3	403.3 ± 63.5	323.3 ± 45.1	386.7 ± 51.3	0.075	0.389
CC (%)						
PC	24.3 ± 0.9	28.2 ± 1.1	30.2 ± 1.2	29.2 ± 2.3	0.869	0.000
PE	16.7 ± 0.7	18.6 ± 0.5	13.6 ± 0.3	11.2 ± 1.0	0.701	0.001
PS	5.4 ± 0.5	6.1 ± 0.4	3.9 ± 0.2	2.7 ± 0.2	0.551	0.006
PI	10.2 ± 0.7	9.3 ± 0.3	7.4 ± 0.4	6.4 ± 0.3	0.741	0.000
PA/CL	2.1 ± 0.4	3.6 ± 0.1	2.4 ± 0.2	1.5 ± 0.4	0.926	0.000
SM	4.0 ± 0.4	4.0 ± 0.2	2.6 ± 0.2	1.6 ± 0.1	0.151	0.212
TP	62.7 ± 2.3	69.8 ± 0.4	60.1 ± 0.7	52.6 ± 1.2	0.493	0.011
TN	37.3 ± 2.3	30.2 ± 0.4	39.9 ± 0.7	47.4 ± 1.2	0.493	0.011
TAG	4.5 ± 1.2	1.2 ± 0.2	8.8 ± 1.3	14.3 ± 3.6	0.502	0.010
CHOL	32.8 ± 0.2	27.2 ± 0.3	28.6 ± 1.6	30.5 ± 2.4	0.018	0.674
FFA	ND	1.8 ± 0.1	2.5 ± 0.2	2.6 ± 0.2	-	-

Footnotes: Results are expressed as mean ± 1 SD (n=3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* EPA = eicosapentaenoic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA composition of CHSE-214 cells incubated with EPA is presented in Table 3.22. The graded supplementation of EPA increased the proportion of EPA itself in the cells (R^2 = 0.928; p = 0.000), along with its direct elongation product, 22:5n-3 up to 50 µM (R^2 = 0.504; p = 0.010), and increased LNA was also detected, but no increased proportions of DHA were observed (R^2 = 0.462; p = 0.015). The effects of increased total n-3 PUFA (R^2 = 0.874; p = 0.000) were balanced by decreased proportions of total SFA (R^2 = 0.483; p =

0.012), MUFA (R^2 = 0.867; p = 0.087), and n-9 PUFA (R^2 = 0.786; p = 0.000), it also decreased the percentages of total n-6 PUFA at concentrations up to 50 µM (R^2 = 0.083; p = 0.364).

Table 3.22. Fatty acid composition (%) of CHSE-214 incubated with 20, 50 and 100 µM of EPA

Fatty acid	Control	20 µM	50 µM	100 µM	R^2	P-value
14:0	1.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	0.159	0.200
15:0	0.4 ± 0.0	0.9 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.009	0.775
16:0	13.0 ± 0.7	15.7 ± 0.2	8.4 ± 0.5	7.3 ± 1.0	0.556	0.006
17:0	ND	0.7 ± 0.1	0.7 ± 0.1	0.5 ± 0.0	0.550	0.006
18:0	7.8 ± 0.2	7.0 ± 0.1	6.1 ± 0.2	5.4 ± 0.1	0.693	0.001
20:0	ND	0.2 ± 0.0	0.3 ± 0.1	0.6 ± 0.2	0.239	0.107
22:0	0.3 ± 0.1	0.5 ± 0.0	0.6 ± 0.1	0.9 ± 0.1	0.875	0.000
Σ SFA	**23.1 ± 0.4**	**25.6 ± 0.3**	**17.0 ± 0.6**	**15.8 ± 0.9**	**0.483**	**0.012**
16:1n-9	3.2 ± 0.1	2.2 ± 0.1	2.1 ± 0.2	1.7 ± 0.4	0.774	0.000
16:1n-7	3.1 ± 0.4	2.1 ± 0.1	2.0 ± 0.4	2.2 ± 0.3	0.264	0.087
18:1n-9	46.6 ± 1.3	26.7 ± 0.1	20.5 ± 0.4	18.5 ± 0.9	0.829	0.000
18:1n-7	2.1 ± 0.1	2.6 ± 0.6	1.7 ± 0.1	1.5 ± 0.1	0.460	0.015
24:1n-9	1.1 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.107	0.299
Σ MUFA	**56.2 ± 1.0**	**34.6 ± 0.5**	**27.3 ± 0.3**	**24.7 ± 1.1**	**0.867**	**0.000**
18:2n-6	2.3 ± 0.3	1.5 ± 0.1	1.8 ± 0.2	1.9 ± 0.2	0.372	0.035
18:3n-6	0.5 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	1.2 ± 0.5	0.517	0.008
20:2n-6*	1.0 ± 0.2	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.487	0.012
20:3n-6	1.3 ± 0.5	ND	ND	ND	-	-
20:4n-6	1.1 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	1.1 ± 0.0	0.002	0.901
22:4n-6	ND	0.3 ± 0.1	0.2 ± 0.1	1.0 ± 0.2	0.840	0.000
22:5n-6	ND	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.888	0.000
Σ n-6 PUFA	**6.2 ± 0.7**	**3.3 ± 0.2**	**3.8 ± 0.1**	**6.1 ± 0.6**	**0.083**	**0.364**
18:3n-3	ND	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.558	0.005
20:5n-3	0.5 ± 0.2	20.1 ± 0.5	33.8 ± 0.2	39.9 ± 1.5	0.928	0.000
22:5n-3	0.7 ± 0.1	9.1 ± 0.1	12.0 ± 0.3	8.0 ± 0.5	0.504	0.010
22:6n-3	1.5 ± 0.3	1.5 ± 0.2	1.3 ± 0.1	1.1 ± 0.1	0.462	0.015
Σ n-3 PUFA	**2.7 ± 0.7**	**31.0 ± 0.6**	**47.4 ± 0.4**	**49.4 ± 2.0**	**0.874**	**0.000**
18:2n-9	6.6 ± 0.6	3.6 ± 0.3	3.1 ± 0.1	2.6 ± 0.1	0.831	0.000
20:2n-9	5.2 ± 0.7	1.9 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	0.727	0.000
Σ n-9 PUFA	**11.8 ± 1.2**	**5.5 ± 0.3**	**4.5 ± 0.1**	**4.0 ± 0.1**	**0.786**	**0.000**
Σ PUFA	**20.7 ± 0.4**	**39.8 ± 0.2**	**55.7 ± 0.4**	**59.4 ±2.0**	**0.941**	**0.000**

Footnotes: Results are expressed as mean ± 1 SD (n=3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* EPA = eicosapentaenoic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; ND = not detected; *contains 20:3n-9.

3.3.4.3. Supplementation with 22:5n-3

The cell lipid content and lipid class composition of CHSE-214 cells incubated with increasing concentrations of 22:5n-3 are presented in Table 3.23. The cell total lipid

content did not show a clear trend with graded supplementation of 22:5n-3 (R^2 = 0.322; p = 0.055). Increased proportions of total neutral lipids (R^2 = 0.676; p = 0.001) and, particularly, TAG (R^2 = 0.875; p = 0.000) were observed, increasing over two-fold at 50 µM and over four-fold at 100 µM. The remaining of the lipid class composition data was rather variable and showed no obvious consistent trends with graded supplementation of 22:5n-3 other than reciprocal changes driven by TAG levels.

Table 3.23. Lipid content and lipid class composition of CHSE-214 incubated with 22:5n-3

Lipid	Control	20 µM	50 µM	100 µM	R^2	P-value
LC (µg)	430.1 ± 50.0	431.4 ± 30.6	431.3 ± 30.6	353.1 ± 70.2	0.322	0.055
CC (%)						
PC	22.5 ± 0.8	22.4 ± 0.8	25.5 ± 1.0	24.6 ± 0.9	0.714	0.001
PE	15.8 ± 0.6	17.4 ± 0.4	15.3 ± 0.2	12.1 ± 0.5	0.568	0.005
PS	5.7 ± 0.6	6.6 ± 0.4	4.8 ± 0.2	3.5 ± 0.2	0.483	0.012
PI	10.3 ± 0.6	10.4 ± 0.2	7.6 ± 0.3	5.7 ± 0.1	0.664	0.001
PA/CL	2.8 ± 0.4	1.9 ± 0.4	2.3 ± 0.5	1.7 ± 0.3	0.879	0.000
SM	5.4 ± 0.3	4.9 ± 0.1	4.9 ± 0.1	3.4 ± 0.5	0.373	0.035
TP	62.5 ± 1.8	63.6 ± 1.3	60.4 ± 2.1	51.0 ± 0.7	0.676	0.001
TN	37.5 ± 1.8	36.4 ± 1.3	39.6 ± 2.1	49.0 ± 0.7	0.676	0.001
TAG	4.3 ± 0.3	4.5 ± 0.4	9.6 ± 1.2	20.2 ± 0.9	0.875	0.000
CHOL	33.2 ± 1.7	31.9 ± 1.4	30.0 ± 1.3	28.8 ± 1.6	0.643	0.002
FFA	ND	ND	ND	ND	-	-

Footnotes: Results are expressed as mean ± 1 SD (n=3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA compositions of CHSE-214 cells incubated with increasing concentrations of 22:5n-3 are presented in Table 3.24. The graded supplementation of 22:5n-3 led to increased deposition of 22:5n-3 itself in the cells (R^2 = 0.950; p = 0.000). There was evidence of some production of DHA at 20 µM and 50 µM supplementation. There was apparent retro-conversion of 22:5n-3 to EPA (R^2 = 0.827; p = 0.000), and some low but increased percentages of 20:4n-3 were also detected. The effects of increased total n-3 PUFA (R^2 = 0.936; p = 0.000) were balanced by decreased total MUFA (R^2 = 0.906; p =

0.000) and n-9 PUFA (R^2 = 0.917; p = 0.000), but the percentages of total SFA (R^2 = 0.242; p = 0.104) and n-6 PUFA were not affected (R^2 = 0.542; p = 0.006).

Table 3.24. Fatty acid composition (%) of CHSE-214 incubated with 20, 50 and 100µM of 22:5n-3

Fatty acid	Control	20 µM	50 µM	100 µM	R^2	P-value
14:0	1.2 ± 0.2	0.8 ± 0.2	1.3 ± 0.3	1.1 ± 0.2	0.031	0.583
15:0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.397	0.028
16:0	12.5 ± 0.9	15.3 ± 0.1	14.2 ± 0.5	10.8 ± 0.7	0.151	0.211
17:0	ND	0.6 ± 0.0	0.3 ± 0.0	0.6 ± 0.1	0.143	0.312
18:0	6.9 ± 0.1	8.4 ± 0.4	6.9 ± 0.2	4.9 ± 0.2	0.282	0.075
22:0	0.4 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.793	0.000
Σ SFA	**21.3 ± 1.2**	**25.8 ± 0.4**	**23.3 ± 0.6**	**17.9 ± 1.0**	**0.242**	**0.104**
16:1n-9	5.0 ± 0.8	3.1 ± 0.3	2.6 ± 0.1	2.3 ± 0.4	0.781	0.000
16:1n-7	3.3 ± 0.7	2.1 ± 0.1	1.9 ± 0.5	1.4 ± 0.1	0.750	0.000
18:1n-9	45.4 ± 1.9	28.7 ± 0.1	20.4 ± 0.3	19.1 ± 0.7	0.902	0.000
18:1n-7	2.5 ± 0.1	2.3 ± 0.1	2.0 ± 0.1	1.7 ± 0.1	0.916	0.000
20:1n-11	ND	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	-	-
20:1n-9	ND	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	-	-
22:1n-11	ND	0.9 ± 0.1	0.6 ± 0.2	0.3 ± 0.2	-	-
22:1n-9	ND	0.2 ± 0.0	0.1 ± 0.0	0.4 ± 0.0	-	-
24:1n-9	1.7 ± 0.4	0.8 ± 0.2	1.2 ± 0.3	0.7 ± 0.0	0.427	0.021
Σ MUFA	**57.9 ± 1.3**	**38.4 ± 0.4**	**29.1 ± 0.9**	**26.3 ± 1.4**	**0.906**	**0.000**
18:2n-6	2.5 ± 0.2	1.7 ± 0.1	1.5 ± 0.0	2.5 ± 0.1	0.009	0.771
18:3n-6	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.813	0.000
20:2n-6*	1.3 ± 0.1	0.6 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.914	0.000
20:3n-6	1.2 ± 0.5	1.5 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	0.611	0.003
20:4n-6	1.1 ± 0.3	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.649	0.002
22:4n-6	ND	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.233	0.112
22:5n-6	ND	0.3 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.843	0.000
Σ n-6 PUFA	**6.5 ± 0.7**	**5.0 ± 0.2**	**4.1 ± 0.1**	**4.9 ± 0.1**	**0.542**	**0.006**
20:4n-3	ND	0.3 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.749	0.000
20:5n-3	0.5± 0.1	6.7 ± 0.1	11.1 ± 0.4	11.3 ± 0.6	0.827	0.000
22:5n-3	0.7 ± 0.1	13.1 ± 0.1	24.4 ± 0.5	33.2 ± 1.9	0.950	0.000
22:6n-3	1.5 ± 0.2	2.1 ± 0.0	2.0 ± 0.1	1.7 ± 0.1	0.107	0.300
Σ n-3 PUFA	**2.7 ± 0.2**	**22.2 ± 0.1**	**38.1 ± 0.9**	**46.6 ± 2.3**	**0.936**	**0.000**
18:2n-9	6.4 ± 0.4	4.4 ± 0.2	2.8 ± 0.2	2.4 ± 0.1	0.916	0.000
20:2n-9	5.2 ± 0.1	3.1 ± 0.1	1.8 ± 0.1	1.5 ± 0.1	0.903	0.000
22:2n-9	ND	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.339	0.047
22:3n-9	ND	0.8 ± 0.2	0.7 ± 0.1	0.2 ± 0.1	-	-
Σ n-9 PUFA	**11.6 ± 0.5**	**7.8 ± 0.2**	**4.8 ± 0.3**	**4.1 ± 0.2**	**0.917**	**0.000**
Σ PUFA	**20.8 ± 0.7**	**35.0 ± 0.4**	**47.0 ± 1.3**	**55.6 ± 2.3**	**0.987**	**0.000**

Footnotes: Results are expressed as mean ± 1 SD (n=3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; ND = not detected; *contains 20:3n-9.

3.3.4.4. Supplementation with DHA

The lipid content and the lipid class composition of CHSE-214 cells incubated with increasing concentrations of DHA are presented in Table 3.25. The cellular total lipid content graded increased with the supplementation of DHA up to 50 µM, and then decreased at 100 µM, albeit results were not statistically significant (R^2 = 0.322; p = 0.055). The lipid class composition data was variable, and generally showed no obvious or consistent trends with increasing supplementation of DHA. However, as with EPA supplementation, there was a significant reduction in cellular TAG when the cells were incubated with 20 µM DHA. Furthermore, at 100 µM supplementation, cellular TAG level increased by almost four-fold (R^2 = 0.489; p = 0.011).

Table 3.25. Lipid content and lipid class composition of CHSE-214 incubated with DHA

Lipid	Control	20µM	50µM	100 µM	R^2	P-value
LC (µg)	423.0 ± 65.6	465.0 ± 70.0	607.0 ± 91.7	517.0 ± 43.6	0.322	0.055
CC (%)						
PC	20.1 ± 0.5	20.0 ± 1.3	22.8 ± 0.9	18.4 ± 1.8	0.068	0.415
PE	16.9 ± 0.9	19.2 ± 1.3	17.0 ± 2.4	11.0 ± 1.4	0.420	0.023
PS	6.8 ± 0.3	7.1 ± 0.5	6.0 ± 0.3	3.6 ± 0.3	0.025	0.627
PI	11.4 ± 0.7	11.4 ± 0.8	9.5 ± 0.6	6.3 ± 0.4	0.727	0.000
PA/CL	2.7 ± 0.2	1.8 ± 0.2	1.7 ± 0.2	1.6 ± 0.1	0.782	0.000
SM	4.4 ± 1.0	4.9 ± 0.8	6.0 ± 0.6	3.1 ± 0.3	0.622	0.002
TP	62.3 ± 0.5	64.4 ± 2.2	63.0 ± 1.2	44.0 ± 0.3	0.539	0.007
TN	37.7 ± 0.5	35.6 ± 2.2	37.0 ± 1.2	56.0 ± 0.3	0.539	0.007
TAG	4.1 ± 0.2	1.4 ± 0.2	3.2 ± 0.4	16.0 ± 0.5	0.489	0.011
CHOL	33.6 ± 0.3	32.2 ± 0.9	31.3 ± 1.0	36.9 ± 0.5	0.213	0.131
FFA	ND	2.0 ± 0.3	2.5 ± 0.2	3.1 ± 0.4	-	-

Footnotes: Results are expressed as mean ± 1 SD (n=3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* DHA = docosahexaenoic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA composition of CHSE-214 cells incubated with DHA is presented in Table 3.26. The graded supplementation of DHA increased the cellular proportion of DHA itself

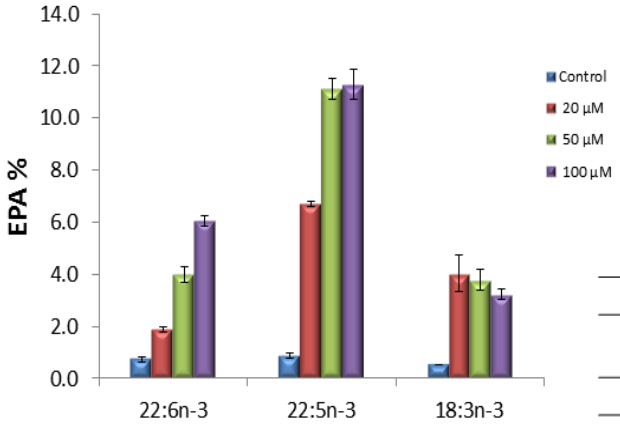
(R^2 = 0.977; p = 0.087), along with increased percentages of EPA (R^2 = 0.992; p = 0.000) and, to a lesser extent, 22:5n-3 (R^2 = 0.951; p = 0.000). The effects of increased total n-3 PUFA (R^2 = 0.978; p = 0.000) were balanced by decreased proportions of total MUFA (R^2 = 0.948; p = 0.000) and n-9 PUFA (R^2 = 0.935; p = 0.000), but the percentages of total SFA (R^2 = 0.019; p = 0.666) and n-6 PUFA (R^2 = 0.061; p = 0.438) were generally not affected.

Table 3.26. Fatty acid composition (%) of CHSE-214 incubated with 20, 50 and 100 µM of DHA

Fatty acid	Control	20 µM	50 µM	100 µM	R^2	P-value
14:0	1.5 ± 0.0	1.2 ± 0.1	1.2 ± 0.1	1.5 ± 0.5	0.128	0.254
15:0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.5 ± 0.0	0.800	0.000
16:0	12.9 ± 0.6	13.1 ± 1.3	14.8 ± 1.9	12.2 ± 0.9	0.130	0.250
17:0	ND	0.5 ± 0.1	0.4 ± 0.0	0.3 ± 0.1	-	-
18:0	7.6 ± 0.3	9.8 ± 0.4	10.6 ± 1.9	5.9 ± 0.1	0.031	0.586
20:0	ND	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	-	-
22:0	0.3 ± 0.0	0.6 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.002	0.891
Σ SFA	**22.5 ± 0.9**	**25.5 ± 1.9**	**28.0 ± 4.1**	**21.1 ± 1.3**	**0.019**	**0.666**
16:1n-9	4.1 ± 0.6	3.7 ± 0.3	2.8 ± 0.2	2.5 ± 0.5	0.750	0.000
16:1n-7	4.4 ± 1.7	2.3 ± 0.8	1.8 ± 0.3	1.8 ± 0.2	0.546	0.006
18:1n-9	43.5 ± 0.2	32.9 ± 1.8	23.1 ± 1.0	17.9 ± 1.3	0.970	0.000
18:1n-7	2.0 ± 0.1	1.6 ± 0.1	1.7 ± 0.2	1.8 ± 0.3	0.355	0.041
20:1n-9	ND	0.3 ± 0.0	0.4 ± 0.2	0.7 ± 0.2	-	-
20:1n-7	ND	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	-	-
22:1n-9	ND	0.2 ± 0.1	0.1 ± 0.0	ND	-	-
24:1n-9	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.3	1.4 ± 0.3	0.000	0.983
Σ MUFA	**55.4 ± 1.3**	**42.4 ± 1.8**	**31.16 ± 1.2**	**26.3 ± 2.0**	**0.948**	**0.000**
18:2n-6	2.1 ± 0.2	1.9 ± 0.1	1.6 ± 0.1	2.7 ± 0.2	0.149	0.216
18:3n-6	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.077	0.384
20:2n-6*	1.3 ± 0.2	1.2 ± 0.0	0.8 ± 0.0	0.8 ± 0.2	0.749	0.000
20:3n-6	1.2 ± 0.1	2.2 ± 1.1	2.0 ± 0.5	1.2 ± 0.5	0.007	0.792
20:4n-6	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	0.344	0.045
Σ n-6 PUFA	**5.7 ± 0.3**	**6.5 ± 1.3**	**5.5 ± 0.8**	**5.5 ± 0.8**	**0.061**	**0.438**
20:5n-3	0.7 ± 0.1	1.9 ± 0.1	4.0 ± 0.3	6.0 ± 0.2	0.992	0.000
22:5n-3	0.5 ± 0.1	0.7 ± 0.0	1.2 ± 0.0	1.6 ± 0.1	0.951	0.000
22:6n-3	1.2 ± 0.3	11.4 ± 1.2	22.5 ± 2.1	35.4 ± 2.1	0.977	0.000
Σ n-3 PUFA	**2.4 ± 0.4**	**14.0 ± 1.2**	**27.7 ± 2.3**	**43.0 ± 1.8**	**0.978**	**0.000**
18:2n-9	6.9 ± 0.8	5.5 ± 0.7	3.6 ± 0.2	2.1 ± 0.3	0.946	0.000
20:2n-9	6.6 ± 0.8	5.6 ± 0.3	3.7 ± 0.1	1.8 ± 0.5	0.908	0.000
22:2n-9	0.5 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.914	0.000
Σ n-9 PUFA	**14.0 ± 1.6**	**11.6 ± 1.0**	**7.6 ± 0.3**	**4.2 ± 0.8**	**0.935**	**0.000**
Σ PUFA	**22.1 ± 2.0**	**32.1 ± 3.5**	**40.8 ± 2.8**	**52.7 ± 3.4**	**0.953**	**0.000**

Footnotes: Results are expressed as mean ± 1 SD (n=3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* DHA = docosahexaenoic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; ND = not detected; *contains 20:3n-9.

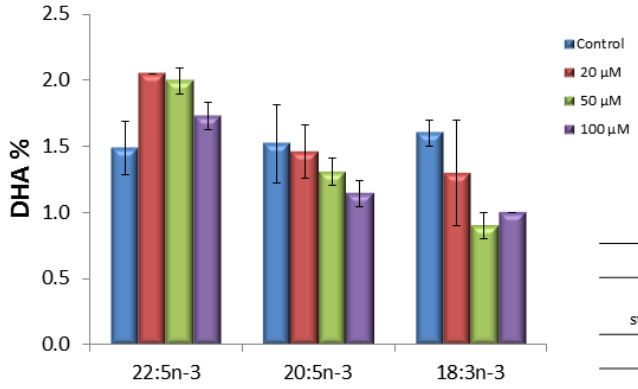
The following figures are presented as a summary to highlight results generated from the individual FA supplementation experiments (section 3.2.2) and the effects observed in cellular LC-PUFA (*i.e.* EPA, DHA and ARA). In all the cases, ANOVA showed that there was an effect of the FA supplementation (treatment), the concentration and the interaction of the last two on the cellular EPA, DHA and ARA levels ($p < 0.05$). Figure 3.1 shows the effects of n-3 PUFA supplementation on EPA production in CHSE-214 cells. There was apparent retro-conversion of 22:5n-3 to EPA observed, and this was higher at 50 µM than at 20 µM, but not really further increased at 100 µM 22:5n-3 supplementation. It is also clear that there was increased cellular EPA when cells were supplemented with LNA, particularly at 20 µM. The proportion of EPA in the cells was also clearly increased in a graded manner by the graded supplementation of DHA.



ANOVA		
Fatty acid supplemented	Concentration	Interaction
<0.000	<0.000	<0.000

Figure 3.1. Percentage of eicosapentaenoic acid (EPA) in CHSE-214 cell line after being incubated for five days with increasing concentrations of fatty acids, *i.e.* 0 (control), 20, 50 and 100 µM. Data were analysed by two-way ANOVA ($p < 0.05$). The interaction between the two factors (fatty acid supplemented and concentration) was also analysed (inlet table).

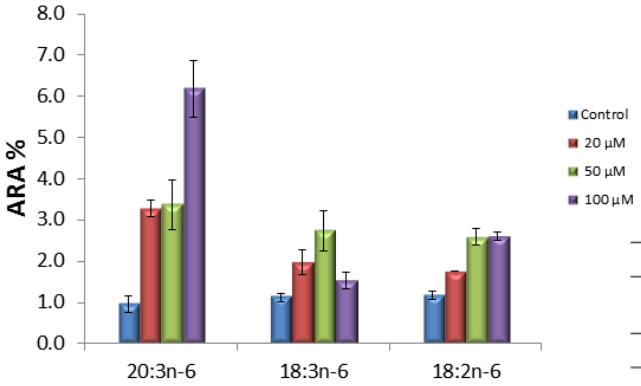
Figure 3.2 shows DHA levels in CHSE-214 cells after supplementation with n-3PUFA. Most of the FA supplemented did not increase the percentage of DHA in the cells, apart from 22:5n-3 which showed and increment of DHA, especially at 20 µM. The percentages of DHA were not significantly affected with supplementation by EPA or LNA.



ANOVA		
Fatty acid supplemented	Concentration	Interaction
<0.000	0.011	<0.000

Figure 3.2. Percentage of docosahexaenoic acid (DHA) in CHSE-214 cell line after being incubated for five days with increasing concentrations of fatty acids, *i.e.* 0 (control), 20, 50 and 100 µM. Data were analysed by two-way ANOVA ($p < 0.05$). The interaction between the two factors (fatty acid supplemented and concentration) was also analysed (inlet table).

Figure 3.3 shows the ARA levels of CHSE-214 cells after being supplemented with n-6 PUFA. The percentage of ARA in the cells was clearly increased by graded increased 20:3n-6, particularly at 100 µM. In addition, supplementing 18:3n-6 and LOA also increased the proportions of cellular ARA, with the highest percentages of ARA observed at 50 µM supplementation of the C_{18} n-6 PUFA.



ANOVA		
Fatty acid supplemented	Concentration	Interaction
<0.000	<0.000	<0.000

Figure 3.3. Percentage of arachidonic acid (ARA) in CHSE-214 cell line after being incubated for five days with increasing concentrations of fatty acids, *i.e.* 0 (control), 20, 50 and 100 µM. Data were analysed by two-way ANOVA ($p < 0.05$). The interaction between the two factors (fatty acid supplemented and concentration) was also analysed (inlet table).

Figure 3.4 shows the effects of supplementing n-3 FA at the different concentrations on the cellular levels of all the n-3 PUFA, including both LC-PUFA and pathway intermediates. The highest percentages of total n-3 PUFA were observed with the supplementation of EPA at 100 µM and 50 µM, with most of the EPA supplemented that was metabolised being elongated to 22:5n-3. There was apparent retro-conversion of DHA to EPA, but very little apparent biosynthesis of DHA from EPA. Similarly, the apparent retro-conversion of 22:5n-3 to EPA was higher than elongation of 22:5n-3 to DHA. There was some apparent biosynthesis of EPA from LNA. However, traveling down the pathway, the production of the metabolites from LNA was lower (18:4n-3 > 20:4n-3 > EPA). In addition, as the concentration of LNA supplementation increased, the increased deposition of LNA increased greatly. Therefore, supplementing LNA at 50 µM and 100 µM very likely masked any potential increments in the proportions of 20:4n-3 and EPA. This probably reflected the fact that the deposition of LNA was higher than the production of EPA.

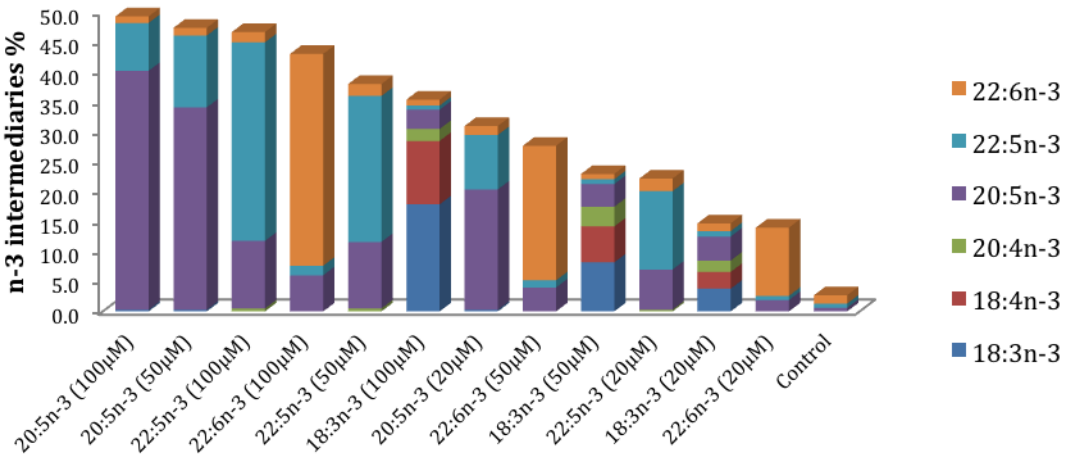


Figure 3.4. Total n-3 fatty acid intermediaries levels in CHSE-214 cell line after being incubated for 5 days with increasing concentrations of fatty acids, *i.e.* 0 (control), 20, 50 and 100 µM.

Figure 3.5 shows the effect of FA supplementation on cellular TAG levels in CHSE-214 cells. For most of the FA there was a trend for TAG to increase with the graded supplementation of FA. Supplementing 18:1n-9, 20:1n-9, LOA, 20:3n-6, LNA and 22:5n-3 at 20 µM did not affect the percentage of TAG, but at 50 µM and 100 µM there were clear trends of increasing TAG. ARA was the only FA that increased TAG immediately at 20 µM supplementation, whereas DHA and EPA had the opposite effect, decreasing cellular TAG levels when supplemented at 20 µM. However, both EPA and DHA at 50 µM and 100 µM increased cellular TAG levels. There was no a clear influence of 18:3n-6 on cell TAG levels, but 16:0 tended to decreased TAG at 20 µM and slightly increased cell TAG at 50 µM and 100 µM.

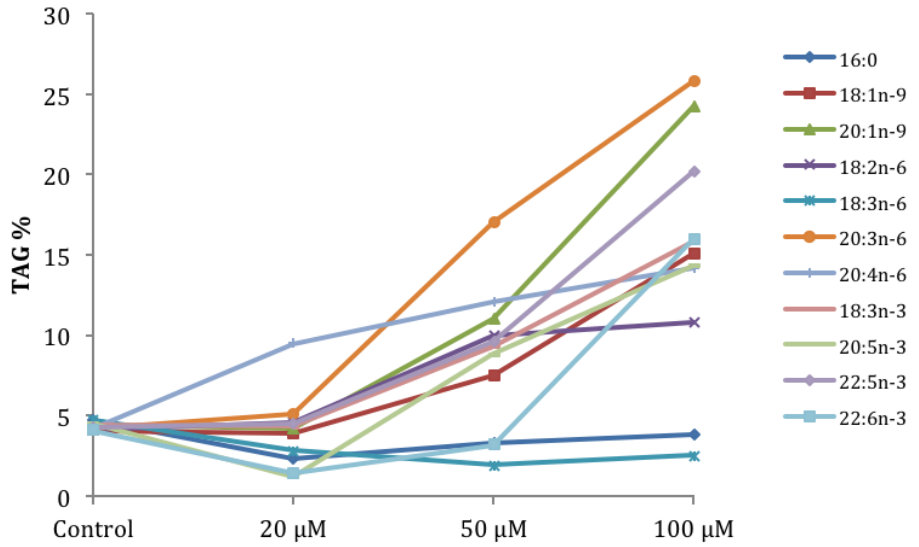


Figure 3.5. TAG (triacylglycerol) percentages in CHSE-214 supplemented with increasing concentrations of fatty acids.

Specifically focussing on the effects of the major PUFA (LOA and LNA) and LC-PUFA (ARA, EPA and DHA) on cellular TAG levels showed that supplementing LOA and ARA at 20 µM had relatively little effect on the percentage of TAG in CHSE-214 cells with LOA increasing it slightly, but ARA increased TAG in the cells by over two-fold

(Figure 3.6). In contrast, EPA and DHA supplemented at 20 µM decreased cellular TAG around three-fold.

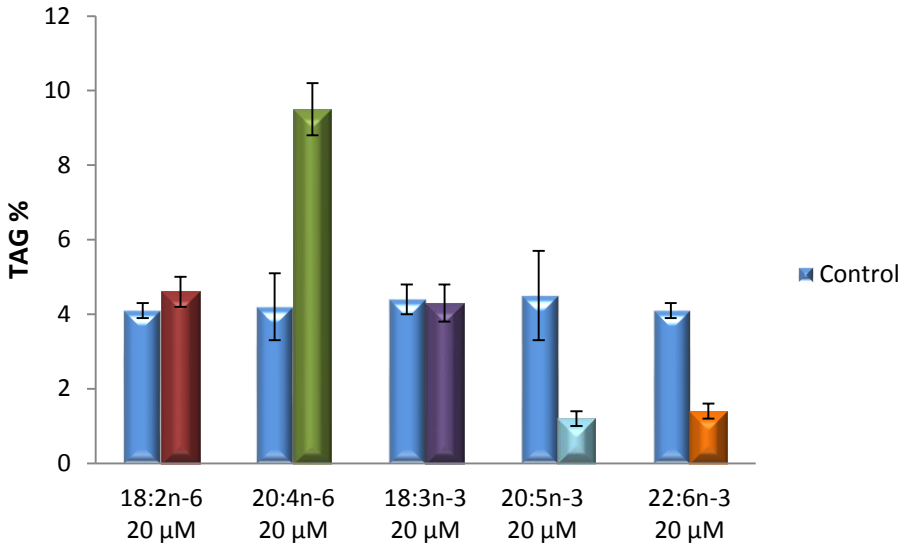


Figure 3.6. TAG (triacylglycerol) percentages in CHSE-214 with graded supplementation of fatty acids.

3.3.5. Effect of supplemental FA on growth of CHSE-214 cells: Toxicity of supplemental FA

The effects of supplemental FA on cell growth are presented in Figure 3.7. The results represent the absorbance obtained from the MTT assay as a proxy for cell number and therefore growth of cells or, alternatively, toxicity of the FA supplementation. The growth/toxicity experiments were described earlier in this Chapter. Thus, in this analysis the absorbance is positively related with the number of live cells. Data shown in Figure 3.7 are relative to un-supplemented cells (control), so less absorbance means a lower number of cells in comparison with the control (*i.e*. lower growth). The decreasing values showed a clear trend with FA supplementation, resulting in decreased absorbance with graded supplementation of FA. The figure shows the absorbance of cells incubated with 20 µM FA, which was 90–95% in relation to unsupplemented cells (control). The absorbance decreased considerably at 100 µM particularly with the supplementation of 22:5n-3, DHA,

20:3n-6, and EPA (70–80%), while supplementing 18:1n-9, LNA, and 16:0 at 100 µM the absorbance did not greatly decreased (~95%).

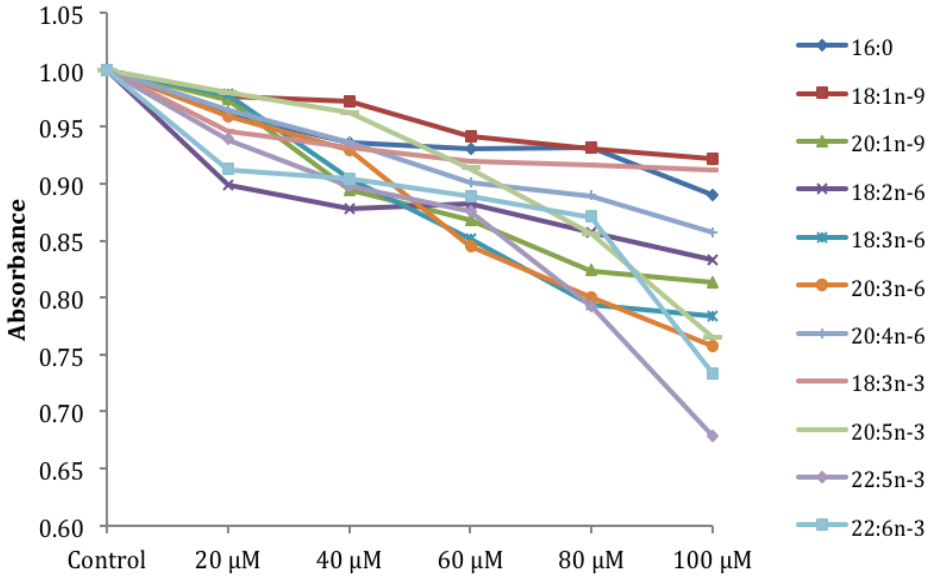


Figure 3.7. Absorbance of the MTT assay in CHSE-214 cell line after being incubated for five days with increasing concentrations of fatty acids, *i.e.* 0 (control), 20, 40, 60, 80 and 100 µM.

3.4. Discussion

The primary aim of the studies described in the present Chapter was to determine the effects of supplemental FA on lipid and FA metabolism in the CHSE-214 cell line and to assess their toxicity. To achieve this goal CHSE-214 cells were incubated for 5 d with increasing concentrations of FA, added as complexes, and the effects on cellular lipid content, lipid class and FA compositions, and survival were determined. The data produced confirmed that CHSE-214 cells incorporated supplemented FA in a concentration-dependent manner and, furthermore, established that this cell line was capable of some, albeit limited LC-PUFA biosynthesis via FA desaturation and/or elongation.

3.4.1. Lipid content, lipid class and FA composition of CHSE-214 growing under routine culture conditions (control)

Tocher *et al.* (1988) determined the FA composition of six fish cell lines growing in media supplemented with 10% FBS, and reported that the most abundant FA found in the cells were not the same as those most commonly found in most fish tissues, *i.e.* 16:0, 18:0, 16:1, 18:1n-9, 18:2, and 20:4, with only low levels of n-3 LC-PUFA. In the current study, the CHSE-214 cell line growing in the routine conditions for supply of cells (*i.e.* 20 ml of L-15 media containing with 10% FBS) or in the control cultures in the FA supplementation studies (*i.e.* 10 ml of L-15 media containing 5% FBS) were rich in 16:0, 18:0, 18:1n-9, and LOA. The FA profile of the cell line was therefore quite different from the fish tissue it was originally derived from. Regarding LC-PUFA, EPA, DHA and 22:5n-3 were also present in CHSE-214 cells; however, they were found in very low amounts compared with fish tissues. This difference is probably due to their culture using mammalian serum, which is poor in n-3 LC-PUFA compared to fish tissues (Tocher *et al.*, 1988). Ackman (1980) and Henderson and Tocher (1987) previously reported that the main qualitative difference between the FA profiles of fish cell lines and fish tissues was the lack of EPA and DHA, and quantitatively they reported that cell lines contain considerably less n-3 LC-PUFA in comparison with fish tissues. In the present study, it was observed that the FA composition of CHSE-214 cells growing in FBS presented less than 1% of EPA and less than 2% of DHA. Regarding lipid class compositions, total polar lipids were found at almost double the proportion of total neutral lipids in CHSE-214 growing in media containing 5–10% FBS. In the EPC (*Epithelioma papulosum cyprini*) cell line, the proportion of total polar lipids was reported to be about 75% and 25% for neutral lipids (Tocher *et al.*, 1995) and in a range of 72–41% in six fish cell lines, including

RTG-2, TF, AS, CHSE-214, BF-2 and FHM, growing in media supplemented with 10% FBS (Tocher *et al.*, 1988).

3.4.2. Effect of the concentration of FA supplemented on the lipid content of CHSE-214

An increment in cell total lipid content was expected with the graded supplementation of individual FA to the CHSE-214 cells, but this trend was only observed when 18:3n-6 was supplemented. In most of the experiments, no clear trends were observed amongst the treatments ($p > 0.05$). In some cases decreased lipid content with the graded supplementation of FA was observed, particularly at 100 µM. This effect was very clear when supplementing FA with the highest number of double bonds, namely EPA, 22:5n-3, and DHA. Prior to harvesting, it was observed that cells incubated with 100 µM FA were easily detached during the washing phase, which was not observed in cells incubated with lower concentrations of FA. Similarly, during harvesting of cells, it was observed that cells came off the substrate (flask surface) very readily when they had been incubated with 100 µM FA. The data produced from the MTT assay showed lower absorbance in wells where cells were incubated with 100 µM FA, indicating fewer cells in the wells. This effect of high FA concentration may be due to increased production of lipid radicals in cells incubated with 100 µM FA, especially the LC-PUFA, which would likely damage the cell membranes inhibiting growth/division and/or causing cell death and, therefore, a lower amount of total lipid was obtained from these flasks. In this sense Gregory *et al*. (2011) reported that n-3 LC-PUFA are easily attacked by reactive oxygen species (ROS), due to their high number of double bonds, triggering a chain reaction of hydrogen abstraction, and the formation of lipid radicals, causing cell membrane damage, and subsequent cell death. From the lipid content and toxicity/growth assay data it was concluded that at 20 µM the monolayer of CHSE-214 cell line is not adversely affected by

the FA supplementation, and enough lipid can be obtained for further analyse the lipid and FA composition.

3.4.3. Effect of supplementation of FA on lipid class composition of CHSE-214

Apart from proportions of TAG, the lipid class composition of CHSE-214 cells did not show clear, consistent and obvious trends with graded supplementation of FA. TAG often increased with the graded supplementation of FA, particularly at 50 µM and 100 µM concentrations. This increment was likely due to the high uptake and incorporation of the FA supplemented to the cells that was greater than the requirement for the FA either for membrane biosynthesis or energy (oxidation) resulting in deposition of the excess FA in cellular TAG stores. However, TAG level was also increased with the supplementation of ARA at only 20 µM, and the opposite effect was observed with the supplementation of EPA and DHA at the same concentration. This result is consistent with Sekiya *et al*. (2003), who reported that dietary n-3 LC-PUFA reduced TAG deposition in the liver of obese (*ob/ob*) mice. It is known that one effect of n-3 LC-PUFA (EPA and DHA) underpinning their role in cardiovascular disease is to reduce blood TAG, and part of that mechanism occurs by decreasing TAG synthesis in hepatocytes, probably mediated through the down-regulation of SREBP (sterol regulatory element-binding proteins) (Sekiya *et al.*, 2003). It was a noteworthy result from the present study that this differential effect of n-6 (ARA) and n-3 (EPA and DHA) LC-PUFA was clearly observed on the metabolism of CHSE-214 cells as has been previously observed in whole animal and primary cell culture studies (Collier and Collier, 1993; Whelan *et al.*, 1995; Whelan, 1996; Manickam *et al.*, 2010; Kaur *et al.*, 2011; Kajikawa *et al.*, 2011). The data on lipid class composition of the present study generally showed that supplementing FA at 20 µM clearly alters the FA composition of CHSE-214 cell line (see section 3.4.4), but without

swamping the cells with excess FA that has additional effects on lipid metabolism including the increased deposition of FA in TAG-rich lipid droplets in the cells. Consistent with other studies suggesting 20–25 μM as the "adequate" concentration for PUFA supplementation to cells in culture (Geyer, 1967; Moskowitz, 1967; Rosenthal, 1981; Stubbs and Smith, 1984; Tocher *et al.*, 1989; Tocher and Dick, 1990). In the current study, concentrations higher than 20 μM stimulated lipid storage pathways, resulting in the supplemented FA being deposited in lipid droplets in the cytoplasm. This situation affected the lipid class composition, increasing TAG production and, hence, total neutral lipid contents, whereas changes in phospholipid class composition were simply reflecting this primary change in relative lipid class composition.

3.4.4. Effect of supplementation of FA on FA composition of CHSE-214

The present study showed that all supplemented FA changed the FA profile of CHSE-214 cells due to the incorporation of the FA itself but also as a result of subsequent metabolism of the incorporated FA. For all FA supplemented, other than 18:1n-9 (see below), a graded incorporation of the FA into the CHSE-214 cells was observed.

3.4.4.1. Supplementation with SFA and MUFA

When 16:0 was supplemented, this FA was incorporated and deposited in greater amounts, and a large portion was desaturated to 16:1n-7, and to a lesser extent to 16:1n-9. Some of the 16:0 was elongated to 18:0, and there was evidence that 16:1n-7 was further elongated to 18:1n-7. These last effects were clear at 20 μM, but they were hidden at 100 μM because so much 16:0 was being deposited and/or desaturated, decreasing the proportions of all other FA. A similar effect was observed when 20:1n-9; however, a completely different effect was observed with the supplementation of 18:1n-9, which was

only deposited at 100 µM. It is most likely that at 20 µM and 50 µM it was largely oxidised to produce energy, and only when it was supplemented at very high concentrations was there more 18:1n-9 taken up by the cell than could be oxidised effectively, essentially swamping lipid metabolism and, as a result, it was finally deposited in TAG lipid stores in cytoplasmic lipid droplets. As a result of the accumulation of 18:1n-9 at 100 µM, all other FA decreased but, at lower concentrations, supplementation of 18:1n-9 had relatively little effect on cellular FA composition. No effects on n-9 PUFA were detected, therefore Δ6 desaturase was not stimulated, which is different from what was reported in turbot fin (TF) cell line (Ghioni *et al.*, 1999).

3.4.4.2. Supplementation with n-6 PUFA

Graded incorporation was observed when n-6 PUFA were supplemented, *i.e.* LOA 18:3n-6, 20:3n-6 and ARA. There was clear evidence of FA metabolism via the LC-PUFA biosynthesis pathways with n-6 PUFA. Therefore there was evidence of FA desaturation and elongation of the supplemented n-6 PUFA in the biosynthesis pathway, but the production of desaturated and/or elongated intermediates decreased down the pathway, and were progressively more affected by increased deposition of the n-6 PUFA that was supplemented. Therefore, the desaturated and elongated metabolites were easily detected at 20 µM and 50 µM, but not so at 100 µM, because any potential increase in their production would be masked due to the high incorporation and deposition of the n-6 PUFA supplemented. The main product of LOA metabolism was 18:3n-6, while supplemented 18:3n-6 was mainly elongated to 20:3n-6, and supplemented 20:3n-6 was both desaturated and retro-converted to ARA and 18:3n-6, respectively, and ARA was elongated into 22:4n-6 with no further desaturation. The incorporation of the n-6 PUFA and the products of the desaturation and/or elongation increased the proportions of total n-6 PUFA. This effect

was balanced by decreasing the proportions of total MUFA and n-9 PUFA but not SFA. Therefore, supplementing n-6 PUFA affected unsaturated FA, but SFA were unaffected. Overall, the data indicated that the CHSE-214 cell line can synthesise ARA from LOA as previously reported seen for other fish cell lines such as RTG-2 (Rainbow trout gonad) and Turbot fin (Tocher *et al.*, 1989).

3.4.4.3. Supplementation with n-3 PUFA

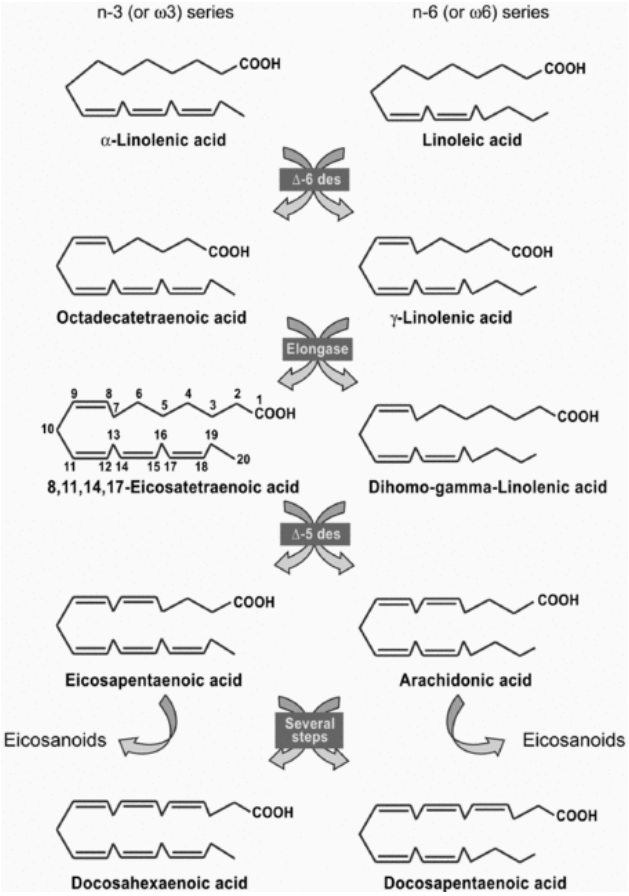
There was graded incorporation of the n-3 PUFA (*i.e.* LNA, EPA, 22:5n-3 and DHA) supplemented to CHSE-214 cells. These supplementations increased the percentage of total n-3 PUFA in CHSE-214 cells, which was balanced by decreasing total MUFA, n-6 PUFA and n-9 PUFA, but not SFA. When CHSE-214 cells were supplemented with LNA the main metabolite produced was 20:4n-3, while 22:5n-3 was the main metabolite produced with the supplementation of EPA, and EPA was the main metabolite when 22:5n-3 and DHA were supplemented. Therefore, there was clear evidence of desaturation and elongation of the n-3 PUFA supplemented by the enzymes of the LC-PUFA biosynthesis pathway. However, as described above for n-6 LC-PUFA, travelling down the pathway, the production of metabolites was lower and progressively more affected by the increased incorporation of the n-3 PUFA supplemented. However, it was clear that CHSE-214 was able to produce EPA from LNA, with the highest percentage observed at 20 µM LNA supplementation. It should be noted that this does not mean that at 50 µM and 100 µM LNA EPA was not produced, but what could have happened is that the high deposition of LNA masked any increased of EPA produced. In contrast the data are quite clear that the CHSE-214 cell line showed only very low levels of DHA irrespective of n-3 FA supplementation other than DHA itself. Therefore, it seems clear that DHA production from LNA is extremely low in CHSE-214 cells and therefore this cell line is not a good

model for DHA biosynthesis. Other fish cell lines have also been reported to lack sufficient enzymatic activity for the production of any biologically meaningful levels of DHA from LNA, at least in the relatively short-term of the assays (up to 7 days usually) in similar FA supplementation studies to those reported in the present study. For example, AS (Atlantic salmon) and RTG-2 cell lines were reported to have high activity of Δ6 and Δ5 desaturase but not Δ4 desaturase, while TF cell line was reported to be active regarding Δ6 desaturase, but showing very low activity of Δ5 desaturase and elongase from C_{18} to C_{20} (Tocher *et al.*, 1989; Ghioni *et al.*, 1999).

The present study revealed that the LC-PUFA biosynthesis pathway is active but limited in the CHSE-214 cell line. Thus, the CHSE-214 cell line is active in EPA synthesis from LNA, and in ARA synthesis from LOA, but the pathway had only very low or no activity for the production of DHA from EPA. Therefore, the CHSE-214 cell line can be regarded as a suitable model for further studies of lipid and FA metabolism in salmonids, specifically EPA production.

Chapter 4

Effects of interaction and competition between supplemented PUFA on lipid and fatty acid compositions of CHSE-214 cells



LC-PUFA biosynthesis pathways [taken from www.biochemsoctrans.org]

"Hunger is not an issue of charity. It is an issue of justice"

Jacques Diouf

4.1. Introduction

The short-chain PUFA, α-linolenic acid (LNA, 18:3n-3) and linoleic acid (LOA, 18:2n-6) are essential fatty acids (EFA), meaning that they have to be obtained from the diet, as fish are not able to synthesise FA with two or more double bounds (Sargent *et al.*, 1989, 2002). LNA and LOA are present in high concentrations in most of the vegetable oil (VO) used for the production of aquafeeds (Tocher, 2003). Depending upon species, fish and vertebrates in general, may have the ability to convert LOA into ARA, and LNA into EPA and DHA. The pathway for the synthesis of LC-PUFA is presented in Figure 4.1. It involves elongase and desaturase enzymes, with the elongation and desaturation steps occurring in endoplasmic reticulum, while the chain shortening required for DHA synthesis is carried out in peroxisomes. The conversion of LNA into DHA requires the participation of the enzyme Δ6 fatty acyl desaturase (Fads2d6) to first produce stearidonic acid (18:4n-3). This has to be elongated to eicosatetraenoic acid (20:4n-3) with the participation of an elongase (Elovl5). The enzyme Δ5 fatty acyl desaturase (Fads2d5) is responsible for the conversion of 20:4n-3 into EPA, followed by two sequential elongation steps to produce docosapentaenoic acid (22:5n-3) and tetracosapentaenoic acid (24:5n-3) with the participation of *elovl2*. The resultant 24:5n-3 is desaturated to tetracosahexaenoic acid (24:6n-3) by a Fads2d6 and, finally, the chain is shorted to DHA (Morais *et al.*, 2009; Gregory *et al.*, 2010). The conversion of LOA into ARA requires the participation of the same enzymes as those required to convert LNA into EPA and DHA. Firstly the Fads2d6 converts LOA into γ-linolenic acid (18:3n-6), which is then elongated to 20:3n-6, with the participation of Elovl5, and finally, the enzyme Fads2d5 converts 20:3n-6 to ARA. Studies of FA metabolism in fish cell lines, have reported high enzymatic desaturation and elongation activity to convert LNA into EPA in lines derived from freshwater or

anadromous fish (*e.g.* common carp, rainbow trout and Atlantic salmon) (Tocher and Dick, 1990, 1999). In contrast, enzymatic limitations have been reported in cell lines derived from marine fish (*e.g.* gilthead sea bream and turbot) including limited ability to elongate C_{18} PUFA, and to desaturate 20:4n-3 to EPA (Tocher and Ghioni, 1999; Ghioni *et al.*, 1999). The synthesis of DHA from EPA seems to be limited in most of the fish cell lines (Tocher and Sargent, 1990; Gregory *et al.*, 2011).

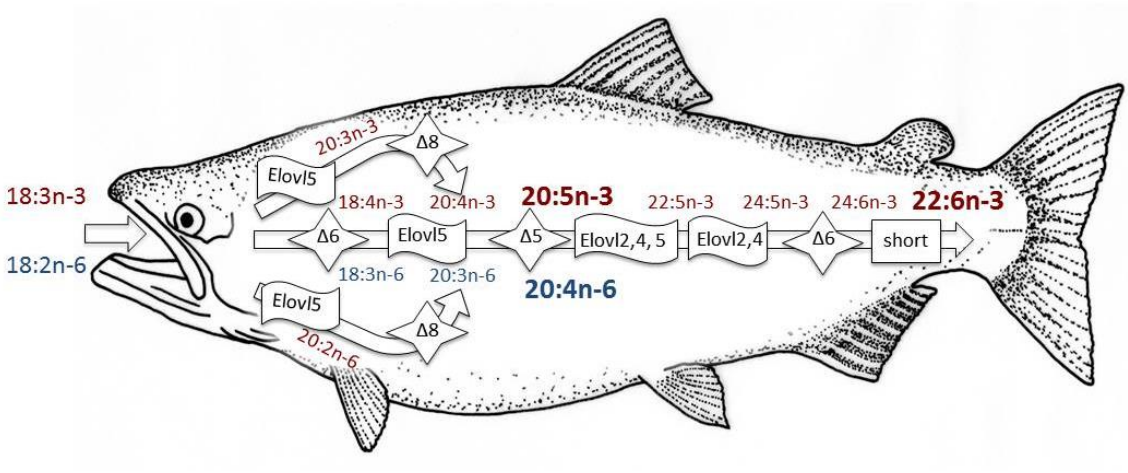


Figure 4.1. Diagram of LC-PUFA synthesis in Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum). Essential fatty acids (EFA), such as α-linolenic (LNA, 18:3n-3) and linoleic (LOA, 18:2n-6) acids, which are the precursors of eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids, and arachidonic acid (ARA, 20:4n-6), respectively, must be obtained in the diet. The synthesis of these important fatty acids is aimed following the action of fatty acyl desaturases (Δ8, Δ6 and Δ5) and elongases (Elovl). The diagram also illustrates the enzyme competition between n-3 and n-6 PUFA series.

As PUFA play important roles in metabolism, it is important to investigate the possible enzyme competition in the well-established pathways of LC-PUFA biosynthesis, LNA to EPA and DHA, and LOA to ARA, given that both pathways require the same enzymes (Figure 4.1). Stubbs and Smith (1984) and Tocher *et al.* (1989) reported that the PUFA preferences of fatty acyl desaturases is in the following rank order: n-3 > n-6 > n-9.

There are some studies in which fish cell lines were incubated with n-3 and n-6 PUFA in order to describe PUFA incorporation and metabolism, and LC-PUFA biosynthesis, but the competition between n-3 and n-6 PUFA was not investigated in detail (Tocher *et al.*, 1989, 1996; Tocher, 1990; Bell *et al.*, 1994; Tocher and Dick, 1999; Ghioni *et al.*, 1999). The study of the interaction between PUFA and LC-PUFA is important, considering the changes in formulation being made to fish and, especially, salmon feeds with fish oil (FO), rich in n-3 LC-PUFA, being increasingly replaced by VO that are rich in C_{18} PUFA. The FA profile of fish flesh largely reflects the FA profile of the diet/feed (Tocher, 2003). Therefore, the replacement of FO with VO in aquafeeds decreases the levels of n-3 LC-PUFA in fish flesh (Zheng *et al.*, 2005; Torstensen *et al.*, 2005; Leaver *et al.*, 2008; Pratoomyot *et al.*, 2008; Trushensk and Boesenberg, 2009). As alluded above, salmonids, such as Atlantic salmon and rainbow trout, are able to synthesise LC-PUFA from C_{18} PUFA (Castell *et al.*, 1972; Bell *et al.*, 1993; Tocher *et al.*, 2000). However, this ability may not be sufficient to compensate the reduced dietary intake of LC-PUFA in fish fed VO compared to fish fed FO (Tocher and Dick, 1990; Tocher and Sargent, 1990; Tocher and Ghioni, 1999; Ghioni *et al.*, 1999).

In the context of the above, the primary aim of the present Chapter was to characterise the uptake, incorporation and metabolism of n-3 and n-6 PUFA when added in combination to the CHSE-214 (Chinook salmon embryo) cell line. The competition between different PUFA substrates and/or pathway intermediates and the influence of pathway products (LC-PUFA) was determined by analysing the effects on the lipid content, class composition and FA composition of cells incubated with different combinations of PUFA and/or LC-PUFA. The maximum concentration of individual PUFA supplemented for the experiments in this competition study was 20 µM based on the results of Chapter 3. This concentration was determined to be sufficient to change the FA

profile of CHSE-214 cells (*i.e.* membrane FA compositions), but to not affect negatively the growth of the monolayer, or to increase deposition of triacylglycerol (TAG), and therefore to not favour the formation of lipid droplets in the cytoplasm.

4.2. Materials and Methods

4.2.1. Cell line and routine culture procedures

The description of CHSE-214 cell line, the media preparation, the routine culture methodology and cell counting have been described in detail in Chapter 2, sections 2.1.1, 2.1.2 and 2.1.2.4, respectively.

4.2.2. PUFA competition experiments

FA were supplemented as bovine serum albumin (BSA) complexes (First Link Ltd., Wolverhampton, UK) in phosphate buffered saline (PBS) (Spector and Hoak, 1969). This procedure has been fully described in Chapter 2, section 2.2.1. The combinations of PUFA and LC-PUFA supplemented to the cells that were investigated in the present Chapter are listed in Table 4.1. The concentration of FA 1 was fixed at 20 µM while the competing FA (FA 2) were added in increasing concentrations of 5, 10, 15 and 20 µM in each experiment. Therefore each experiment consisted of five treatment combinations, namely 20/0 µM, 20/5 µM, 20/10 µM, 20/15 µM and 20/20 µM. The maximum concentration of FA supplemented was 40 µM based on the results obtained in the toxicity test described in Chapter 3, section 3.3.5. The experiments outlined below were designed to establish the effect of the combinations of PUFA supplemented on the cellular FA profile and lipid class composition. Moreover, the potential enzyme competition of C_{18} PUFA supplementation (*i.e.* LNA (as FA 1) / LOA (as FA 2), and LOA (as FA 1) / LNA (as FA 2)) was also established, as well as the product inhibition (*i.e.* LNA (as FA 1) / EPA

(as FA 2); LNA (as FA 1) / DHA (as FA 2); LNA (as FA 1) / EPA+DHA (as FA 2); LOA (as FA 1) / ARA (as FA 2). Flasks were incubated with the combinations of PUFA for 5 d. Further details of the seeding and FA supplementation procedures are provided in Chapter 2, section 2.2.2. After the incubation time, cells were harvested and washed for further lipid analyses.

Table 4.1. Concentration and combinations of FA used as substrates and FA competitors for CHSE-214 cell line

EXPERIMENT	FATTY ACID 1 (substrate)	FATTY ACID 2 (competitor)
1	LNA	LOA
2	LNA	ARA
3	LNA	EPA
4	LNA	DHA
5	LNA	EPA+DHA (1:1)
6	EPA	LNA
7	EPA	DHA
8	LOA	LNA
9	LOA	EPA+DHA (1:1)
10	LOA	ARA

Abbreviations: LNA = α-linolenic acid; LOA = linoleic acid; ARA = arachidonic acid; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid.

4.2.3. Lipid analyses

Cellular lipids were extracted, following the protocol established by Folch *et al.* (1957). Details of these procedures are given in Chapter 2, sections 2.1.2.2 and 2.3.1. High-performance thin-layer chromatography (HPTLC) plates were used to carry out the lipid class composition analysis (Henderson and Tocher, 1992). Full details of the procedures for determining lipid class composition are given in Chapter 2, section 2.3.2.

Fatty acid methyl esters (FAME) of cell total lipid were prepared by acid-catalysed transmethylation according to Christie (2003). Further details of the procedures for FAME preparation and GC analyses are given in Chapter 2, section 2.3.3.

4.3. Results

4.3.1. Effect of competing n-6 PUFA (LOA and ARA) and n-3 LC-PUFA (EPA, DHA and EPA+DHA) in cells supplemented with LNA

4.3.1.1. Supplementation with LNA in presence of increasing LOA

Table 4.2 shows the lipid content and the lipid class composition of CHSE-214 cells incubated with LNA in the presence of increasing concentrations of LOA. No consistent trend was observed in the cell lipid content (R^2 = 0.189; p = 0.158), but the highest level was recorded in cells incubated with 40 µM total PUFA, at 20/20 µM. In the lipid class data there was a clear trend observed, with increasing TAG (R^2 = 0.505; p = 0.010), and total neutral lipids (R^2 = 0.666; p = 0.001), with the graded supplementation of LOA.

The FA composition of CHSE-214 cells incubated with 20 µM LNA and graded supplementation of LOA is presented in Table 4.3. EPA levels decreased with the graded supplementation of LOA (R^2 = 0.366; p = 0.037). LNA was incorporated at the same percentage in all the treatments (R^2 = 0.030; p = 0.589), some of which was desaturated and elongated to 18:4n-3 (R^2 = 0.605; p = 0.003) and 20:4n-3 (R^2 = 0.320; p = 0.055), particularly at 20/20 µM. The percentages of 22:5n-3 (R^2 = 0.047; p = 0.498) and DHA (R^2 = 0.003; p = 0.862) were not affected by LOA supplementation, being essentially the same at all supplemented concentrations. Increasing levels of LOA were incorporated with graded supplementation of the PUFA (R^2 = 0.963; p = 0.000), and some of which was desaturated and elongated to 18:3n-6 (R^2 = 0.804; p = 0.000) and 20:3n-6 (R^2 = 0.659; p = 0.001), respectively, and, to a lesser extent, desaturated to ARA (R^2 = 0.598; p = 0.003). The increment of n-6 PUFA with the supplementation of LOA, was balanced by reduced

MUFA (R^2 = 0.862; p = 0.000) and SFA (R^2 = 0.767; p = 0.000), particularly decreasing the percentages of 18:1n-9 (R^2 = 0.869; p = 0.000) and 16:0 (R^2 = 0.678; p = 0.001), but there was no significant effect on total n-3 PUFA (R^2 = 0.110; p = 0.293).

Table 4.2. Lipid content and lipid class composition of CHSE-214 incubated with LNA and LOA

Lipid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
LC (µg)	490.0 ± 62.4	383.3 ± 30.1	433.3 ± 60.9	623.3 ± 38.0	0.189	0.158
CC (%)						
PC	22.4 ± 1.8	24.6 ± 2.4	22.6 ± 1.1	21.6 ± 1.5	0.075	0.389
PE	19.5 ± 0.7	19.1 ± 0.3	21.6 ± 0.6	21.4 ± 0.6	0.582	0.004
PS	5.2 ± 1.3	4.0 ± 0.1	4.3 ± 0.1	3.5 ± 0.6	0.438	0.019
PI	8.8 ± 0.1	8.8 ± 1.2	8.5 ± 0.4	7.2 ± 0.4	0.450	0.017
PA/CL	1.8 ± 0.5	1.2 ± 0.1	1.5 ± 0.1	0.9 ± 0.1	0.486	0.012
SM	6.7 ± 0.4	6.2 ± 1.0	3.4 ± 0.3	4.2 ± 0.8	0.589	0.004
TP	64.4 ± 2.0	63.9 ± 0.9	61.9 ± 0.9	58.8 ± 2.5	0.666	0.001
TN	35.6 ± 2.0	36.1 ± 0.9	38.1 ± 0.9	41.2 ± 2.5	0.666	0.001
TAG	5.2 ± 0.3	6.5 ± 0.5	6.6 ± 0.2	8.8 ± 1.6	0.505	0.010
CHOL	30.4 ± 1.7	29.6 ± 0.6	31.5 ± 0.8	32.4 ± 1.0	0.409	0.025
FFA	ND	ND	ND	ND	-	-

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LNA = α-linolenic acid; LOA = linoleic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

Table 4.3. Fatty acid composition (%) of CHSE-214 cells incubated with LNA and LOA

Fatty acid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
14:0	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.3	1.2 ± 0.0	0.116	0.280
15:0	0.6 ± 0.1	0.5 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.733	0.000
16:0	14.2 ± 0.3	14.8 ± 0.7	12.2 ± 0.4	11.9 ± 0.1	0.678	0.001
17:0	0.7 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.696	0.001
18:0	11.0 ± 0.5	10.2 ± 0.4	10.5 ± 0.5	10.3 ± 0.7	0.109	0.294
22:0	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	0.5 ± 0.0	0.004	0.848
Σ SFA	**28.3 ± 0.3**	**28.0 ± 0.7**	**25.2 ± 1.0**	**24.3 ± 0.7**	**0.767**	**0.000**
16:1n-9	2.9 ± 0.1	2.6 ± 0.3	2.7 ± 0.1	2.2 ± 0.4	0.474	0.013
16:1n-7	1.3 ± 0.2	1.3 ± 0.2	1.2 ± 0.0	1.1 ± 0.1	0.383	0.032
18:1n-9	28.6 ± 0.7	25.8 ± 0.6	24.4 ± 0.4	22.2 ± 1.8	0.869	0.000
18:1n-7	2.1 ± 0.2	1.6 ± 0.1	1.8 ± 0.4	2.6 ± 0.2	0.214	0.130
24:1n-9	0.7 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	0.9 ± 0.1	0.109	0.295
Σ MUFA	**35.6 ± 0.7**	**32.4 ± 0.5**	**31.2 ± 0.5**	**29.0 ± 1.7**	**0.862**	**0.000**
18:2n-6	3.4 ± 0.1	5.6 ± 0.9	7.3 ± 0.1	11.3 ± 0.6	0.963	0.000
18:3n-6	0.8 ± 0.1	1.0 ± 0.0	1.5 ± 0.2	2.0 ± 0.5	0.804	0.000
20:2n-6*	0.4 ± 0.0	0.6 ± 0.0	0.6 ± 0.1	1.0 ± 0.3	0.730	0.000
20:3n-6	1.6 ± 0.1	3.1 ± 0.2	3.3 ± 0.1	3.6 ± 0.8	0.659	0.001
20:4n-6	0.6 ± 0.0	0.7 ± 0.2	1.0 ± 0.1	1.2 ± 0.2	0.598	0.003
Σ n-6 PUFA	**6.8 ± 0.2**	**11.0 ± 0.6**	**13.7 ± 0.6**	**19.1 ± 2.0**	**0.961**	**0.000**
18:3n-3	5.8 ± 0.2	5.2 ± 0.2	5.8 ± 0.4	5.4 ± 0.2	0.030	0.589
18:4n-3	3.8 ± 0.2	3.5 ± 0.4	4.4 ± 0.3	4.7 ± 0.2	0.605	0.003
20:4n-3	4.0 ± 0.1	3.7 ± 0.2	4.4 ± 0.2	4.7 ± 0.2	0.320	0.055
20:5n-3	5.6 ± 0.2	5.3 ± 0.2	5.3 ± 0.4	4.8 ± 0.7	0.366	0.037
22:5n-3	1.3 ± 0.1	1.3 ± 0.0	1.2 ± 0.1	1.3 ± 0.1	0.047	0.498
22:6n-3	1.2 ± 0.1	1.2 ± 0.2	1.2 ± 0.0	1.2 ± 0.1	0.003	0.862
Σ n-3 PUFA	**21.7 ± 0.7**	**20.2 ± 1.0**	**22.3 ± 1.4**	**22.1 ± 0.7**	**0.110**	**0.293**
18:2n-9	4.3 ± 0.3	4.0 ± 0.1	4.1 ± 0.1	3.2 ± 0.2	0.648	0.002
20:2n-9	2.8 ± 0.2	2.7 ± 0.1	2.3 ± 0.2	2.0 ± 0.2	0.769	0.000
22:2n-9	0.5 ± 0.0	1.7 ± 0.1	1.2 ± 0.4	0.3 ± 0.1	0.069	0.408
Σ n-9 PUFA	**7.6 ± 0.6**	**8.4 ± 0.2**	**7.6 ± 0.5**	**5.5 ± 0.1**	**0.557**	**0.005**
Σ PUFA	**36.1 ± 0.6**	**39.6 ± 0.5**	**43.6 ± 1.5**	**46.7 ± 2.4**	**0.915**	**0.000**

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LNA = α-linolenic acid; LOA = linoleic acid; PUFA = polyunsaturated fatty acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; *contains 20:3n-9.

4.3.1.2. Supplementation with LNA in presence of increasing ARA

Table 4.4 shows the cell lipid content and lipid class composition of CHSE-214 cells after being incubated with LNA and increasing concentrations of ARA. There was no clear trend observed in cell lipid content data ($R^2 = 0.002$; $p = 0.878$). No clear or consistent trends were identified in the lipid class composition data, apart from TAG, which tended to increase with the graded supplementation of ARA ($R^2 = 0.520$; $p = 0.008$).

Table 4.4. Lipid content and lipid class composition of CHSE-214 cells incubated with LNA and ARA

Lipid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
LC (µg)	440.0 ± 75.5	393.3 ± 57.2	473.3 ± 76.2	396.7 ± 36.5	0.002	0.878
CC (%)						
PC	24.3 ± 0.8	22.4 ± 1.0	23.3 ± 0.7	24.8 ± 1.5	0.056	0.458
PE	20.0 ± 0.5	20.3 ± 0.4	20.8 ± 0.4	21.7 ± 0.4	0.720	0.000
PS	5.5 ± 0.3	4.6 ± 0.7	5.4 ± 0.6	5.1 ± 0.5	0.005	0.835
PI	9.0 ± 0.2	8.4 ± 0.4	8.2 ± 0.6	8.0 ± 0.8	0.378	0.033
PA/CL	1.5 ± 0.3	1.3 ± 0.2	1.5 ± 0.1	2.9 ± 0.8	0.448	0.017
SM	4.8 ± 0.7	4.1 ± 0.5	4.3 ± 0.9	4.9 ± 0.4	0.019	0.666
TP	65.1 ± 2.1	61.1 ± 1.4	63.5 ± 0.3	67.4 ± 0.8	0.171	0.181
TN	34.9 ± 2.1	38.9 ± 1.4	36.5 ± 0.3	32.6 ± 0.8	0.171	0.181
TAG	3.2 ± 0.4	3.4 ± 0.3	4.0 ± 0.2	4.1 ± 0.5	0.520	0.008
CHOL	31.7 ± 1.7	35.5 ± 1.5	32.5 ± 0.5	28.5 ± 0.3	0.281	0.076
FFA	ND	ND	ND	ND	-	-

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LNA = α-linolenic acid; ARA = arachidonic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA composition of CHSE-214 cells incubated with 20 µM LNA and graded supplementation of ARA is presented in Table 4.5. There was decrement of LNA (R^2 = 0.931; p = 0.000), 18:4n-3 (R^2 = 0.499; p = 0.010), and EPA (R^2 = 0.385; p = 0.031) with the graded supplementation of ARA, but no overall effects of supplementation with ARA were observed on 22:5n-3 (R^2 = 0.307; p = 0.062) and DHA (R^2 = 0.091; p = 0.340). Increased incorporation of ARA with graded supplementation of the FA was observed (R^2 = 0.960; p = 0.000). Some of the ARA was elongated to 22:4n-6 (R^2 = 0.749; p = 0.000), particularly at 20/20 µM, but no production of 22:5n-6 was observed. Similarly at 20/20 µM there was observed some apparent retro-conversion of incorporated ARA to 20:3n-6 (R^2 = 0.755; p = 0.000) and 18:3n-6 (R^2 = 0.642; p = 0.002). The total n-6 PUFA increased with the graded supplementation of ARA (R^2 = 0.969; p = 0.000), which was balanced by decreased MUFA (R^2 = 0.951; p = 0.000), particularly 18:1n-9 (R^2 = 0.874; p = 0.000), and total n-9 PUFA (R^2 = 0.934; p = 0.000).

Table 4.5. Fatty acid composition (%) of CHSE-214 cells incubated with LNA and ARA

Fatty acid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
14:0	1.3 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.762	0.000
15:0	0.5 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.643	0.002
16:0	13.5 ± 1.2	13.8 ± 1.0	13.2 ± 0.1	13.3 ± 0.7	0.035	0.560
17:0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.672	0.001
18:0	10.6 ± 0.5	11.4 ± 0.9	11.3 ± 0.2	11.2 ± 0.6	0.147	0.218
22:0	0.4 ± 0.1	0.6 ± 0.2	0.4 ± 0.1	0.5 ± 0.2	0.003	0.877
Σ SFA	**26.6 ± 1.7**	**27.4 ± 2.1**	**26.3 ± 0.1**	**26.3 ± 1.4**	**0.031**	**0.582**
16:1n-9	3.1 ± 0.1	2.8 ± 0.2	2.5 ± 0.1	2.4 ± 0.1	0.900	0.000
16:1n-7	1.5 ± 0.2	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	0.572	0.004
18:1n-9	33.9 ± 1.0	31.6 ± 1.0	30.3 ± 1.4	26.3 ± 0.4	0.874	0.000
18:1n-7	1.9 ± 0.2	2.0 ± 0.4	1.6 ± 0.1	1.7 ± 0.0	0.141	0.229
24:1n-9	0.8 ± 0.1	1.1 ± 0.1	0.7 ± 0.0	1.2 ± 0.4	0.227	0.117
Σ MUFA	**41.2 ± 0.6**	**39.0 ± 0.6**	**36.5 ± 1.1**	**33.0 ± 0.2**	**0.951**	**0.000**
18:2n-6	1.9 ± 0.1	1.8 ± 0.1	1.8 ± 0.0	1.7 ± 0.1	0.702	0.001
18:3n-6	0.5 ± 0.1	0.6 ± 0.0	0.6 ± 0.1	0.7 ± 0.0	0.642	0.002
20:2n-6*	0.6 ± 0.0	0.5 ± 0.0	0.5 ± 0.1	0.4 ± 0.1	0.808	0.000
20:3n-6	1.5 ± 0.1	1.9 ± 0.2	2.0 ± 0.2	2.3 ± 0.3	0.755	0.000
20:4n-6	4.4 ± 0.7	6.6 ± 0.6	9.6 ± 0.4	11.6 ± 0.3	0.960	0.000
22:4n-6	0.4 ± 0.1	0.6 ± 0.3	0.8 ± 0.1	3.3 ± 0.1	0.749	0.000
Σ n-6 PUFA	**9.3 ±0.8**	**12.0 ± 0.7**	**15.3 ± 0.9**	**20.0 ± 0.7**	**0.969**	**0.000**
18:3n-3	3.7 ± 0.1	3.1± 0.2	3.1 ± 0.0	2.7 ± 0.0	0.931	0.000
18:4n-3	2.3 ± 0.1	2.1 ± 0.1	2.1 ± 0.0	1.9 ± 0.0	0.499	0.010
20:4n-3	2.5 ± 0.2	2.6 ± 0.2	2.8 ± 0.1	2.7 ± 0.0	0.251	0.097
20:5n-3	3.9 ± 0.1	3.8 ± 0.4	3.7± 0.3	3.3 ± 0.1	0.385	0.031
22:5n-3	0.9 ± 0.1	1.1 ± 0.1	1.8 ± 0.6	1.6 ±1.0	0.307	0.062
22:6n-3	1.1 ± 0.0	1.0 ± 0.1	1.1 ± 0.1	1.6 ± 1.0	0.091	0.340
Σ n-3 PUFA	**14.4 ± 0.5**	**13.7 ± 1.0**	**14.6 ± 0.0**	**13.8 ± 2.0**	**0.013**	**0.726**
18:2n-9	4.6 ± 0.1	4.2 ± 0.1	4.0 ± 0.2	3.7 ± 0.3	0.826	0.000
20:2n-9	3.5 ± 0.1	3.4 ± 0.1	3.0 ± 0.1	2.7 ± 0.1	0.894	0.000
22:2n-9	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.1	0.028	0.603
Σ n-9 PUFA	**8.5 ± 0.1**	**7.9 ± 0.2**	**7.3 ± 0.1**	**6.9 ± 0.2**	**0.934**	**0.000**
Σ PUFA	**32.2 ± 1.1**	**33.6 ± 1.4**	**37.2 ± 1.0**	**40.7 ± 1.4**	**0.940**	**0.000**

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LNA = α-linolenic acid; ARA = arachidonic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; *contains 20:3n-9.

4.3.1.3. Supplementation with LNA in presence of increasing concentrations of EPA

Table 4.6 shows the cell lipid content and the lipid class composition of CHSE-214 cells after being incubated with LNA and increasing concentrations of EPA. Total lipid content clearly decreased with the graded supplementation of EPA ($R^2 = 0.459$; $p = 0.016$). No clear or consistent trends were identified in the lipid class composition data, apart from

TAG, which was found in percentages lower than 3% at 20/5 µM and 20/10 µM, and it was increased at 20/15 µM and 20/20 µM (R^2 = 0.723; p = 0.000).

Table 4.6. Lipid content and lipid class composition of CHSE-214 cells incubated with LNA and EPA

Lipid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
LC (µg)	556.7 ± 75.1	546.7 ± 64.3	500.0 ± 81.9	403.3 ± 58.6	0.459	0.016
CC (%)						
PC	21.0 ± 0.4	21.5 ± 0.7	17.8 ± 1.1	16.9 ± 3.3	0.528	0.007
PE	14.2 ± 0.9	16.4 ± 0.4	14.7 ± 0.4	14.6 ± 0.8	0.075	0.389
PS	4.8 ± 0.2	5.2 ± 0.3	4.1 ± 0.5	4.5 ± 0.7	0.160	0.198
PI	10.7 ± 0.5	11.0 ± 0.1	8.8 ± 0.6	8.9 ± 0.9	0.565	0.005
PA/CL	3.3 ± 0.1	2.9 ± 0.1	2.9 ± 0.1	2.7 ± 0.5	0.031	0.581
SM	3.6 ± 0.7	4.5 ± 0.9	2.1 ± 0.3	2.8 ± 0.5	0.269	0.084
TP	57.6 ± 0.5	61.5 ± 1.5	50.4 ± 1.4	50.4 ± 0.9	0.564	0.005
TN	42.4 ± 0.5	38.5 ± 1.5	49.6 ± 1.4	49.6 ± 0.9	0.564	0.005
TAG	2.4 ± 0.5	2.6 ± 0.4	7.6 ± 1.1	7.0 ± 1.1	0.723	0.000
CHOL	40.0 ± 0.7	35.9 ± 1.9	42.0 ± 0.9	42.6 ± 0.6	0.315	0.057
FFA	ND	ND	ND	ND	-	-

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LNA = α-linolenic acid; EPA = eicosapentaenoic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total neutral; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

Table 4.7 shows the FA composition of CHSE-214 cells after being incubated with LNA and increasing concentrations of EPA. The proportion of the major FA groups in CHSE-214 cells at 20/5 µM was as follows: MUFA > PUFA >SFA, while at 20/10 µM, 20/15 µM and 20/20 µM the FA were decreasing in the following order: PUFA > MUFA > SFA. EPA in cell total lipid, increased with the graded supplementation of the FA itself (R^2 = 0.791; p = 0.000). There was a graded increment in the percentage of 22:5n-3 (R^2 = 0.969; p = 0.000) with supplementation of EPA. However, 18:4n-3 (R^2 = 0.726; p = 0.000) decreased with the graded supplementation of EPA, whereas DHA (R^2 = 0.001; p = 0.942) and other n-3 FA such as LNA (R^2 = 0.122; p = 0.267), and 20:4n-3 (R^2 = 0.239; p = 0.106) were not greatly affected. The increased proportions of total n-3 PUFA were

balanced by decreased MUFA (R^2 = 0.692; p = 0.001) and n-9 PUFA (R^2 = 0.807; p = 0.000).

Table 4.7. Fatty acid composition (%) of CHSE-214 cells incubated with LNA and EPA

Fatty acid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
14:0	2.0 ± 0.5	1.9 ± 0.1	2.0 ± 0.1	1.4 ± 0.5	0.246	0.101
15:0	0.3 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.282	0.076
16:0	14.4 ± 2.2	14.1 ± 0.3	15.2 ± 1.2	15.3 ± 1.2	0.130	0.249
17:0	0.4 ± 0.2	0.4 ± 0.0	0.4 ± 0.1	0.3± 0.1	0.130	0.249
18:0	10.0 ± 1.2	10.0 ± 0.4	10.1 ± 0.4	10.0 ± 0.4	0.004	0.844
22:0	0.4 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.000	0.957
Σ SFA	**27.5 ± 4.3**	**27.1 ± 0.0**	**28.5 ± 1.7**	**27.8 ± 1.3**	**0.012**	**0.737**
16:1n-9	4.3 ± 0.2	4.3 ± 0.1	3.9 ± 0.2	3.8 ± 0.3	0.526	0.008
16:1n-7	2.2 ± 0.2	1.9 ± 0.2	2.0 ± 0.1	1.5 ± 0.7	0.236	0.109
18:1n-9	27.8 ± 1.9	26.9 ± 0.1	25.5 ± 0.9	23.8 ± 2.3	0.570	0.005
18:1n-7	2.1 ± 0.2	2.0 ± 0.0	2.1 ± 0.2	2.1 ± 0.0	0.002	0.904
24:1n-9	0.8 ± 0.1	0.8 ± 0.0	0.8 ± 0.1	1.3 ± 0.8	0.210	0.134
Σ MUFA	**37.2 ± 1.8**	**35.9 ± 0.3**	**34.3 ± 0.9**	**32.5 ± 1.9**	**0.692**	**0.001**
18:2n-6	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.2	1.0 ± 0.0	0.035	0.563
18:3n-6	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.205	0.139
20:2n-6*	1.0 ± 0.1	0.9 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.936	0.000
20:3n-6	1.4 ± 0.1	1.4 ± 0.0	1.4 ± 0.3	1.6 ± 0.1	0.186	0.161
20:4n-6	1.8 ± 0.3	1.8 ± 0.0	1.7 ± 0.1	1.6 ± 0.1	0.387	0.031
22:5n-6	0.4 ± 0.1	0.6 ± 0.3	0.2 ± 0.0	0.3 ± 0.0	0.133	0.244
Σ n-6 PUFA	**5.9 ± 0.4**	**6.0 ± 0.3**	**5.3 ± 0.4**	**5.3 ± 0.2**	**0.368**	**0.037**
18:3n-3	1.5 ± 0.2	1.4 ± 0.0	1.5 ± 0.2	1.7 ± 0.5	0.122	0.267
18:4n-3	2.0 ± 0.3	1.5 ± 0.0	1.3 ± 0.1	1.2 ± 0.2	0.726	0.000
20:4n-3	1.3 ± 0.2	1.3 ± 0.0	1.4 ± 0.1	1.6 ± 0.4	0.239	0.106
20:5n-3	9.2 ± 1.2	11.1 ± 0.3	11.8 ± 0.8	13.1 ± 0.3	0.791	0.000
22:5n-3	2.2 ± 0.4	3.4 ± 0.0	4.8 ± 0.3	6.8 ± 0.6	0.969	0.000
22:6n-3	1.9 ± 0.1	2.0 ± 0.0	1.9 ± 0.2	1.9 ± 0.1	0.001	0.942
Σ n-3 PUFA	**18.1 ± 2.4**	**20.7 ± 0.4**	**22.7 ± 1.2**	**26.3 ± 1.8**	**0.823**	**0.000**
18:2n-9	6.3 ± 0.2	5.4 ± 0.3	4.9 ± 0.2	4.2 ± 0.9	0.743	0.000
20:2n-9	4.3 ± 0.2	4.2 ± 0.0	3.6 ± 0.3	3.2 ± 0.6	0.625	0.002
22:2n-9	0.7 ± 0.3	0.7 ± 0.0	0.7 ± 0.3	0.7 ± 0.4	0.002	0.878
Σ n-9 PUFA	**11.3 ± 0.3**	**10.3 ± 0.4**	**9.2 ± 0.3**	**8.1 ± 1.2**	**0.807**	**0.000**
Σ PUFA	**35.3 ± 2.5**	**37.0. ± 0.3**	**37.2 ± 1.2**	**39.7 ± 0.8**	**0.594**	**0.003**

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LNA = α -linolenic acid; EPA = eicosapentaenoic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; * contains 20:3n-9.

4.3.1.4. Supplementation with LNA and increasing percentages of DHA

Table 4.8 shows the lipid content and the lipid class composition of CHSE-214 cells after being incubated with LNA and DHA at concentrations of 20/5 µM, 20/10 µM,

20/15 µM, and 20/20 µM. Cell lipid content decreased from 20/5 to 20/10 µM and then increased from 20/10 to 20/20 µM, however there were no consistent trends observed (R^2 = 0.183; p = 0.166). A slight increase of TAG following graded supplementation of DHA up to 20/15 µM was observed, but results were not statistically significant (R^2 = 0.065; p = 0.424). At all concentrations there were some free fatty acids (FFA) detected.

Table 4.8. Lipid content and lipid class composition of CHSE-214 cells incubated with LNA and DHA

Lipid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
LC (µg)	443.3 ± 47.4	330.0 ± 50.0	476.7 ± 32.1	503.3 ± 28.9	0.183	0.166
CC (%)						
PC	14.1 ± 2.5	16.1 ± 1.0	16.4 ± 0.9	16.4 ± 2.8	0.180	0.169
PE	25.4 ± 1.5	22.8 ± 0.5	22.0 ± 0.9	23.0 ± 0.8	0.359	0.040
PS	7.2 ± 0.2	7.2 ± 0.6	5.7 ± 0.6	7.0 ± 0.6	0.107	0.300
PI	10.3 ± 0.7	9.0 ± 0.6	7.8 ± 0.4	7.6 ± 0.6	0.775	0.000
PA/CL	1.5 ± 0.1	1.6 ± 0.2	1.8 ± 0.4	1.9 ± 0.6	0.307	0.062
SM	3.1 ± 0.7	4.8 ± 1.0	4.6 ± 1.3	3.0 ± 0.1	0.002	0.892
TP	61.6 ± 1.6	61.5 ± 0.4	58.3 ± 1.6	58.9 ± 1.3	0.471	0.014
TN	38.4 ± 1.6	38.5 ± 0.4	41.7 ± 1.6	41.1 ± 1.3	0.471	0.014
TAG	5.8 ± 0.8	6.2 ± 0.4	6.5 ± 0.5	6.0 ± 0.1	0.065	0.424
CHOL	30.3 ± 1.9	30.4 ± 0.9	33.7 ± 1.2	33.2 ± 0.8	0.505	0.010
FFA	2.3 ± 0.2	1.9 ± 0.1	1.5 ± 0.2	1.9 ± 0.5	0.245	0.102

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05).*Abbreviations:* LNA = α-linolenic acid; DHA = docosahexaenoic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids.

The FA composition of CHSE-214 cells incubated with 20 µM LNA and graded supplementation of DHA is presented in Table 4.9. The proportion of DHA in the cell total lipid was increased with the graded supplementation of the FA itself (R^2 = 0.969; p = 0.000). In addition, the highest percentages of 22:5n-3 and EPA were observed at 20/20 µM, which may reflect some retroconversion of DHA at the highest concentration supplemented. The percentage of LNA was similar at all concentrations (R^2 = 0.032; p = 0.579), and 18:4n-3 decreased with the graded supplementation of DHA (R^2 = 0.599; p =

0.003). The increased proportions of total n-3 PUFA were balanced by decreased total MUFA (R^2 = 0.927; p = 0.000), n-9 PUFA (R^2 = 0.839; p = 0.000) and n-6 PUFA (R^2 = 0.477; p = 0.013).

Table 4.9. Fatty acid composition (%) of CHSE-214 cells incubated with LNA and DHA

Fatty acid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
14:0	1.5 ± 0.2	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	0.210	0.134
15:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.262	0.089
16:0	12.9 ± 0.3	13.5 ± 0.9	13.8 ± 1.0	14.3 ± 0.6	0.381	0.032
17:0	0.4 ± 0.1	0.7 ± 0.1	0.5 ± 0.2	0.2 ± 0.0	0.148	0.217
18:0	8.8 ± 0.2	9.4 ± 0.7	9.8 ± 0.7	9.7 ± 0.4	0.351	0.042
20:0	0.2 ± 0.1	0.1 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.115	0.280
22:0	0.4 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.006	0.818
Σ SFA	**24.4 ± 0.9**	**25.5 ± 1.7**	**26.4 ± 1.4**	**26.3 ± 1.1**	**0.313**	**0.059**
16:1n-9	3.7 ± 0.1	3.6 ± 0.1	3.6 ± 0.0	3.2 ± 0.2	0.688	0.001
16:1n-7	2.5 ± 0.3	2.1 ± 0.1	1.9 ± 0.1	1.7 ± 0.2	0.784	0.000
18:1n-9	34.5 ± 1.2	33.5 ± 0.2	30.6 ± 0.2	27.7 ± 1.1	0.903	0.000
18:1n-7	2.1 ± 0.2	2.2 ± 0.1	2.1 ± 0.2	2.1 ± 0.1	0.025	0.625
24:1n-9	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	0.5 ± 0.0	0.002	0.896
Σ MUFA	**43.3 ± 0.7**	**41.9 ± 0.2**	**38.8 ± 0.2**	**35.2 ± 1.4**	**0.927**	**0.000**
18:2n-6	1.7 ± 0.2	1.6 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	0.526	0.008
18:3n-6	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.407	0.026
20:2n-6*	1.3 ± 0.5	0.9 ± 0.1	0.8 ± 0.0	0.7 ± 0.1	0.534	0.007
20:3n-6	1.3 ± 0.2	1.0 ± 0.1	1.1 ± 0.2	1.2 ± 0.0	0.010	0.759
20:4n-6	1.4 ± 0.1	1.4 ± 0.2	1.4 ± 0.2	1.3 ± 0.1	0.065	0.424
22:5n-6	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	0.140	0.231
Σ n-6 PUFA	**6.2 ± 0.7**	**5.4 ± 0.4**	**5.3 ± 0.0**	**5.0 ± 0.2**	**0.477**	**0.013**
18:3n-3	1.8 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	2.0 ± 0.4	0.032	0.579
18:4n-3	2.0 ± 0.1	1.6 ± 0.1	1.2 ± 0.1	1.4 ± 0.3	0.599	0.003
20:4n-3	1.8 ± 0.1	1.7 ± 0.1	1.5 ± 0.1	2.3 ± 0.7	0.107	0.299
20:5n-3	4.8 ± 0.2	4.5 ± 0.2	4.7 ± 0.2	5.4 ± 0.6	0.174	0.177
22:5n-3	1.4 ± 0.2	1.3 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	0.095	0.329
22:6n-3	5.1 ± 0.4	7.5 ± 0.6	10.6 ± 0.6	13.6 ± 1.1	0.969	0.000
Σ n-3 PUFA	**16.9 ± 0.4**	**18.2 ± 1.1**	**20.7 ± 1.0**	**26.2 ± 2.8**	**0.820**	**0.000**
16:2n-9	0.2 ± 0.0	0.5 ± 0.0	0.4 ± 0.1	0.5 ± 0.0	0.504	0.010
18:2n-9	4.6 ± 0.4	4.1 ± 0.1	3.9 ± 0.0	3.3 ± 0.1	0.859	0.000
20:2n-9	4.2 ± 0.1	4.2 ± 0.2	4.2 ± 0.2	3.2 ± 0.3	0.680	0.001
22:2n-9	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.154	0.207
Σ n-9 PUFA	**9.2 ± 0.5**	**9.0 ± 0.3**	**8.8 ± 0.2**	**7.3 ± 0.4**	**0.839**	**0.000**
Σ PUFA	**32.3 ± 0.5**	**32.6 ± 1.7**	**34.8 ± 1.3**	**38.5 ± 2.5**	**0.635**	**0.002**

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LNA = α-linolenic acid; DHA = docosahexaenoic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; * contains 20:3n-9.

4.3.1.5. Supplementation with LNA and increasing concentrations of EPA+DHA combined

Table 4.10 shows the cell lipid content and lipid class composition of CHSE-214 cells after being incubated with LNA and increasing concentrations of an equimolar (1:1)

mix of EPA+DHA. The cell lipid content was highly variable and there were no significant differences observed (R^2 = 0.016; p = 0.693). There was a clear and consistent effect on TAG, which decreased significantly with the graded supplementation of EPA+DHA (R^2 = 0.714; p = 0.022). There were small percentages of FFA detected at all concentrations.

Table 4.10. Lipid content and lipid class composition of CHSE-214 cells incubated with LNA and EPA+DHA (1:1)

Lipid	20/5µM	20/10µM	20/15µM	20/20µM	R^2	P-value
LC (µg)	303.3 ± 92.4	260.0 ± 87.2	223.3 ± 75.7	346.7 ± 75.1	0.016	0.693
CC (%)						
PC	11.4 ± 1.3	17.6 ± 3.3	16.1 ± 3.1	16.8 ± 0.3	0.308	0.061
PE	19.8 ± 0.9	22.3 ± 0.8	26.5 ± 2.0	26.9 ± 0.9	0.345	0.044
PS	7.3 ± 1.3	6.4 ± 0.9	8.2 ± 1.8	5.7 ± 0.5	0.067	0.416
PI	7.2 ± 0.5	8.1 ± 1.0	7.7 ± 0.2	7.9 ± 0.7	0.099	0.319
PA/CL	1.5 ± 0.6	2.4 ± 0.1	1.4 ± 0.6	1.3 ± 0.5	0.103	0.308
SM	5.6 ± 0.1	4.3 ± 0.3	4.4 ± 1.2	2.8 ± 0.2	0.694	0.001
TP	52.8 ± 1.7	61.1 ± 2.2	64.3 ± 0.2	61.4 ± 1.4	0.177	0.174
TN	47.2 ± 1.7	38.9 ± 2.2	35.7 ± 0.2	38.6 ± 1.4	0.177	0.174
TAG	10.6 ± 1.2	7.1 ± 0.4	5.6 ± 0.1	4.9 ± 0.6	0.714	0.022
CHOL	34.2 ± 1.7	30.4 ± 1.7	27.7 ± 1.5	32.1 ± 2.2	0.125	0.260
FFA	2.4 ± 0.7	1.4 ± 0.4	2.4 ± 0.7	1.6 ± 0.4	0.019	0.670

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LNA = α-linolenic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

Table 4.11 shows the FA composition of CHSE-214 cells after being incubated with LNA and increasing concentrations of the EPA+DHA mix (1:1). There was increased incorporation of DHA with the graded supplementation of the EPA+DHA mix (R^2 = 0.855; p = 0.000), but the proportion of EPA was not significantly affected by the supplementation (R^2 = 0.001; p = 0.905). LNA and 20:4n-3 were present at less than 2% and not greatly affected by supplementation with EPA+DHA (R^2 = 0.022; p = 0.642 and R^2 = 0.002; p = 0.896, respectively). SFA, particularly 16:0 (R^2 = 0.753; p = 0.000), increased with the graded supplementation of EPA+DHA. The increment in total n-3 PUFA was

balanced by decreased total MUFA (R^2 = 0.751; p = 0.000), n-6 PUFA (R^2 = 0.840; p = 0.000) and n-9 PUFA (R^2 = 0.532; p = 0.007).

Table 4.11. Fatty acid composition (%) of CHSE-214 cells incubated with LNA and EPA+DHA (1:1)

Fatty acid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
14:0	1.4 ± 0.1	1.4 ± 0.2	1.5 ± 0.0	1.4 ± 0.4	0.025	0.626
15:0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.391	0.030
16:0	12.7 ± 0.1	15.0 ± 1.0	15.4 ± 1.3	16.8 ± 0.2	0.753	0.000
17:0	0.3 ± 0.0	0.5 ± 0.2	0.3 ± 0.0	0.5 ± 0.1	0.111	0.289
18:0	9.8 ± 0.4	11.3 ± 0.7	11.0 ± 0.4	11.2 ± 0.2	0.357	0.040
20:0	0.2 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.097	0.324
22:0	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.292	0.070
Σ SFA	**24.9 ± 0.3**	**29.2 ± 1.7**	**29.2 ± 2.0**	**30.8 ± 0.4**	**0.641**	**0.002**
16:1n-9	4.1 ± 0.5	3.2 ± 0.4	3.5 ± 0.1	3.0 ± 0.3	0.419	0.023
16:1n-7	1.6 ± 0.2	1.7 ± 0.2	1.8 ± 0.1	1.8 ± 0.2	0.274	0.080
18:1n-9	34.0 ± 0.1	32.4 ± 2.2	29.0 ± 0.3	28.5 ± 0.2	0.807	0.000
18:1n-7	1.7 ± 0.0	1.6 ± 0.2	2.0 ± 0.1	2.1 ± 0.2	0.453	0.017
24:1n-9	0.5 ± 0.0	0.8 ± 0.1	0.6 ± 0.0	0.8 ± 0.1	0.358	0.040
Σ MUFA	**41.9 ± 0.2**	**39.7 ± 2.9**	**36.9 ± 0.2**	**36.2 ± 0.5**	**0.751**	**0.000**
18:2n-6	1.7 ± 0.2	1.6 ± 0.3	1.8 ± 0.0	1.6 ± 0.1	0.031	0.583
18:3n-6	0.4 ± 0.0	0.5 ± 0.1	0.6 ± 0.0	0.3 ± 0.0	0.136	0.238
20:2n-6*	1.0 ± 0.0	0.7 ± 0.1	0.7 ± 0.0	0.5 ± 0.0	0.750	0.000
20:3n-6	1.6 ± 0.0	1.8 ± 0.4	1.6 ± 0.1	1.9 ± 0.1	0.145	0.221
20:4n-6	1.1 ± 0.0	1.2 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	0.591	0.003
22:5n-6	0.5 ± 0.1	0.4 ± 0.1	0.6 ± 0.0	0.3 ± 0.0	0.221	0.123
Σ n-6 PUFA	**6.3 ± 0.0**	**6.2 ± 0.1**	**6.1 ± 0.2**	**5.4 ± 0.1**	**0.840**	**0.000**
18:3n-3	1.4 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1.5 ± 0.1	0.022	0.642
18:4n-3	1.7 ± 0.1	1.9 ± 0.5	1.0 ± 0.1	1.3 ± 0.1	0.388	0.030
20:4n-3	1.9 ± 0.1	1.2 ± 0.1	1.5 ± 0.1	1.9 ± 0.2	0.002	0.896
20:5n-3	7.1 ± 0.2	7.9 ± 0.4	7.6 ± 0.6	7.2 ± 0.7	0.001	0.905
22:5n-3	1.3 ± 0.3	2.1 ± 0.2	2.2 ± 0.2	1.7 ± 0.1	0.147	0.219
22:6n-3	2.9 ± 0.2	2.9 ± 0.1	5.7 ± 0.6	6.4 ± 0.1	0.855	0.000
Σ n-3 PUFA	**16.3 ± 0.2**	**16.9 ± 1.1**	**19.1 ± 1.7**	**20.0 ± 0.8**	**0.707**	**0.001**
16:2n-9	0.7 ± 0.0	0.6 ± 0.0	0.8 ± 0.2	0.5 ± 0.1	0.066	0.419
18:2n-9	5.0 ± 0.2	3.8 ± 0.2	4.3 ± 0.1	3.7 ± 0.2	0.444	0.018
20:2n-9	4.7 ± 0.1	3.5 ± 0.2	3.5 ± 0.1	3.3 ± 0.0	0.645	0.002
22:2n-9	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.231	0.114
Σ n-9 PUFA	**10.6 ± 0.1**	**8.0 ± 0.4**	**8.7 ± 0.0**	**7.6 ± 0.3**	**0.532**	**0.007**
Σ PUFA	**33.2 ± 0.1**	**31.1 ± 1.5**	**33.9 ± 1.9**	**33.0 ± 0.7**	**0.033**	**0.570**

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LNA = α-linolenic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; * contains 20:3n-9.

4.3.2. Supplementation of CHSE-214 cells with EPA in combination with other n-3 PUFA (LNA and DHA)

4.3.2.1. Supplementation with EPA in presence of increasing concentrations of LNA

The cell lipid content and lipid class composition of CHSE-214 cells after being incubated with EPA and LNA at concentrations of 20/5 µM, 20/10 µM, 20/15 µM, and 20/20 µM is shown in Table 4.12. The cell lipid content data was highly variable and no significant differences were apparent (R^2 = 0.268; p = 0.085). The proportion of TAG increased with increasing supplementation of LNA (R^2 = 0.797; p = 0.000), apart from that no other clear trends were observed in the lipid class composition data.

Table 4.12. Lipid content and lipid class composition of CHSE-214 cells incubated with EPA and LNA

Lipid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
LC (µg)	493.3 ± 64.5	546.7 ± 58.7	343.3 ± 58.6	403.3 ± 58.6	0.268	0.085
CC (%)						
PC	21.8 ± 0.1	19.6 ± 1.0	22.2 ± 2.0	16.9 ± 3.3	0.273	0.082
PE	19.2 ± 0.3	18.1 ± 0.8	20.6 ± 1.2	14.6 ± 0.8	0.300	0.065
PS	6.5 ± 0.1	5.3 ± 0.2	6.1 ± 0.7	4.5 ± 0.7	0.431	0.020
PI	11.6 ± 0.2	11.4 ± 0.1	11.9 ± 1.5	8.9 ± 0.9	0.379	0.033
PA/CL	0.9 ± 0.0	0.8 ± 0.1	0.9 ± 0.2	2.7 ± 0.5	0.549	0.006
SM	3.2 ± 0.6	3.3 ± 0.1	2.1 ± 0.3	2.8 ± 0.5	0.182	0.167
TP	63.2 ± 0.7	58.5 ± 1.2	63.8 ± 1.6	50.4 ± 0.9	0.460	0.015
TN	36.8 ± 0.7	41.5 ± 1.2	36.2 ± 1.6	49.6 ± 0.9	0.460	0.015
TAG	1.1 ± 0.2	1.0 ± 0.4	3.3 ± 1.6	7.0 ± 1.1	0.797	0.000
CHOL	35.7 ± 0.6	40.5 ± 1.5	32.9 ± 0.1	42.6 ± 0.6	0.135	0.241
FFA	ND	ND	ND	ND	-	-

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* EPA = eicosapentaenoic acid; LNA = α-linolenic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA compositions of CHSE-214 cells after being incubated with EPA and LNA at concentrations of 20/5 µM, 20/10 µM, 20/15 µM, and 20/20 µM are shown in Table 4.13. In all treatments the proportion of the major FA groups was in the rank order PUFA

> MUFA > SFA. Similar levels of EPA were detected in cell total lipid in all treatments (R^2 = 0.084; p = 0.362).

Table 4.13. Fatty acid composition (%) of CHSE-214 cells incubated with EPA and LNA

Fatty acid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
14:0	1.6 ± 0.1	1.6 ± 0.3	1.4 ± 0.2	1.4 ± 0.5	0.126	0.257
15:0	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.4 ± 0.1	0.074	0.393
16:0	14.9 ± 1.5	15.1 ± 1.3	16.0 ± 1.5	15.3 ± 1.2	0.044	0.512
17:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.401	0.027
18:0	10.5 ± 1.0	10.8 ± 1.0	11.4 ± 1.5	10.0 ± 0.4	0.016	0.698
22:0	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.007	0.790
Σ SFA	**27.9 ± 2.8**	**28.3 ± 2.6**	**29.5 ± 3.2**	**27.8 ± 1.3**	**0.002**	**0.897**
16:1n-9	3.9 ± 0.3	3.9 ± 0.1	3.4 ± 0.3	3.8 ± 0.3	0.079	0.377
16:1n-7	2.1 ± 0.1	2.0 ± 0.2	1.8 ± 0.1	1.5 ± 0.7	0.304	0.063
18:1n-9	26.2 ± 1.4	26.5 ± 0.9	24.5 ± 0.9	23.8 ± 2.3	0.381	0.032
18:1n-7	2.0 ± 0.2	2.2 ± 0.1	2.2 ± 0.2	2.1 ± 0.0	0.011	0.749
24:1n-9	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	1.3 ± 0.8	0.175	0.176
Σ MUFA	**35.0 ± 1.6**	**35.3 ± 1.0**	**32.6 ± 1.5**	**32.5 ± 1.9**	**0.395**	**0.029**
18:2n-6	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.0	1.0 ± 0.0	0.249	0.099
18:3n-6	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.051	0.482
20:2n-6*	0.7 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	0.245	0.102
20:3n-6	1.5 ± 0.1	1.4 ± 0.2	1.3 ± 0.2	1.6 ± 0.1	0.022	0.648
20:4n-6	1.7 ± 0.2	1.6 ± 0.2	1.6 ± 0.1	1.6 ± 0.1	0.125	0.259
22:5n-6	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.148	0.217
Σ n-6 PUFA	**5.3 ± 0.2**	**5.1 ± 0.4**	**4.9 ± 0.3**	**5.3 ± 0.2**	**0.001**	**0.911**
18:3n-3	0.5 ± 0.0	0.7 ± 0.1	1.8 ± 0.7	1.7 ± 0.5	0.707	0.001
18:4n-3	0.5 ± 0.0	0.6 ± 0.0	1.1 ± 0.3	1.2 ± 0.2	0.839	0.000
20:4n-3	0.5 ± 0.0	0.7 ± 0.0	1.3 ± 0.4	1.6 ± 0.4	0.848	0.000
20:5n-3	12.2 ± 0.6	11.9 ± 0.7	12.4 ± 2.3	13.1 ± 0.3	0.084	0.362
22:5n-3	7.0 ± 0.2	6.2 ± 0.2	6.8 ± 1.5	6.8 ± 0.6	0.000	0.990
22:6n-3	2.0 ± 0.1	1.8 ± 0.1	1.7 ± 0.2	1.9 ± 0.1	0.020	0.664
Σ n-3 PUFA	**22.7 ± 0.6**	**21.9 ± 1.1**	**25.1 ± 1.4**	**26.3 ± 1.8**	**0.287**	**0.073**
18:2n-9	4.7 ± 0.3	4.7 ± 0.3	4.1 ± 0.2	4.2 ± 0.9	0.222	0.122
20:2n-9	4.1 ± 0.3	4.1 ± 0.3	3.2 ± 0.4	3.2 ± 0.6	0.490	0.011
22:2n-9	0.3 ± 0.2	0.6 ± 0.3	0.6 ± 0.3	0.7 ± 0.4	0.127	0.256
Σ n-9 PUFA	**9.1 ± 0.7**	**9.4 ± 0.2**	**7.9 ± 0.8**	**8.1 ± 1.2**	**0.332**	**0.050**
Σ PUFA	**37.1 ± 1.4**	**36.4 ± 1.6**	**37.9 ± 4.5**	**39.7 ± 0.8**	**0.189**	**0.158**

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* EPA = eicosapentaenoic acid; LNA = α-linolenic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; *contains 20:3n-9.

The percentage of LNA increased with the graded supplementation of the FA itself (R^2 = 0.707; p = 0.001), and in addition, 18:4n-3 and 20:4n-3, increased with graded supplementation of LNA (R^2 = 0.839; p = 0.000 and R^2 = 0.848; p = 0.000, respectively).

The increased percentage of total n-3 PUFA was balanced by decreased proportions of total MUFA (R^2 = 0.395; p = 0.029) and n-9 PUFA (R^2 = 0.332; p = 0.050), without affecting the proportions of total n-6 PUFA or SFA (R^2 = 0.002; p = 0.897).

4.3.2.2. Supplementation with EPA and increasing concentrations of DHA

Table 4.14 shows the cell lipid content and lipid class composition of CHSE-214 cells incubated with EPA and DHA at concentrations of 20/5 µM, 20/10 µM, 20/15 µM, and 20/20 µM. There were no significant effects on total cell lipid content (R^2 = 0.101; p = 0.314). There was a consistent trend for the percentage of TAG to decrease with increasing supplementation of DHA (R^2 = 0.639; p = 0.039). However, total neutral lipids showed a significant increasing trend with DHA supplementation due to a clear, significant and graded increase in the proportion of cholesterol (R^2 = 0.607; p = 0.003).

Table 4.14. Lipid content and lipid class composition of CHSE-214 cells incubated with EPA and DHA

Lipid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
LC (µg)	343.3 ± 20.8	330.0 ± 45.8	353.3 ± 61.1	286.7 ± 76.4	0.101	0.314
CC (%)						
PC	22.8 ± 1.0	21.2 ± 0.4	20.8 ± 2.1	19.0 ± 0.7	0.429	0.021
PE	16.8 ± 0.4	19.1 ± 0.7	16.8 ± 1.0	14.1 ± 1.4	0.430	0.021
PS	7.6 ± 0.8	6.8 ± 0.4	6.2 ± 1.2	5.5 ± 0.4	0.597	0.003
PI	5.6 ± 0.4	4.9 ± 0.4	4.2 ± 0.7	7.6 ± 0.4	0.182	0.167
PA/CL	1.6 ± 0.4	1.5 ± 0.2	1.1 ± 0.5	1.5 ± 0.1	0.066	0.419
SM	2.9 ± 0.2	2.0 ± 0.3	1.4 ± 0.3	2.2 ± 0.5	0.204	0.141
TP	57.3 ± 2.3	55.5 ± 0.5	50.5 ± 1.9	49.9 ± 1.1	0.446	0.018
TN	42.7 ± 2.3	44.5 ± 0.5	49.5 ± 1.9	50.1 ± 1.1	0.446	0.018
TAG	6.5 ± 2.0	6.2 ± 0.1	5.5 ± 0.9	5.4 ± 0.9	0.639	0.039
CHOL	33.9 ± 0.4	36.6 ± 0.1	42.5 ± 2.4	43.3 ± 0.2	0.607	0.003
FFA	2.3 ± 0.8	1.7 ± 0.4	1.5 ± 0.3	1.4 ± 0.1	0.387	0.031

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids.

Table 4.15 shows the FA composition of CHSE-214 cells after being incubated with EPA and increasing concentrations of DHA. The proportions of the major FA groups at 20/5 µM were in the rank order: MUFA > PUFA > SFA, while at 20/10 µM and 20/15 µM it was: PUFA > MUFA > SFA, and at 20/20 µM it was: PUFA > SFA > MUFA.

Table 4.15. Fatty acid composition (%) of CHSE-214 cells incubated with EPA and DHA

Fatty acid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
14:0	1.8 ± 0.2	1.7 ± 0.5	1.6 ± 0.1	1.8 ± 0.2	0.000	0.992
15:0	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.135	0.240
16:0	16.2 ± 0.3	16.4 ± 2.1	15.8 ± 0.7	17.5 ± 1.5	0.086	0.355
17:0	0.5 ± 0.1	0.3 ± 0.2	0.5 ± 0.1	0.4 ± 0.1	0.018	0.674
18:0	8.9 ± 0.5	9.2 ± 0.5	9.0 ± 0.2	9.6 ± 0.6	0.234	0.111
20:0	0.3 ± 0.1	0.3 ± 0.2	0.2 ± 0.1	0.3 ± 0.0	0.157	0.202
22:0	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.1	0.6 ± 0.1	0.657	0.001
Σ SFA	**28.3 ± 0.6**	**28.4 ± 2.9**	**27.9 ± 0.9**	**30.6 ± 2.2**	**0.132**	**0.247**
16:1n-9	2.7 ± 0.2	2.6 ± 0.1	2.5 ± 0.1	2.2 ± 0.2	0.569	0.005
16:1n-7	2.6 ± 0.3	2.4 ± 0.7	2.1 ± 0.1	2.0 ± 0.3	0.381	0.033
18:1n-9	27.7 ± 0.3	27.4 ± 0.6	24.1 ± 0.9	20.1 ± 1.2	0.887	0.000
18:1n-7	2.4 ± 0.1	2.5 ± 0.5	2.1 ± 0.1	2.1 ± 0.3	0.223	0.121
24:1n-9	0.6 ± 0.1	0.7 ± 0.2	0.8 ± 0.3	0.8 ± 0.1	0.242	0.104
Σ MUFA	**36.0 ± 0.3**	**35.6 ± 0.5**	**31.6 ± 1.2**	**27.2 ± 0.8**	**0.909**	**0.000**
18:2n-6	2.4 ± 0.2	2.3 ± 0.1	2.7 ± 0.3	2.5 ± 0.3	0.106	0.301
18:3n-6	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	0.001	0.917
20:2n-6*	0.5 ± 0.0	0.5 ± 0.2	0.7 ± 0.2	0.5 ± 0.2	0.021	0.649
20:3n-6	1.4 ± 0.0	1.1 ± 0.2	1.8 ± 0.2	2.1 ± 0.2	0.515	0.009
20:4n-6	1.2 ± 0.0	1.1 ± 0.2	1.5 ± 0.1	1.1 ± 0.1	0.010	0.754
Σ n-6 PUFA	**6.0 ± 0.1**	**5.4 ± 0.5**	**7.2 ± 1.2**	**6.7 ± 0.2**	**0.246**	**0.101**
18:3n-3	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.095	0.331
18:4n-3	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.003	0.859
20:4n-3	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.5 ± 0.0	0.718	0.001
20:5n-3	10.0 ± 0.1	10.2 ± 1.2	11.3 ± 0.9	11.0 ± 0.9	0.272	0.082
22:5n-3	5.5 ± 0.1	5.0 ± 0.7	5.1 ± 0.6	4.9 ± 0.5	0.164	0.192
22:6n-3	6.4 ± 0.4	8.5 ± 1.1	10.0 ± 1.3	13.8 ± 0.8	0.899	0.000
Σ n-3 PUFA	**22.8 ± 0.3**	**24.5 ± 2.1**	**27.3 ± 0.3**	**30.8 ± 2.1**	**0.771**	**0.000**
16:2n-9	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	0.4 ± 0.2	0.005	0.827
18:2n-9	3.4 ± 0.3	3.3 ± 0.2	2.9 ± 0.2	2.5 ± 0.1	0.756	0.000
20:2n-9	2.8 ± 0.2	2.0 ± 0.7	2.3 ± 0.2	1.6 ± 0.2	0.378	0.033
22:2n-9	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.066	0.421
Σ n-9 PUFA	**6.9 ± 0.4**	**6.1 ± 0.5**	**6.0 ± 0.3**	**4.7 ± 0.2**	**0.715**	**0.001**
Σ PUFA	**35.7 ± 0.2**	**36.0 ± 3.4**	**40.5 ± 1.1**	**42.2 ± 2.0**	**0.687**	**0.001**

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; * contains 20:3n-9.

The percentage of DHA in cell total lipid increased with graded supplementation of the FA itself ($R^2 = 0.899$; $p = 0.000$). Similar percentages of EPA were detected in all

treatments (R^2 = 0.272; p = 0.082), but the percentages of 22:5n-3 decreased with increasing supplementation with DHA (R^2 = 0.164; p = 0.192). LNA (R^2 = 0.095; p = 0.331), 18:4n-3 (R^2 = 0.003; p = 0.859), and 20:4n-3 (R^2 = 0.718; p = 0.001) were detected at percentages lower than 0.5% and were not influenced by supplementation. The increased percentages of total n-3 PUFA were compensated by decreased proportions of total MUFA (R^2 = 0.909; p = 0.000) and n-9 PUFA (R^2 = 0.715; p = 0.001), without affecting the percentages of total SFA (R^2 = 0.132; p = 0.247) and n-6 PUFA (R^2 = 0.246; p = 0.101).

4.3.3. Effect of competing n-3 PUFA (LNA), LC-PUFA (EPA+DHA) and n-6 LC-PUFA (ARA) in cells supplemented with LOA

4.3.3.1. Supplementation with LOA in combination with increasing concentrations of LNA

Table 4.16 shows the cell lipid content and lipid class composition of CHSE-214 cells incubated with LOA and LNA at concentrations of 20/5 µM, 20/10 µM, 20/15 µM, and 20/20 µM. The lipid content initially decreased from 20/5 to 20/10 then progressively increased with the highest lipid content recorded at 20/20 µM, although there was no obvious explanation for this pattern (R^2 = 0.303; p = 0.064). The only clear trend in the lipid class data was the proportion of TAG increased with the graded supplementation of LNA (R^2 = 0.655; p = 0.026).

Table 4.17 shows the FA composition of CHSE-214 cells incubated with LOA and increasing concentrations of LNA. The proportions of the major FA groups at 20/5 µM, 20/10 µM, and 20/15 µM were in the rank order: MUFA > PUFA > SFA, while at 20/20 µM it changed to: PUFA > MUFA > SFA. The levels of EPA were increased in treatments 20/10 µM to 20/20 µM in comparison to treatment 20/5 µM (R^2 = 0.643; p = 0.002). There were increased percentages of LNA in cell total lipid with the graded supplementation of LNA (R^2 = 0.792; p = 0.000), some of which was desaturated to 18:4n-3 (R^2 = 0.888; p =

0.000) and elongated into 20:4n-3 (R^2 = 0.877; p = 0.000). The proportions of total n-3 PUFA increased with the graded supplementation of LNA (R^2 = 0.930; p = 0.000). The percentage of LOA was the similar at 20/5 µM, 20/10 µM and 20/15 µM, but its level abruptly increased at 20/20 (R^2 = 0.545; p = 0.006) and there was some evidence of desaturation to 18:3n-6 in that treatment, however the proportions of 20:3n-6 (R^2 = 0.192; p = 0.155) and ARA (R^2 = 0.598; p = 0.003) decreased with increasing supplementation of LNA. The proportion of n-3 PUFA increased with LNA supplementation (R^2 = 0.930; p = 0.000) with the percentages of MUFA (R^2 = 0.781; p = 0.000) and n-9 PUFA (R^2 = 0.749; p = 0.000) correspondingly decreased.

Table 4.16. Lipid content and lipid class composition of CHSE-214 cells incubated with LOA and LNA

Lipid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
LC (µg)	530.0 ± 26.5	450.0 ± 50.0	530.0 ± 79.4	623.3 ± 38.0	0.303	0.064
CC (%)						
PC	21.1 ± 0.6	20.1 ± 0.2	17.5 ± 1.0	21.6 ± 1.5	0.008	0.787
PE	19.5 ± 0.3	19.0 ± 1.9	20.2 ± 3.2	21.4 ± 0.6	0.192	0.154
PS	4.9 ± 0.7	3.6 ± 0.5	3.8 ± 0.5	3.5 ± 0.6	0.402	0.027
PI	10.9 ± 0.6	9.1 ± 0.2	9.2 ± 0.9	7.2 ± 0.4	0.783	0.000
PA/CL	0.6 ± 0.2	0.8 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	0.024	0.627
SM	4.0 ± 0.8	3.4 ± 0.1	3.0 ± 0.2	4.2 ± 0.8	0.003	0.856
TP	61.0 ± 0.7	56.0 ± 2.1	54.6 ± 3.5	58.8 ± 2.5	0.026	0.618
TN	39.0 ± 0.7	44.0 ± 2.1	45.4 ± 3.5	41.2 ± 2.5	0.026	0.618
TAG	6.3 ± 0.7	7.4 ± 0.8	8.5 ± 1.9	8.8 ± 1.6	0.655	0.026
CHOL	32.7 ± 0.7	36.6 ± 2.6	36.9 ± 1.9	32.4 ± 1.0	0.001	0.918
FFA	ND	ND	ND	ND	-	-

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LOA = linoleic acid; LNA = α-linolenic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

Table 4.17. Fatty acid composition (%) of CHSE-214 cells incubated with LOA and LNA

Fatty acid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
14:0	1.9 ± 0.1	1.5 ± 0.3	1.3 ± 0.2	1.2 ± 0.0	0.702	0.001
15:0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.061	0.438
16:0	12.6 ± 0.5	12.5 ± 0.9	13.2 ± 1.0	11.9 ± 0.1	0.059	0.446
17:0	0.4 ± 0.2	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.268	0.085
18:0	10.1 ± 0.3	10.5 ± 0.5	11.6 ± 0.7	10.3 ± 0.7	0.067	0.417
22:0	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	0.5 ± 0.0	0.230	0.115
Σ SFA	**25.6 ± 1.0**	**25.4 ± 1.9**	**27.1 ± 2.0**	**24.3 ± 0.7**	**0.022**	**0.643**
16:1n-9	5.2 ± 0.0	4.7 ± 0.0	4.6 ± 0.3	2.2 ± 0.4	0.726	0.000
16:1n-7	2.3 ± 0.2	2.1 ± 0.1	1.6 ± 0.4	1.1 ± 0.1	0.797	0.000
18:1n-9	29.7 ± 0.7	29.0 ± 0.9	28.0 ± 0.6	22.2 ± 1.8	0.707	0.001
18:1n-7	2.1 ± 0.2	1.9 ± 0.3	2.1 ± 0.2	2.6 ± 0.2	0.396	0.028
24:1n-9	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	0.400	0.027
Σ MUFA	**39.9 ± 0.7**	**38.3 ± 0.6**	**37.0 ± 0.8**	**29.0 ± 1.7**	**0.781**	**0.000**
18:2n-6	4.6 ± 0.1	4.2 ± 0.1	4.2 ± 0.2	11.3 ± 0.6	0.545	0.006
18:3n-6	1.6 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	2.0 ± 0.5	0.195	0.151
20:2n-6*	2.1 ± 0.1	1.8 ± 0.0	1.6 ± 0.1	1.0 ± 0.3	0.837	0.000
20:3n-6	4.1 ± 0.0	3.8 ± 0.2	3.8 ± 0.3	3.6 ± 0.8	0.192	0.155
20:4n-6	2.9 ± 0.1	2.7 ± 0.2	1.1 ± 0.1	1.0 ± 0.2	0.598	0.003
22:4n-6	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.169	0.184
Σ n-6 PUFA	**15.5 ± 0.2**	**14.1 ± 0.6**	**12.3 ± 1.4**	**19.1 ± 2.0**	**0.121**	**0.268**
18:3n-3	0.3 ± 0.0	0.7 ± 0.0	1.0 ± 0.1	5.4 ± 0.2	0.792	0.000
18:4n-3	0.8 ± 0.0	1.4 ± 0.1	1.8 ± 0.1	4.7 ± 0.2	0.888	0.000
20:4n-3	0.4 ± 0.0	0.8 ± 0.0	1.5 ±0.8	4.7 ± 0.2	0.877	0.000
20:5n-3	2.3± 0.1	4.1 ± 0.4	5.3 ±0.6	4.8 ± 0.7	0.643	0.002
22:5n-3	1.2 ± 0.1	1.4 ± 0.0	1.5 ±0.2	1.3 ± 0.1	0.191	0.156
22:6n-3	1.7 ± 0.1	1.8 ± 0.1	1.7 ±0.2	1.2 ± 0.1	0.529	0.007
Σ n-3 PUFA	**6.7 ± 0.3**	**10.2 ± 0.7**	**12.8 ±1.3**	**22.1 ± 0.7**	**0.930**	**0.000**
18:2n-9	6.5 ± 0.1	6.8 ± 0.5	5.9 ±0.1	3.2 ± 0.2	0.701	0.001
20:2n-9	5.5 ± 0.1	5.1 ± 0.1	4.6 ±0.2	2.0 ± 0.2	0.761	0.000
22:2n-9	0.3 ± 0.1	0.1 ± 0.0	0.3 ±0.1	0.3 ± 0.1	0.045	0.508
Σ n-9 PUFA	**12.3 ± 0.1**	**12.0 ± 0.5**	**10.8 ±0.4**	**5.5 ± 0.1**	**0.749**	**0.000**
Σ PUFA	**34.5 ± 0.3**	**36.3 ± 1.4**	**35.9 ±2.5**	**46.7± 2.4**	**0.627**	**0.002**

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LOA = linoleic acid; LNA = α-linolenic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; *contains 20:3n-9.

4.3.3.2. Supplementation with LOA and increasing concentrations of EPA+DHA

The cell lipid content and lipid class composition of CHSE-214 cells incubated with LOA and EPA+DHA (1:1) at concentrations of 20/5 µM, 20/10 µM, 20/15 µM, and 20/20 µM are shown in Table 4.18. The FA supplementations had no significant effect on cell total lipid content ($R^2 = 0.004$; $p = 0.843$). There was a clear effect of EPA+DHA supplementation on the level of cellular TAG, which decreased with the graded supplementation of the n-3 LC-PUFA mix ($R^2 = 0.818$; $p = 0.000$).

Table 4.18. Lipid content and lipid class composition of CHSE-214 cells incubated with LOA and EPA+DHA (1:1)

Lipid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
LC (µg)	293.3 ± 66.6	296.7 ± 49.3	290.0 ± 20.0	303.3 ± 50.3	0.004	0.843
CC (%)						
PC	10.1 ± 1.0	10.5 ± 1.1	13.5 ± 1.4	11.3 ± 1.5	0.197	0.148
PE	18.6 ± 1.6	19.6 ± 0.7	20.3 ± 0.5	22.5 ± 1.1	0.684	0.001
PS	5.4 ± 0.6	7.1 ± 0.5	6.7 ± 0.2	9.0 ± 1.9	0.608	0.033
PI	11.3 ± 1.2	12.0 ± 0.7	11.0 ± 0.0	12.0 ± 1.5	0.017	0.683
PA/CL	1.3 ± 0.0	1.5 ± 0.1	1.3 ± 0.0	1.1 ± 0.3	0.186	0.162
SM	6.3 ± 0.8	8.3 ± 0.6	7.5 ± 0.6	6.6 ± 0.8	0.000	0.973
TP	53.0 ± 2.1	59.0 ± 1.2	60.3 ± 1.1	62.5 ± 0.8	0.817	0.000
TN	47.0 ± 2.1	41.0 ± 1.2	39.7 ± 1.1	37.5 ± 0.8	0.817	0.000
TAG	11.1 ± 1.4	5.6 ± 0.5	4.5 ± 0.4	3.7 ± 0.5	0.818	0.000
CHOL	32.7 ± 0.6	32.9 ± 1.2	31.8 ± 0.6	31.3 ± 1.2	0.351	0.042
FFA	3.2 ± 0.9	2.5 ± 0.4	3.4 ± 0.3	2.5 ± 0.1	0.047	0.499

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LOA = linoleic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids.

Table 4.19 shows the FA composition of CHSE-214 cells incubated with LOA and increasing concentrations of the EPA+DHA mix. In all treatments the proportions of the major FA groups were in the rank order: MUFA > PUFA > SFA. The percentages of both EPA and DHA in the cell total lipid increased with the graded supplementation of the n-3 LC-PUFA mixture ($R^2 = 0.877$; $p = 0.000$ and $R^2 = 0.897$; $p = 0.000$, respectively). In addition the percentage of 22:5n-3 also increased with the graded supplementation of EPA+DHA ($R^2 = 0.901$; $p = 0.000$), whilst the proportions of LOA ($R^2 = 0.582$; $p = 0.004$) and the other n-6 FA decreased. The increased proportions of total n-3 PUFA ($R^2 = 0.927$; $p = 0.000$) was balanced by decreased proportions of total MUFA ($R^2 = 0.178$; $p = 0.172$), n-6 PUFA ($R^2 = 0.843$; $p = 0.000$), and n-9 PUFA ($R^2 = 0.630$; $p = 0.002$), with minimal effects on total SFA ($R^2 = 0.044$; $p = 0.513$).

Table 4.19. Fatty acid composition (%) of CHSE-214 cells incubated with LOA and EPA+DHA

Fatty acid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
14:0	1.5 ± 0.3	1.3 ± 0.2	1.6 ± 0.3	1.3 ± 0.2	0.016	0.699
15:0	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.159	0.198
16:0	13.6 ± 0.6	14.2 ± 1.0	12.0 ± 1.2	14.6 ± 0.5	0.000	0.996
17:0	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	0.079	0.377
18:0	8.9 ± 0.2	10.1 ± 0.5	12.0 ± 1.7	10.0 ± 0.5	0.196	0.149
20:0	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.173	0.179
22:0	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.009	0.771
Σ SFA	**25.4 ± 0.8**	**26.9 ± 1.7**	**27.0 ± 1.6**	**27.2 ± 1.0**	**0.044**	**0.513**
16:1n-9	3.3 ± 0.1	3.8 ± 0.4	3.8 ± 0.5	3.5 ± 0.2	0.031	0.586
16:1n-7	2.3 ± 0.3	2.5 ± 0.2	2.8 ± 0.2	2.4 ± 0.2	0.086	0.355
18:1n-9	33.7 ± 0.2	30.8 ± 1.9	28.3 ± 6.6	30.2 ± 2.4	0.159	0.199
18:1n-7	2.0 ± 0.0	2.1 ± 0.2	2.0 ± 0.3	1.6 ± 0.2	0.327	0.052
24:1n-9	0.7 ± 0.1	0.9 ± 0.1	0.6 ± 0.0	0.7 ± 0.0	0.176	0.175
Σ MUFA	**42.0 ± 0.1**	**40.1 ± 1.7**	**37.5 ± 6.8**	**38.4 ± 2.7**	**0.178**	**0.172**
18:2n-6	7.0 ± 0.8	5.4 ± 0.3	5.7 ± 0.1	4.9 ± 0.2	0.582	0.004
18:3n-6	1.7 ± 0.2	1.6 ± 0.3	1.4 ± 0.1	1.1 ± 0.1	0.626	0.002
20:2n-6*	0.9 ± 0.1	0.9 ± 0.3	0.7 ± 0.0	0.6 ± 0.0	0.464	0.015
20:3n-6	3.9 ± 0.4	3.3 ± 0.2	3.2 ± 0.1	2.7 ± 0.2	0.776	0.000
20:4n-6	2.2 ± 0.3	1.6 ± 0.2	1.7 ± 0.0	1.5 ± 0.1	0.561	0.005
22:4n-6	0.2 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.113	0.284
22:5n-6	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.125	0.259
Σ n-6 PUFA	**16.1 ± 0.4**	**13.3 ± 1.0**	**13.3 ± 0.3**	**11.3 ± 0.4**	**0.843**	**0.000**
18:3n-3	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.786	0.000
18:4n-3	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.169	0.185
20:5n-3	2.7 ± 0.6	4.0 ± 0.3	5.4 ± 0.3	6.0 ± 0.6	0.877	0.000
22:5n-3	1.1 ± 0.1	1.3 ± 0.1	1.9 ± 0.1	2.2 ± 0.3	0.901	0.000
22:6n-3	3.0 ± 0.2	5.2 ± 0.4	6.3 ± 0.3	7.2 ± 0.5	0.897	0.000
Σ n-3 PUFA	**7.3 ± 0.5**	**10.9 ± 0.7**	**13.9 ± 0.6**	**15.7 ± 1.4**	**0.927**	**0.000**
16:2n-9	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	0.344	0.045
18:2n-9	4.9 ± 0.5	4.7 ± 0.2	4.5 ± 0.2	3.8 ± 0.2	0.630	0.002
20:2n-9	3.4 ± 0.5	3.3 ± 0.2	3.1 ± 0.2	2.7 ± 0.1	0.538	0.007
22:2n-9	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.209	0.135
Σ n-9 PUFA	**9.2 ± 0.9**	**8.8 ± 0.4**	**8.4 ± 0.5**	**7.4 ± 0.3**	**0.630**	**0.002**
Σ PUFA	**32.6 ± 0.9**	**33.0 ± 1.5**	**35.5 ± 0.3**	**34.4 ± 2.2**	**0.282**	**0.076**

Footnotes: Results are expressed as means ± SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LOA = linoleic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; *contains 20:3n-9.

4.3.3.3. Supplementation with LOA in the presence of increasing concentrations of ARA

Table 4.20 shows the cell lipid content and lipid class composition of CHSE-214 cells incubated with LOA and ARA at concentrations of 20/5 µM, 20/10 µM, 20/15 µM, and 20/20 µM. Cell total lipid content increased from 20/5 µM to 20/15 µM and there was observed a decrement from 20/15 µM to 20/20 µM, however results were not significant

(R^2 = 0.016; p = 0.694). In the lipid class data, there was a slight but consistent increase in the proportion of TAG with the graded supplementation of ARA (R^2 = 0.682; p = 0.036).

Table 4.20. Lipid content and lipid class composition of CHSE-214 cells incubated with LOA and ARA

Lipid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
LC (µg)	493.3 ± 40.4	523.3 ± 59.7	543.3 ± 76.4	510.0 ± 36.1	0.016	0.694
CC (%)						
PC	22.5 ± 0.4	22.6 ± 0.2	22.0 ± 0.4	16.8 ± 0.7	0.014	0.717
PE	21.4 ± 0.4	22.3 ± 0.2	22.9 ± 1.0	24.5 ± 1.3	0.603	0.003
PS	3.9 ± 0.2	5.7 ± 0.3	4.1 ± 0.4	4.1 ± 0.2	0.023	0.641
PI	8.2 ± 0.1	8.0 ± 0.4	8.1 ± 0.4	8.1 ± 0.1	0.041	0.530
PA/CL	0.4 ± 0.2	0.6 ± 0.1	0.4 ± 0.2	0.6 ± 0.1	0.065	0.422
SM	3.3 ± 0.3	5.5 ± 0.2	5.4 ± 0.3	3.5 ± 0.4	0.005	0.822
TP	59.7 ± 0.6	64.7 ± 0.5	62.9 ± 1.5	57.6 ± 2.4	0.033	0.571
TN	40.3 ± 0.6	35.3 ± 0.5	37.1 ± 1.5	42.4 ± 2.4	0.033	0.571
TAG	8.8 ± 1.3	9.3± 0.2	9.8 ± 0.2	11.3 ± 1.2	0.682	0.036
CHOL	31.5 ± 0.9	26.0 ± 0.6	27.3± 1.4	31.1 ± 1.2	0.020	0.661
FFA	ND	ND	ND	ND	-	-

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LOA = linoleic acid; ARA = arachidonic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

Table 4.21 shows the FA composition of CHSE-214 cells incubated with LOA and ARA at concentrations of 20/5 µM, 20/10 µM, 20/15 µM, and 20/20 µM. In all treatments the proportions of the major FA groups were in the rank order: PUFA > MUFA > SFA. The proportion of ARA increased with the graded supplementation of the FA itself (R^2 = 0.881; p = 0.000). There was some apparent elongation as the percentage of 22:4n-6 also increased with the graded supplementation of ARA (R^2 = 0.776; p = 0.000). The percentages of the other n-6 PUFA, LOA (R^2 = 0.762; p = 0.000), 18:3n-6 (R^2 = 0.685; p = 0.001) and 20:3n-6 (R^2 = 0.640; p = 0.002) decreased with the graded supplementation of ARA. In consequence, total n-6 PUFA did not show a clear trend with the graded supplementation of ARA (R^2 = 0.231; p = 0.114), and similarly there were clear trends

observed in the proportions of total SFA (R^2 = 0.004; p = 0.849), MUFA (R^2 = 0.194; p = 0.151), n-3 PUFA (R^2 = 0.146; p = 0.221) or n-9 PUFA (R^2 = 0.065; p = 0.425).

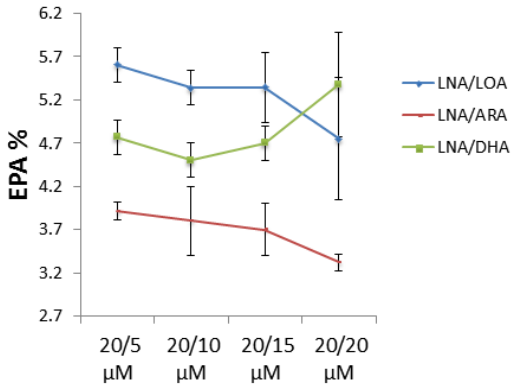
Table 4.21. Fatty acid composition (%) of CHSE-214 cells incubated with LOA and ARA

Fatty acid	20/5 µM	20/10 µM	20/15 µM	20/20 µM	R^2	P-value
14:0	1.8 ± 0.3	1.6 ± 0.0	1.8 ± 0.4	1.7 ± 0.1	0.001	0.913
15:0	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.002	0.902
16:0	14.0 ± 1.3	13.7 ± 0.7	14.5 ± 0.9	13.9 ± 1.0	0.006	0.811
17:0	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.215	0.129
18:0	11.4 ± 0.8	11.1 ± 0.7	11.0 ± 0.6	11.1 ± 0.8	0.022	0.643
22:0	0.6 ± 0.3	0.6 ± 0.3	0.6 ± 0.3	0.4 ± 0.1	0.048	0.494
Σ SFA	**28.3 ± 2.3**	**27.5 ± 1.8**	**28.4 ± 2.2**	**27.5 ± 1.9**	**0.004**	**0.849**
16:1n-9	3.8 ± 0.3	4.3 ± 0.6	4.4 ± 0.6	5.0 ± 0.9	0.397	0.028
16:1n-7	1.7 ± 0.2	2.0 ± 0.2	2.1 ± 0.6	1.8 ± 0.8	0.002	0.895
18:1n-9	21.2 ± 1.2	23.9 ± 2.5	23.3 ± 3.4	23.9 ± 1.4	0.138	0.234
18:1n-7	2.2 ± 0.2	2.2 ± 0.2	2.1 ± 0.2	2.0 ± 0.1	0.079	0.376
20:1n-9	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.087	0.353
24:1n-9	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.036	0.557
Σ MUFA	**29.6 ± 1.5**	**33.2 ± 3.0**	**32.6 ± 4.3**	**33.5 ± 1.3**	**0.194**	**0.151**
18:2n-6	7.1 ± 0.2	4.6 ± 1.3	3.7 ± 1.6	2.5 ± 0.3	0.762	0.000
18:3n-6	4.2 ± 0.2	2.5 ± 0.9	2.1 ± 0.3	1.3 ± 0.2	0.685	0.001
20:2n-6*	1.2 ± 0.0	1.5 ± 0.3	1.4 ± 0.3	1.2 ± 0.1	0.022	0.644
20:3n-6	7.0 ± 0.1	5.3 ± 1.2	4.7 ± 1.5	3.8 ± 0.3	0.640	0.002
20:4n-6	9.5 ± 0.4	10.5 ± 0.7	11.7 ± 0.8	13.5 ± 0.6	0.881	0.000
22:4n-6	1.4 ± 0.1	1.8 ± 0.1	2.3 ± 0.8	4.0 ± 0.1	0.776	0.000
Σ n-6 PUFA	**30.4 ± 0.8**	**26.2 ± 2.6**	**25.9 ± 4.7**	**26.3 ± 0.2**	**0.231**	**0.114**
20:5n-3	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.0	0.7 ± 0.1	0.153	0.208
22:5n-3	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.210	0.134
22:6n-3	1.5 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	0.012	0.730
Σ n-3 PUFA	**3.0 ± 0.3**	**3.1 ± 0.1**	**3.1 ± 0.1**	**3.3 ± 0.2**	**0.146**	**0.221**
18:2n-9	4.7 ± 0.1	5.0 ± 0.4	4.7 ± 0.6	4.6 ± 0.3	0.023	0.637
20:2n-9	3.8 ± 0.1	4.7 ± 0.5	4.5 ± 0.9	4.6 ± 0.3	0.182	0.167
22:2n-9	0.2 ± 0.0	0.3 ± 0.1	0.8 ± 0.2	0.2 ± 0.1	0.037	0.551
Σ n-9 PUFA	**8.7 ± 0.2**	**10.0 ± 0.9**	**10.0 ± 1.5**	**9.4 ± 0.7**	**0.065**	**0.425**
Σ PUFA	**42.1 ± 1.0**	**39.3 ± 1.7**	**39.0 ± 3.5**	**39.0 ± 0.7**	**0.266**	**0.086**

Footnotes: Results are expressed as means ± SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LOA = linoleic acid; ARA = arachidonic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; *contains 20:3n-9.

The following figures represent a summary of the results generated from the PUFA competition experiments (section 4.2.2), and the effects observed in cellular LC-PUFA levels. Figure 4.2 shows the EPA levels of CHSE-214 incubated with 20 µM LNA in

presence of graded concentration of LOA, ARA and DHA. EPA levels decreased with the graded supplementation of LOA and ARA. In the combination LNA/DHA, there was observed an increment of EPA with graded supplementation of DHA, particularly from 20/10 to 20/20 µM. The ANOVA shows that cellular EPA levels were affected by the FA combination supplemented (treatment) and the interaction of this with the concentration of FA ($p < 0.05$) but not the concentration itself ($p = 0.293$).



ANOVA		
Fatty acid supplemented	Concentration	Interaction
<0.000	0.293	0.009

Figure 4.2. Percentages of EPA in CHSE-214 incubated for five days with different combinations of PUFA. *Abbreviations:* EPA = eicosapentaenoic acid; LNA = α-linolenic acid; LOA = linoleic acid; ARA = arachidonic acid; DHA = docosahexaenoic acid; PUFA = polyunsaturated fatty acid. Data were analysed by two-way ANOVA ($p < 0.05$). The interaction between the two factors (fatty acid supplemented and concentration) was also analysed (inlet table).

Figure 4.3 shows the DHA levels of CHSE-214 incubated with 20 µM EPA in presence of graded concentration of LNA. DHA levels were lower than 2% in the four concentrations supplemented, and the data did not show a clear trend.

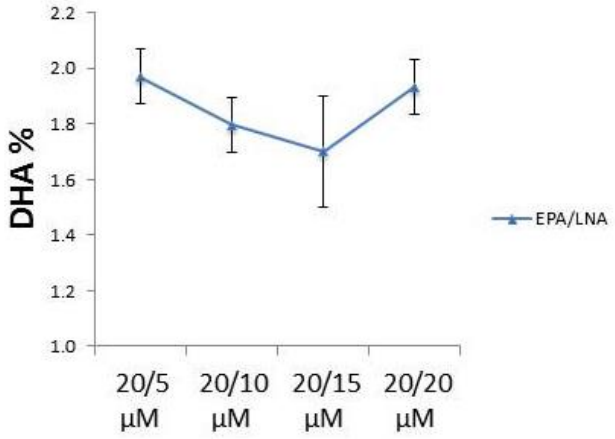
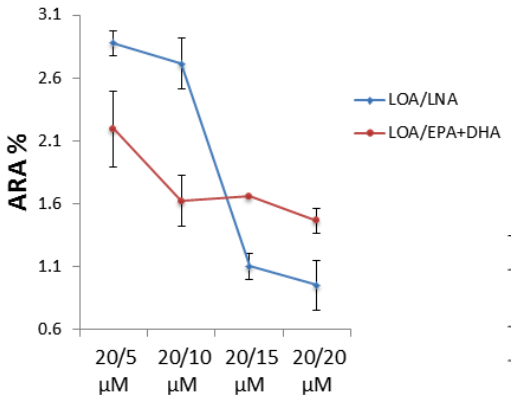


Figure 4.3. Percentages of DHA in CHSE-214 incubated for five days with increasing concentrations of EPA/LNA. *Abbreviations:* DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; LNA = α-linolenic acid.

Figure 4.4 shows the ARA levels of CHSE-214 incubated with 20 µM LOA in presence of graded concentration of LNA and EPA+DHA. ARA levels decreased with the graded supplementation of LNA or EPA+DHA. However, cells supplemented with LOA/LNA at 20/5 and 20/10 µM, and LOA/EPA+DHA at 20/5 µM exhibited higher ARA levels than the supplementation of 20 µM LOA alone (1.8%). The highest ARA level was recorded in cells incubated with LOA/LNA at 20/5 µM. There was not significant evidence to confirm that ARA cellular levels were affected by the FA supplemented ($p = 0.918$), but the concentration supplemented and the interaction between the two variables had an effect on the ARA levels ($p < 0.05$).



ANOVA		
Fatty acid supplemented	Concentration	Interaction
0.918	0.001	0.016

Figure 4.4. Percentages of ARA in CHSE-214 incubated for five days with different combinations of PUFA. *Abbreviations:* LOA = linoleic acid; LNA = α-linolenic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; PUFA = polyunsaturated fatty acid. Data analysed by two-way ANOVA ($p < 0.05$). The interaction between the two factors (fatty acid supplemented and concentration) was also analysed (inlet table).

Figure 4.5 shows the 19 PUFA combinations and their concentrations supplemented that resulted in the highest total n-3 PUFA intermediaries. The maximum level of n-3 intermediaries was reported with the supplementation of EPA/DHA at 20/20 µM. The treatment with highest EPA level was EPA/LNA at 20/20 µM, while the treatment with highest DHA level was EPA/DHA at 20/20 µM. The total n-3 intermediaries were higher when supplementing the PUFA combinations compared with the supplementation of just LNA at 20 µM (14.7%).

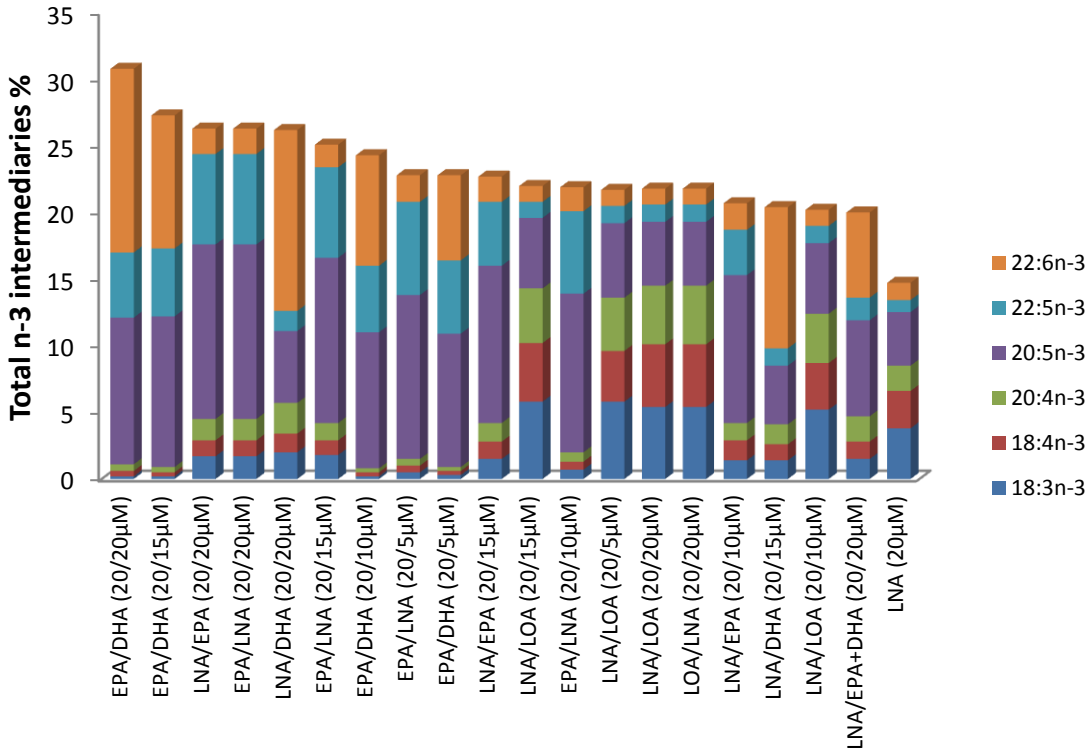
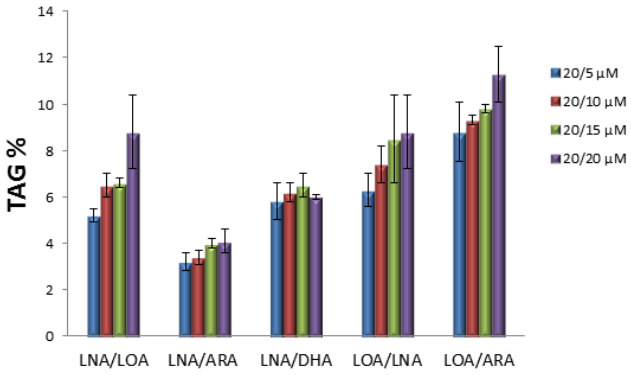


Figure 4.5. PUFA combinations that showed the highest total n-3 PUFA percentages in CHSE-214. *Abbreviations:* EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; LNA = α-linolenic acid; LOA = linoleic acid; PUFA = polyunsaturated fatty acid.

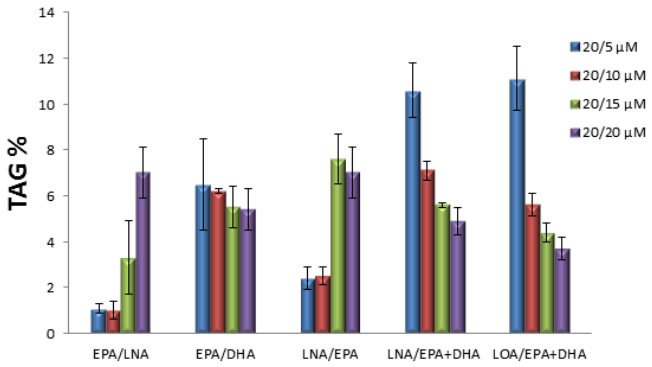
From the lipid class data, TAG was the only lipid class that showed clear trends, as it increased with the graded supplementation of the FA, meaning that any excess of FA was stored as TAG. Other changes in lipid classes occurred mainly to balance the increment of TAG. Figure 4.6 and 4.7 show that cellular TAG was affected by the combination of FA supplemented (treatment), the concentration and the interaction of these two variables ($p < 0.05$). Figure 4.6 shows the highest increment of TAG, reported when CHSE-214 were incubated with LOA/ARA at 20/20 µM. Apart from LNA/DHA, the PUFA combinations LNA/LOA, LNA/ARA, LOA/LNA, and LOA/ARA increased the TAG with the graded supplementation of the PUFA.



ANOVA		
Fatty acid supplemented	Concentration	Interaction
<0.000	<0.000	<0.000

Figure 4.6. TAG (%) reported in CHSE-214 incubated with different combinations of n-3 and n-6 PUFA. *Abbreviations:* LNA = α-linolenic acid; LOA = linoleic acid; ARA = arachidonic acid; DHA = docosahexaenoic acid; PUFA = polyunsaturated fatty acid. Data were analysed by two-way ANOVA ($p < 0.05$). The interaction between the two factors (fatty acid supplemented and concentration) was also analysed (inlet table).

Figure 4.7 shows the reduction of TAG with the graded supplementation of EPA+DHA, and fixed supplementation of LNA and LOA. A similar situation was observed when supplementing EPA/DHA. However, supplementing EPA/LNA and LNA/EPA at 20/5 µM and 20/10 µM resulted in lower TAG levels compared with 20/15 µM and 20/20 µM.



ANOVA		
Fatty acid supplemented	Concentration	Interaction
<0.000	<0.000	<0.000

Figure 4.7. TAG (%) reported in CHSE-214 cells incubated with different combinations of n-3 and n-6 PUFA. *Abbreviations:* TAG = triacylglycerol; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; LNA = α-linolenic acid; LOA = linoleic acid; PUFA = polyunsaturated fatty acid. Data were analysed by two-way ANOVA ($p < 0.05$). The interaction between the two factors (fatty acid supplemented and concentration) was also analysed (inlet table).

4.4 Discussion

The present study attempted to establish the effect of the supplementation of combinations of PUFA on the FA profile and lipid class composition of CHSE-214 cells, and the potential competition between PUFA of the series n-3 and n-6 for enzyme pathways. Experiments were specifically designed in order to determine how competing PUFA, *i.e.* LOA, or the pathway end products, *i.e.* EPA, DHA and ARA, affected the production of EPA from LNA. To achieve this goal, CHSE-214 cells were incubated with different PUFA combinations at various concentrations for 5 d, followed by lipid and FA analyses. This study is the first to deal with the simultaneous supplementation of PUFA using a fish cell line.

4.4.1 Effect the supplementation of PUFA on lipid content of CHSE-214 cells

It was perhaps expected that there would be an increase in cell lipid content with the graded supplementation of PUFA; however, this trend was only observed when LOA was supplemented at 20 µM in presence of graded concentrations of ARA. The lipid content was lower when n-3 PUFA combinations were supplemented, *i.e*. LNA/EPA, LNA/EPA+DHA, EPA/DHA and LOA/EPA+DHA. However, lipid content was based on a per flask basis and so cell number was a confounding factor. Therefore, differential effects of different PUFA on growth performance as discussed in Chapter 3 would affect absolute levels of lipid recovered. EPA and DHA are highly unsaturated FA, which means that they have several double bonds, making them more susceptible to the attack of reactive oxygen species, triggering a chain reaction of hydrogen abstraction, and the formation of lipid radicals (Mourente *et al.*, 2007; Siddiqui *et al.*, 2008; Di Nunzio *et al.*, 2011). These processes damage and can cause death of the cells, which will consequently result in lower lipid recovered, as shown in the current study. Gregory *et al.* (2011)

observed more peroxidation when the FHM (fathead minnow, *Pimephales promelas* Rafinesque) cell line was supplemented with EPA and DHA in comparison with the supplementation of LNA or SFA. Several studies have reported a detrimental effect on the viability of human cell lines due to the supplementation of LC-PUFA (Colquhoun and Schumacher, 2001; Bianchi *et al.*, 2004; Shirota *et al.*, 2005; Li *et al.*, 2006; Toit-Kohn *et al.*, 2009; Di Nunzio *et al.*, 2011).

4.4.2 Effect of supplementation of FA on lipid class composition of CHSE-214

Studies using cell culture systems reported that supplementing PUFA at 25 µM was sufficient to significantly change the PUFA composition of the cells without adversely altering the lipid class composition or inducing the formation of lipid droplets in the cytoplasm (Geyer, 1967; Moskowitz, 1967; Rosenthal, 1981; Stubbs and Smith, 1984; Tocher and Dick, 1990). Tocher and Dick (1990) reported cytoplasmic lipid droplets when the AS cell line was supplemented with PUFA concentrations of 50 µM or higher. For all the aforementioned and based on the results in Chapter 3 of the present study, the mixtures of PUFA and LC-PUFA supplemented to the CHSE-214 cell line were 20/5 µM, 20/10 µM, 20/15 µM and 20/20 µM. Regarding lipid class composition, TAG was the lipid class that was likely directly affected by FA supplementation, and other changes in lipid class composition were consequent to that. In the experiments supplementing graded concentrations of n-6 PUFA, *i.e.* LNA/LOA, LNA/ARA, LOA/LNA and LOA/ARA TAG increased with the graded supplementation of the competing PUFA, which was likely due to the incorporation and acylation of the PUFA into TAG leading to increased lipid deposition. Previous studies have reported that supplementation of ARA increased cellular TAG levels (Collier and Collier, 1993; Whelan *et al.*, 1995; Whelan, 1996). *In vivo* (Kajikawa *et al.*, 2011) and *in vitro* (Manickam *et al.*, 2010) studies reported that n-3 LC-

PUFA have the effect of lowering tissue/cellular TAG levels, probably through a mechanism whereby EPA down-regulates sterol regulating element binding protein (SREBP-1c) thereby reducing the expression of genes involved with lipid biosynthesis/lipogenesis (Kaur *et al.*, 2011). In the current study, there was a clear trend of decreasing cellular TAG levels with the graded supplementation of the mixture of EPA+DHA (1:1). Furthermore, in the treatment EPA/DHA (constant EPA + increasing DHA), TAG decreased with the graded supplementation of DHA. In the treatments LNA/EPA and EPA/LNA at 20/5 µM and 20/10 µM levels of TAG were lower, in comparison with the levels recorded at 20/15 µM and 20/20 µM. These combinations suggest that n-3 LC-PUFA reduce cellular TAG, particularly at lower levels of supplementation. At higher concentrations of PUFA supplementation, the rate of normal lipid metabolism becomes overwhelmed with FA such that they cannot be metabolised fast enough and the FA have to be stored, increasing the percentages of TAG in the cells.

4.4.3 Effect of supplementation of FA on FA composition of CHSE-214 cell line

All the combinations of PUFA supplemented changed the FA profile of the CHSE-214 cells. In some cases this change reflected the incorporation of the PUFA supplemented, while in other occasions n-3 and n-6 PUFA metabolites (pathway intermediates) were synthesised from the precursors LNA and LOA. In all treatments the increment of PUFA in the cells was balanced by reduced proportions of MUFA; particularly, the percentage of 18:1n-9 decreased with the graded supplementation of PUFA, in agreement with results previously reported by Tocher *et al.* (1996) in studies using the EPC-EFAD (Epithelioma papulosum cyprini-essential fatty acid-deficient) cell line derived from common carp (*Cyprinus carpio* L.).

4.4.3.1 EPA production from LNA mixed with n-6 PUFA (LOA and ARA) and n-3 LC-PUFA (DHA)

The EPA levels of CHSE-214 cells incubated with the combinations LNA/LOA and LNA/ARA, showed a clear decreasing trend with the graded supplementation of the n-6 PUFA. The supplementation of LNA/LOA at 20/5 μM showed a higher EPA level (5.6%), in comparison with cells incubated with only LNA at 20 μM (4%) (see Chapter 3), whereas the treatment LNA/ARA showed lower EPA levels at all supplemented concentrations ARA (3.3–3.9%). The EPA levels of CHSE-214 cells incubated with the combination LNA/DHA showed a trend to increase with the graded supplementation of DHA, perhaps suggesting some retroconversion of DHA to EPA (4.5-5.4%) (Grønn *et al.*, 1991). It is noteworthy that the effect of the LNA:LOA ratio observed in the experiments using the combination LNA/LOA, where supplementation of LOA at 5 μM enhanced the conversion of 20 μM LNA to EPA, whereas when the concentration of LOA was increased to 20 μM supplementation, EPA levels were considerably decreased. In addition, LNA/LOA supplemented at equal concentrations (20/20 μM) gave lower total n-6 PUFA (19.1%) than total n-3 PUFA (22.1%). This may be explained because at concentrations of 20/20 μM, the supplemented LOA was only metabolised to a small amount, being mainly incorporated and stored (11.3%) without any further conversion. The data from the current study were consistent with the enzymes (elongases and desaturases) involved in the conversion of PUFA to LC-PUFA having a preference for the n-3 series rather than the n-6 series, as reported in previous studies (Stubbs and Smith, 1984; Tocher *et al.*, 1989; Gregory *et al.*, 2011). In CHSE-214 cells incubated with LNA/ARA, total cellular n-6 PUFA levels at the 20/15 μM and 20/20 μM combinations were 15.3% and 20.0%, respectively, which were higher than total n-3 PUFA at 14.6% and 13.8%, respectively. In contrast, at lower ARA supplements (LNA/ARA combinations of 20/5 μM and 20/10 μM)

total n-6 PUFA were 9.3% and 12%, respectively, which were lower than the total n-3 PUFA (14.4% and 13.7%, respectively). Therefore, the pathway of n-3 LC-PUFA synthesis from LNA was observed when LNA was supplemented in combination with ARA; however, the percentages of n-3 PUFA intermediaries decreased with the increment of ARA.

4.4.3.2 DHA production from EPA mixed with n-3 PUFA (LNA)

In the experiment supplementing combinations of EPA/LNA, CHSE-214 cells showed elongation of EPA to 22:5n-3; however, the cells did not show conversion of the latter to DHA at high levels. The maximum percentage of cellular DHA observed was 2.0%, recorded at the EPA/LNA combination of 20/5 μM. This was perhaps not surprising as it had been reported that fish cell lines have a limited ability to synthesise DHA from precursors (*i.e.* LNA and EPA) (Tocher and Sargent, 1990; Gregory *et al.*, 2011).

4.4.3.3 ARA production from LOA mixed with n-3 PUFA and LC-PUFA (LNA and EPA+DHA)

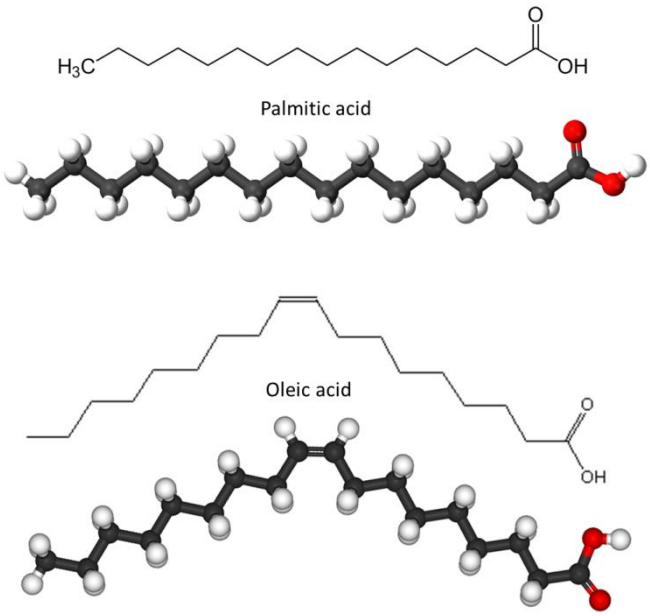
The ARA levels of CHSE-214 cells supplemented with combinations of LOA/LNA and LOA/EPA+DHA (1:1 ratio) decreased with the graded supplementation of the competing FA, LNA and EPA+DHA, respectively. When LOA was supplemented at 20 μM along with a low concentration of LNA (5 μM) it was possible to observe the pathway of synthesis of LOA to ARA with 2.9% production. However, when LOA and LNA were supplemented at equal concentrations (20/20), LOA was incorporated but not greatly converted to other n-6 PUFA metabolites (total n-6 PUFA = 19.1%), while the n-3 pathway (*i.e.* the conversion of LNA to EPA) was still clear and consistent (total n-3 PUFA = 22.1%), which is possibly simply reflecting the fact that n-3 PUFA series are the

preferred substrates of the enzymes involved in LC-PUFA synthesis (Stubbs and Smith, 1984; Tocher *et al.*, 1989).

The current study confirmed that LNA and LOA compete for the same enzymes in the LC-PUFA biosynthesis pathway, having antagonistic effects when they are added together; therefore, the ratio of LNA:LOA is an important factor to consider when fish diets are being formulated, in order to enhance and optimise the production of EPA from LNA.

Chapter 5

Effects of interaction between C_{18} PUFA and saturated/monounsaturated fatty acids on lipid and fatty acid compositions of CHSE-214 cells



Molecular structures of palmitic and oleic acids [modified from Wikimedia]

"The doctor of the future will give no medicine, but will interest his patient in the care of the human frame, in diet and in the cause and prevention of disease"

Thomas Alva Edison

5.1. Introduction

Atlantic salmon (*Salmo salar* L.), herring (*Clupea harengus* L.) and mackerel (*Scomber scombrus* L.) are "oily" fish species rich in n-3 long-chain polyunsaturated fatty acids (LC-PUFA), used for human consumption (Bell *et al.*, 2001; Tocher, 2009). As the human population is constantly increasing, and wild capture fisheries are static or decreasing, it has been estimated that by 2030 about 2/3 of the global demand for fish will be supplied by the aquaculture sector (FAO, 2014). However, one of the main challenges for aquaculture is the generation of new sustainable ingredients to replace fishmeal and fish oil (FO) included in aquafeeds, while still covering the n-3 LC-PUFA requirements (Bendiksen *et al.*, 2011). Currently, the main sustainable alternatives to FO are vegetable oils (VO), and their use can consequently reduce feed production costs due to their ready availability in large volumes (Naylor *et al.*, 2009). Studies have reported that growth, feed conversion and survival are not affected with partial replacement of FO in aquafeeds (Torstensen *et al.*, 2000; Bell *et al.*, 2001; Rosenlund *et al.*, 2001; Bransden *et al.*, 2003; Liland *et al.*, 2013). The main limiting factor in the use of VO is their fatty acid (FA) profile, since they can be rich in saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and short chain (C_{18}) PUFA, while they completely lack LC-PUFA, such as eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3), and arachidonic acid (ARA, 20:4n-6) (Jobling, 2004). Fish fed diets formulated with 100% VO presented lower n-3 LC-PUFA levels in their flesh, reducing the nutritional quality of the final product to the human consumer (Bell *et al.*, 2003b; Menoyo *et al.*, 2005; Tocher, 2010; Alves Martins *et al.*, 2011).

According to Bell *et al.* (2002), an ideal FO substitute should maintain as high levels of EPA and DHA as possible, not promote linoleic acid (LOA, 18:2n-6) deposition, while it should supply the necessary SFA and MUFA in order to provide the energy

required for optimal growth. The main VO that are currently being used in aquafeeds around the world include palm oil (Bell *et al.*, 2002), soybean oil (Peng *et al.*, 2008) and rapeseed oil (Bell *et al.*, 2001, 2003a; b), all of which cover the energy requirements due to their richness in SFA and MUFA including palmitic acid (16:0) and oleic acid (18:1n-9) (Miller *et al.*, 2008). However, the main VO being used in salmon feeds in Scotland (and Norway) is rapeseed oil which has a high 18:1n-9 content and a good n-6/n-3 PUFA ratio of around two with relatively moderate level of LOA (20%) and reasonable α-linolenic acid (LNA, 18:3n-3) (10%) (Tocher *et al.*, 2000; Bell *et al.*, 2001, 2003b; Ng *et al.*, 2004; Torstensen *et al.*, 2004). In Atlantic salmon diets, up to 50% of dietary FO can be replaced by VO without a major detrimental effect on the levels of n-3 LC-PUFA and/or the organoleptic characteristics of the flesh (Bell *et al.*, 2002). Some studies have reported some health and welfare issues when replacing FO with a single VO (Montero *et al.*, 2003), and better results have been achieved using a blend of VO, rather than with just one VO source, covering the energy and n-3 LC-PUFA requirements (Torstensen *et al.*, 2005). One option to ensure high levels of n-3 LC-PUFA in the final product in fish fed high levels of dietary VO during the large part of the production cycle is to feed a FO-rich "finishing" diet to supply n-3 LC-PUFA at the end of the production phase prior to harvesting the fish (Bell *et al.*, 2003b; Benedito-Palos *et al.*, 2009).

The study of lipid requirements in fish is important as they provide the FA required for the development of cells, tissues, and normal growth (Sargent *et al.*, 1995b). Lipids represent the principal source of energy for fish (Sargent *et al.*, 1989; Frøyland *et al.*, 1998), particularly SFA and MUFA (Henderson, 1996), but the digestion and absorption of PUFA is generally higher compared with SFA and MUFA (Lie *et al.*, 1987; Olsen *et al.*, 1998). When formulating fish diets, therefore, particular attention should be paid to the energy availability including SFA and MUFA and the ratio of these non-essential energy-

providing FA with the essential PUFA and LC-PUFA (EPA, DHA and ARA) (Bell *et al.*, 2002). The optimal balance of the energy-providing FA and essential FA will be positively reflected in the productive parameters, such as growth, survival, feed conversion efficiency, quality of fillet, and also in fish health, *i.e.* immune competence and disease resistance (Peng *et al.*, 2008).

The two sources of FA for fish, as all vertebrates are: 1) dietary lipid intake, and 2) synthesis *de novo*, using carbon from non-lipid sources, particularly amino acids, as there is a limited availability of carbohydrates in aquatic environments. Fish can synthesise SFA through the action of the enzymes including acetyl-CoA carboxylase and fatty acid synthase, using acetyl-CoA derived from the protein and amino acid catabolism. In salmonids, *de novo* FA synthesis take place mainly in the liver, while adipose tissue is the main tissue for the deposition and storage of excess lipid (FA), either obtained from the diet or endogenously synthesised (Henderson, 1996). Fish can further convert SFA into MUFA via Δ9 or stearoyl-CoA desaturase. However, they cannot synthesise PUFA, which must be obtained from the diet (Henderson, 1996). Some fish species, including salmonids, can endogenously produce LC-PUFA from dietary C_{18} PUFA precursors depending upon their complement of fatty acyl desaturase and elongase enzymes (Tocher, 2003).

Tocher *et al.* (1989) suggested that fish cell culture can show some aspects of lipid metabolism, which also occur in the whole fish, making fish cell lines a potential tool for the study of lipid and FA metabolism. Castell *et al.* (1972) reported that EFA requirements of rainbow trout (*Oncorhynchus mykiss* Walbaum) were satisfied with the supplementation of LNA, whereas marine species, such as turbot (*Scophthalmus maximus* L.), have specific LC-PUFA requirements to satisfy EFA requirements (Owen *et al.*, 1975). These results were later confirmed by Tocher *et al.* (1989) in a study using fish cell lines, showing that the rainbow trout cell line, RTG-2, exhibited desaturation and elongation activities and,

therefore, could convert LOA and LNA into ARA and EPA, respectively. However, these enzymatic activities were not observed in the TF (turbot fin) cell line. In this sense would be ideal to obtain a salmon cell line, which would allow the study of the molecular regulation of lipid and FA metabolism in Atlantic salmon, saving time and money.

A recent study in Atlantic salmon suggested that LC-PUFA synthesis is negatively affected by increased dietary lipid content (Martinez-Rubio *et al.*, 2013). Therefore, the aim of the present Chapter was to investigate how SFA (16:0) and MUFA (18:1n-9) affected the conversion of C_{18} PUFA to LC-PUFA (with particular emphasis on the conversion of LNA to EPA) *in vitro*, using the CHSE-214 cell line.

5.2. Materials and Methods

5.2.1. Cell line and routine culture procedures

The general description of CHSE-214 cell line, the media and the routine culture methodologies are provided in Chapter 2, sections 2.1.1 and 2.1.2.

5.2.2. C_{18} PUFA interaction with SFA and MUFA experiments

FA were supplemented to the CHSE-214 cell line as complexes, bound to bovine serum albumin (BSA) (First Link (UK) Ltd., Wolverhampton, UK) and suspended in phosphate buffered saline (PBS), according to Spector and Hoak (1969). For further details refer to Chapter 2, section 2.2.1. Table 5.1 shows the combinations and concentrations of FA supplemented in the current study. The combinations of PUFA and "competing" SFA and MUFA (mimicking dietary lipid content in *in vivo* trials) are listed in Table 5.1, these were added directly into each flask 24 h after splitting; cells were incubated for further 5 d, and then harvested for lipid and FA analyses, further details are provided in Chapter 2,

section 2.2.2). After the incubation period cells were harvested as described in Chapter 2, section 2.1.2.2 for further lipid analyses.

Table 5.1. Combinations and concentrations of PUFA supplemented to CHSE-214 cells

PUFA (substrate, FA1)	LIPID (competitor, FA2)	COMBINATIONS (FA1/FA2, μM/μM)			
LNA	16:0	20/20	20/40	20/60	20/80
LNA	18:1n-9	20/20	20/40	20/60	20/80
LNA	16:0+18:1n-9 (1:1)	20/20	20/40	20/60	20/80
LOA	16:0	20/20	20/40	20/60	20/80
LOA	18:1n-9	20/20	20/40	20/60	20/80
LOA	16:0+18:1n-9 (1:1)	20/20	20/40	20/60	20/80
EPA	16:0	20/20	20/40	20/60	20/80
EPA	18:1n-9	20/20	20/40	20/60	20/80
EPA	16:0+18:1n-9 (1:1)	20/20	20/40	20/60	20/80
LNA/EPA (1:1)	16:0	40/20	40/40	40/60	
LNA/EPA (1:1)	18:1n-9	40/20	40/40	40/60	
LNA/EPA (1:1)	16:0+18:1n-9 (1:1)	40/20	40/40	40/60	

Abbreviations: LNA = α-linolenic acid; EPA = eicosapentaenoic acid; LOA = linoleic acid; PUFA = polyunsaturated fatty acid; FA = fatty acid.

5.2.3. Lipid analyses

Cellular lipids were extracted according to Folch *et al.* (1957) as described in detail in Chapter 2, section 2.3.1. Lipid class analyses were carried out using high-performance thin-layer chromatography (HPTLC) plates. All details of this procedure are provided in Chapter 2, section 2.3.2. Fatty acid methyl esters (FAME) were prepared by acid-catalysed transmethylation essentially according to Christie (2003). Full details of the FAME preparation, extraction, purification and GC analysis are provided in Chapter 2, section 2.3.3.

5.3. Results

5.3.1. Effects of SFA and MUFA (lipid content) on the metabolism of LNA

5.3.1.1. Supplementation with LNA in presence of increasing levels of 16:0

Table 5.2 shows the lipid content and lipid class composition of CHSE-214 cells after incubation with 20 µM LNA in the presence of increasing concentrations of supplemental 16:0. Supplementation with increasing levels of 16:0 increased lipid content of the cells at concentrations from 20/20 µM to 20/60 µM and then lipid content slightly decreased with the concentrations 20/60 µM to 20/80 µM, although results were not statistically significant (R^2 = 0.197; p = 0.149). Lipid class data showed a clear increase in the proportion of triacylglycerol (TAG) with the graded supplementation of 16:0 (R^2 = 0.968; p = 0.000). The increased TAG was most obviously reflected in concomitant decreased proportions of the main phospholipid classes, PC (R^2 = 0.564; p = 0.005) and particularly PE (R^2 = 0.974; p = 0.000), but the driver to changes in lipid class composition was the increased proportion of TAG.

The FA compositions of CHSE-214 cells incubated with 20 µM LNA in the presence of increasing concentrations of supplemental 16:0 is presented in Table 5.3. EPA levels decreased with the graded supplementation of 16:0 (R^2 = 0.683; p = 0.001). The levels of LNA were relatively unaffected by supplementation with increasing concentrations of 16:0 (R^2 = 0.296; p = 0.068). Interestingly, the proportions of 18:4n-3 increased with the graded supplementation of 16:0 (R^2 = 0.630; p = 0.002), whereas the proportions of 20:4n-3 were negatively affected (R^2 = 0.746; p = 0.000), with the latter possibly simply reflecting the increasing proportions of 16:0 and its metabolites. The CHSE-214 cells incorporated the supplemented 16:0 to some extent although its level was

only slightly increased in the cells (R^2 = 0.557; p = 0.005). However, it was clear that the main fate of incorporated 16:0, other than probable oxidation, was that a substantial amount of the incorporated 16:0 was desaturated to 16:1n-7 (R^2 = 0.987; p = 0.000), which was then elongated to 18:1n-7 as this also increased (R^2 = 0.723; p = 0.000). The levels of 16:1n-9 were also increased with increasing supplementation with 16:0 (R^2 = 0.725; p = 0.000) although the precise metabolic pathway was less obvious. The increments of 16:1n-7, 18:1n-7 and 16:1n-9 were balanced primarily by decreased proportions of 18:1n-9 (R^2 = 0.757; p = 0.000).

Table 5.2. Lipid contents and class compositions of CHSE-214 cells incubated with 20 µM LNA and increasing concentrations of 16:0

Lipid	20/20 µM	20/40 µM	20/60 µM	20/80 µM	R^2	P-value
LC (µg)	256.7 ± 51.3	296.7 ± 64.3	316.7 ± 15.3	310.0 ± 45.8	0.197	0.149
(%)						
PC	24.0 ± 1.4	22.6 ± 0.2	20.9 ± 0.8	21.4 ± 0.8	0.564	0.005
PE	21.3 ± 0.1	19.6 ± 0.3	18.5 ± 0.2	16.6 ± 0.3	0.974	0.000
PS	6.9 ± 0.3	5.5 ± 0.3	5.1 ± 0.6	5.5 ± 0.2	0.441	0.018
PI	10.6 ± 0.8	10.4 ± 0.4	9.8 ± 0.6	8.5 ± 0.6	0.645	0.002
PA/CL	0.9 ± 0.1	1.6 ± 0.4	0.9 ± 0.1	0.9 ± 0.1	0.051	0.482
SM	7.6 ± 0.9	6.5 ± 0.1	6.1 ± 0.3	3.3 ± 0.4	0.790	0.000
TP	71.3 ± 0.4	66.2 ± 0.7	61.3 ± 0.8	56.2 ± 0.7	0.991	0.000
TN	28.7 ± 0.4	33.8 ± 0.7	38.7 ± 0.8	43.8 ± 0.7	0.991	0.000
TAG	3.5 ± 0.4	7.3 ± 0.4	11.7 ± 0.8	16.4 ± 1.6	0.968	0.000
CHOL	22.5 ± 0.8	25.0 ± 0.4	25.5 ± 0.6	26.5 ± 0.9	0.770	0.000
FFA	2.7 ± 0.6	1.5 ± 0.1	1.5 ± 0.2	0.9 ± 0.1	0.760	0.000

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LNA = α-linolenic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

Table 5.3. Fatty acid composition (%) of CHSE-214 cells incubated with 20 µM LNA and increasing concentrations of 16:0

Fatty acid	20/20 µM	20/40 µM	20/60 µM	20/80 µM	R^2	P-value
14:0	0.9 ± 0.2	1.6 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.086	0.355
15:0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.190	0.156
16:0	12.4 ± 1.5	14.7 ± 0.5	14.4 ± 0.8	15.6 ± 0.2	0.557	0.005
17:0	0.6 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.661	0.001
18:0	7.6 ± 0.5	8.0 ± 1.1	6.8 ± 0.5	6.0 ± 0.4	0.503	0.010
22:0	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.181	0.168
Σ SFA	**22.0 ± 2.2**	**25.3 ± 1.5**	**22.9 ± 1.2**	**23.4 ± 0.7**	**0.013**	**0.722**
16:1n-9	2.7 ± 0.1	2.7 ± 0.2	3.1 ± 0.1	4.0 ± 0.4	0.725	0.000
16:1n-7	2.5 ± 0.1	4.3 ± 0.3	6.5 ± 0.6	9.2 ± 0.1	0.987	0.000
18:1n-9	34.4 ± 2.1	34.2 ± 0.5	30.7 ± 1.6	28.6 ± 0.5	0.757	0.000
18:1n-7	2.3 ± 0.2	2.6 ± 0.1	3.0 ± 0.3	3.0 ± 0.1	0.723	0.000
24:1n-9	0.9 ± 0.0	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.299	0.066
Σ MUFA	**42.8 ± 2.0**	**44.5 ± 0.3**	**44.0 ± 1.1**	**45.5 ± 0.3**	**0.411**	**0.025**
18:2n-6	3.5 ± 0.1	3.4 ± 0.0	3.4 ± 0.0	3.2 ± 0.1	0.721	0.000
18:3n-6	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	0.019	0.666
20:2n-6*	0.5 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.273	0.082
20:3n-6	1.7 ± 0.0	1.4 ± 0.2	1.5 ± 0.1	1.4 ± 0.1	0.348	0.043
20:4n-6	1.8 ± 0.2	1.5 ± 0.3	1.6 ± 0.2	1.6 ± 0.1	0.041	0.529
Σ n-6 PUFA	**8.2 ± 0.2**	**7.2 ± 0.5**	**7.5 ± 0.1**	**7.3 ± 0.3**	**0.349**	**0.043**
18:3n-3	7.5 ± 0.7	6.6 ± 0.1	8.4 ± 1.1	8.4 ± 0.1	0.296	0.068
18:4n-3	4.1 ± 0.3	3.7 ± 0.1	5.5 ± 0.9	5.8 ± 0.1	0.630	0.002
20:4n-3	3.2 ± 0.8	1.9 ± 0.2	1.9 ± 0.2	1.2 ± 0.1	0.746	0.000
20:5n-3	3.7 ± 0.2	2.5 ± 0.3	2.7 ± 0.2	2.0 ± 0.2	0.683	0.001
22:5n-3	1.0 ± 0.0	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.532	0.007
22:6n-3	1.3 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	0.277	0.079
Σ n-3 PUFA	**20.8 ± 1.2**	**16.5 ± 0.9**	**20.5 ± 2.0**	**19.2 ± 0.7**	**0.001**	**0.924**
18:2n-9	3.5 ± 0.3	3.8 ± 0.2	3.1 ± 0.2	2.9 ± 0.0	0.574	0.004
20:2n-9	2.7 ± 0.3	2.7 ± 0.1	2.0 ± 0.4	1.7 ± 0.1	0.693	0.001
Σ n-9 PUFA	**6.2 ± 0.6**	**6.5 ± 0.3**	**5.1 ± 0.6**	**4.6 ± 0.1**	**0.654**	**0.001**
Σ PUFA	**35.2 ± 1.0**	**30.2 ± 1.2**	**33.1 ± 1.6**	**31.1 ± 0.8**	**0.237**	**0.109**

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LNA = α-linolenic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; *contains 20:3n-9.

5.3.1.2. Supplementation with LNA in presence of increasing levels of 18:1n-9

Table 5.4 shows the lipid contents and lipid class compositions of CHSE-214 cells incubated with 20 µM LNA and increasing levels of 18:1n-9. Cell total lipid content did not show a clear trend with increasing concentrations of supplemented 18:1n-9 (R^2 = 0.091; p = 0.341). The lipid class data showed an increased proportion of TAG with the graded supplementation of 18:1n-9 (R^2 = 0.713; p = 0.001). This increment was balanced by decreasing proportions of most polar lipid classes but especially the main phospholipid class, PC (R^2 = 0.671; p = 0.001).

Table 5.4. Lipid contents and class compositions of CHSE-214 cells incubated with 20 µM LNA and increasing concentrations of 18:1n-9

Lipid	20/20 µM	20/40 µM	20/60 µM	20/80 µM	R^2	P-value
LC (µg)	470.0 ± 70.0	436.7 ± 65.1	506.7 ± 83.3	376.7 ± 81.4	0.091	0.341
CC (%)						
PC	22.6 ± 0.0	20.0 ± 1.4	18.2 ± 0.9	18.6 ± 0.7	0.671	0.001
PE	21.2 ± 0.3	19.0 ± 0.5	19.8 ± 0.3	19.5 ± 0.5	0.294	0.068
PS	6.2 ± 0.2	5.1 ± 0.2	4.9 ± 0.3	5.0 ± 0.1	0.544	0.006
PI	10.3 ± 0.2	9.0 ± 0.2	8.9 ± 0.5	8.8 ± 0.1	0.611	0.003
PA/CL	0.9 ± 0.0	1.0 ± 0.3	1.1 ± 0.1	1.1 ± 0.0	0.306	0.062
SM	4.1 ± 1.2	4.1 ± 0.4	2.4 ± 0.2	2.6 ± 0.4	0.500	0.010
TP	65.3 ± 0.5	58.2 ± 1.8	55.3 ± 1.8	55.6 ± 1.2	0.722	0.000
TN	34.7 ± 0.5	41.8 ± 1.8	44.7 ± 1.8	44.4 ± 1.2	0.722	0.000
TAG	8.7 ± 0.1	14.3 ± 2.1	15.2 ± 1.3	16.2 ± 1.1	0.713	0.001
CHOL	23.9 ± 0.2	24.9 ± 0.5	27.3 ± 0.9	25.7 ± 0.4	0.455	0.016
FFA	2.1 ± 0.3	2.6 ± 0.3	2.2 ± 0.5	2.5 ± 0.3	0.031	0.585

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LNA = α-linolenic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA composition of CHSE-214 incubated with 20 µM of LNA and graded concentrations of 18:1n-9 is presented in Table 5.5. The rank order was MUFA > PUFA > SFA in all treatments. EPA levels were unaffected by the graded supplementation of 18:1n-9 ($R^2 = 0.052$; $p = 0.476$), and at all concentrations of 18:1n-9 the levels of EPA recorded were lower than 2% of total FA. The incorporation of LNA was similar at all concentrations supplemented ($R^2 = 0.240$; $p = 0.106$) and no effects were observed in the n-3 PUFA intermediates, being essentially the same at all concentrations. However, total n-3 PUFA was slightly higher in cells given the 20/20 µM combination. The 18:1n-9 was incorporated with the graded supplementation of the FA ($R^2 = 0.539$; $p = 0.040$) and a small amount was further desaturated to 18:2n-9 ($R^2 = 0.382$; $p = 0.032$). The increment of 18:1n-9 was primarily balanced by decreased proportions of total SFA.

Table 5.5. Fatty acid compositions (%) of CHSE-214 cells incubated with 20 µM LNA and increasing concentrations of 18:1n-9

Fatty acid	20/20 µM	20/40 µM	20/60 µM	20/80 µM	R^2	P-value
14:0	1.1 ± 0.2	1.1 ± 0.2	1.0 ± 0.1	1.1 ± 0.1	0.002	0.903
15:0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.1	0.011	0.745
16:0	10.9 ± 0.7	10.4 ± 1.0	10.5 ± 0.3	9.9 ± 0.2	0.263	0.088
17:0	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.3	0.075	0.388
18:0	7.8 ± 0.5	7.0 ± 0.8	7.4 ± 0.3	6.5 ± 0.2	0.396	0.028
20:0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.0	0.370	0.036
22:0	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.253	0.095
Σ SFA	**21.1 ± 1.5**	**19.8 ± 1.7**	**20.4 ± 0.9**	**18.8 ± 0.7**	**0.259**	**0.091**
16:1n-9	2.2 ± 0.9	3.1 ± 0.1	3.1 ± 0.1	3.2 ± 0.2	0.271	0.083
16:1n-7	1.3 ± 0.2	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.2	0.072	0.400
18:1n-9	44.0 ± 0.9	47.6 ± 0.3	46.7 ± 2.1	47.5 ± 1.2	0.359	0.040
18:1n-7	1.6 ± 0.1	1.6 ± 0.2	1.6 ± 0.4	1.2 ± 0.0	0.240	0.106
24:1n-9	0.9 ± 0.1	0.8 ± 0.1	0.7 ± 0.0	0.9 ± 0.0	0.050	0.483
Σ MUFA	**50.0 ± 0.2**	**54.2 ± 0.3**	**53.4 ± 2.4**	**54.0 ± 1.3**	**0.373**	**0.035**
18:2n-6	3.3 ± 0.2	3.0 ± 0.1	3.0 ± 0.2	2.9 ± 0.2	0.421	0.022
18:3n-6	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.2	0.4 ± 0.0	0.001	0.916
20:2n-6*	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.064	0.428
20:3n-6	1.4 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.0	0.118	0.273
20:4n-6	1.3 ± 0.1	1.1 ± 0.1	1.2 ± 0.2	1.3 ± 0.1	0.006	0.811
22:5n-6	0.6 ± 0.1	0.8 ±0.1	0.6 ± 0.2	0.6 ± 0.1	0.093	0.335
Σ n-6 PUFA	**7.5 ± 0.4**	**7.0 ± 0.5**	**6.8 ± 0.2**	**6.9 ± 0.1**	**0.318**	**0.056**
18:3n-3	6.8 ± 0.6	6.3 ± 0.4	6.0 ± 0.7	6.1 ± 0.3	0.240	0.106
18:4n-3	1.9 ± 0.3	2.0 ± 0.2	1.8 ± 0.3	2.0 ± 0.1	0.002	0.879
20:3n-3	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.187	0.160
20:4n-3	3.0 ± 0.2	2.3 ± 0.2	2.5 ± 0.4	2.4 ± 0.2	0.190	0.157
20:5n-3	1.9 ± 0.2	1.6 ± 0.2	1.7 ± 0.2	1.7 ± 0.1	0.052	0.476
22:5n-3	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.067	0.417
22:6n-3	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.067	0.418
Σ n-3 PUFA	**15.6 ± 1.4**	**13.8 ± 1.2**	**13.7 ± 1.9**	**14.2 ± 0.7**	**0.142**	**0.227**
18:2n-9	2.8 ± 0.0	2.8 ± 0.1	2.8 ± 0.0	3.1 ± 0.3	0.382	0.032
20:2n-9	3.0 ± 0.2	2.5 ± 0.1	2.9 ± 0.2	3.0 ± 0.2	0.068	0.413
Σ n-9 PUFA	**5.8 ± 0.2**	**5.3 ± 0.2**	**5.7 ± 0.2**	**6.1 ± 0.4**	**0.218**	**0.126**
Σ PUFA	**28.9 ± 1.7**	**26.0 ± 1.9**	**26.2 ± 2.1**	**27.2 ± 0.6**	**0.095**	**0.328**

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LNA = α-linolenic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; *contains 20:3n-9.

5.3.1.3. Supplementation with LNA in presence of increasing levels of a 1:1 mix of 16:0 and 18:1n-9 combined

Table 5.6 shows the lipid content and lipid class composition of CHSE-214 cells incubated with 20 µM LNA and graded increased concentrations of a 1:1 mix of 16:0 + 18:1n-9 combined. The cell total lipid content decreased at concentrations 20/20 µM to 20/40 µM, but then at concentrations 20/40–20/80 µM showed a trend to increase with

graded supplementation of 16:0+18:1n-9, but data were not statistically significant (R^2 = 0.144; p = 0.225). There were no clear trends observed in the lipid class composition data. TAG levels were higher than 15% in all treatments, and no increasing trend with the graded supplementation of the FA was observed (R^2 = 0.042; p = 0.525).

Table 5.6. Lipid contents and class compositions of CHSE-214 cells incubated with 20 µM LNA and increasing concentrations of a 1:1 mix of 16:0 and 18:1n-9 combined

Lipid	20/20 µM	20/40 µM	20/60 µM	20/80 µM	R^2	P-value
LC (µg)	330.0 ± 25.7	226.7 ± 5.8	263.3 ± 20.8	266.7 ± 20.8	0.144	0.225
CC (%)						
PC	15.2 ± 1.7	15.9 ± 2.3	15.7 ± 3.3	21.9 ± 0.9	0.440	0.019
PE	21.5 ± 1.3	22.1 ± 2.6	19.0 ± 1.8	17.1 ± 0.6	0.567	0.005
PS	4.5 ± 0.5	4.3 ± 0.9	5.8 ± 0.4	5.3 ± 0.1	0.336	0.048
PI	7.7 ± 1.0	8.7 ± 0.2	10.7 ± 0.9	9.3 ± 0.2	0.377	0.034
PA/CL	3.1 ± 0.4	3.1 ± 0.8	1.3 ± 0.3	1.0 ± 0.0	0.765	0.000
SM	3.5 ± 0.2	3.8 ± 0.3	4.5 ± 0.4	3.3 ± 0.3	0.003	0.874
TP	55.5 ± 1.6	57.9 ± 3.1	57.1 ± 1.2	57.9 ± 1.7	0.135	0.240
TN	44.5 ± 1.6	42.1 ± 3.1	42.9 ± 1.2	42.1 ± 1.7	0.135	0.240
TAG	16.4 ± 2.2	16.6 ± 2.1	15.6 ± 0.8	15.8 ± 1.4	0.042	0.525
CHOL	26.4 ± 0.5	23.9 ± 1.4	25.7 ± 0.5	25.2 ± 0.4	0.032	0.581
FFA	1.7 ± 0.2	1.6 ± 0.2	1.6 ± 0.2	1.1 ± 0.1	0.524	0.008

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid content is given in µg. Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis ($p < 0.05$). LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA composition of CHSE-214 cells incubated with 20 µM of LNA and graded concentrations of 16:0+18:1n-9 is presented in Table 5.7. The proportions of EPA decreased with the graded supplementation of 16:0+18:1n-9 (R^2 = 0.870; p = 0.000). The percentage of LNA did not show a clear trend (R^2 = 0.379; p = 0.033) whereas 18:4n-3 increased (R^2 = 0.819; p = 0.000), and 20:4n-3 (R^2 = 0.873; p = 0.000) decreased with the graded supplementation of the mix 16:0+18:1n-9.

Table 5.7. Fatty acid compositions (%) of CHSE-214 cells incubated with 20 µM LNA and increasing concentrations of a 1:1 mix of 16:0 and 18:1n-9 combined

Fatty acid	20/20 µM	20/40 µM	20/60 µM	20/80 µM	R^2	P-value
14:0	1.1 ± 0.2	1.4 ± 0.2	1.1 ± 0.1	1.0 ± 0.1	0.134	0.243
15:0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.412	0.024
16:0	11.6 ± 0.4	12.7 ± 1.2	12.3 ± 0.1	12.4 ± 0.3	0.110	0.292
17:0	0.8 ± 0.0	0.3 ± 0.0	0.7 ± 0.1	0.4 ± 0.0	0.101	0.314
18:0	7.3 ± 0.2	6.8 ± 0.1	5.8 ± 0.3	5.4 ± 0.1	0.923	0.000
20:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.344	0.045
22:0	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.654	0.001
Σ SFA	**21.8 ± 0.5**	**22.2 ± 1.3**	**20.6 ± 0.5**	**19.9 ± 0.5**	**0.546**	**0.006**
16:1n-9	3.2 ±0.2	3.7 ± 0.1	4.1 ± 0.2	4.0 ± 0.1	0.654	0.001
16:1n-7	1.9 ± 0.2	2.5 ± 0.4	2.7 ± 0.2	3.0 ± 0.1	0.740	0.000
18:1n-9	38.8 ± 1.0	40.2 ± 0.5	41.4 ± 0.7	42.1 ± 0.4	0.811	0.000
18:1n-7	2.0 ± 0.1	2.2 ± 0.2	1.7 ± 0.3	1.8 ± 0.1	0.235	0.110
24:1n-9	0.8 ± 0.1	0.8 ± 0.0	0.7 ± 0.1	0.6 ± 0.0	0.685	0.001
Σ MUFA	**46.7 ± 1.2**	**49.4 ± 0.2**	**50.6 ± 0.5**	**51.5 ± 0.3**	**0.856**	**0.000**
18:2n-6	3.5 ± 0.1	3.0 ± 0.2	3.1 ± 0.1	3.1 ± 0.1	0.174	0.177
18:3n-6	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.561	0.005
20:2n-6*	0.6 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.644	0.002
20:3n-6	1.5 ± 0.1	1.3 ± 0.1	1.2 ± 0.0	1.1 ± 0.0	0.821	0.000
20:4n-6	1.3 ± 0.2	1.4 ± 0.2	1.2 ± 0.1	1.2 ± 0.0	0.114	0.284
22:5n-6	0.7 ± 0.1	0.5 ± 0.1	0.2 ± 0.0	0.6 ± 0.1	0.107	0.299
Σ n-6 PUFA	**8.1 ± 0.2**	**7.1 ± 0.5**	**6.6 ± 0.1**	**6.9 ± 0.1**	**0.555**	**0.005**
18:3n-3	5.4 ± 0.4	4.9 ± 0.3	6.5 ± 0.3	6.1 ± 0.3	0.379	0.033
18:4n-3	3.3 ± 0.2	3.4 ± 0.1	4.1 ± 0.4	4.6 ± 0.2	0.819	0.000
20:4n-3	2.5 ± 0.2	1.8 ± 0.2	1.5 ± 0.1	1.4 ± 0.1	0.873	0.000
20:5n-3	3.6 ± 0.3	3.0 ± 0.2	2.3 ± 0.1	2.2 ± 0.2	0.870	0.000
22:5n-3	0.9 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.556	0.005
22:6n-3	1.1 ± 0.0	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.386	0.031
Σ n-3 PUFA	**16.8 ± 1.0**	**14.8 ± 1.0**	**16.1 ± 0.2**	**16.0 ± 0.7**	**0.027**	**0.612**
18:2n-9	3.6 ± 0.1	3.7 ± 0.1	3.8 ± 0.2	3.6 ± 0.1	0.078	0.380
20:2n-9	3.0 ± 0.1	2.8 ± 0.2	2.3 ± 0.2	2.1 ± 0.1	0.880	0.000
Σ n-9 PUFA	**6.6 ± 0.1**	**6.5 ± 0.3**	**6.1 ± 0.2**	**5.7 ± 0.2**	**0.766**	**0.000**
Σ PUFA	**31.5 ± 1.3**	**28.4 ± 1.5**	**28.8 ± 0.3**	**28.6 ± 0.7**	**0.375**	**0.034**

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LNA = α-linolenic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; *contains 20:3n-9.

The proportions of 22:5n-3 and DHA were detected in levels lower than 1.2%, being similar at all concentrations of supplemented 16:0+18:1n-9. Cellular percentages of 18:1n-9 increased with the graded supplementation of the FA ($R^2 = 0.811$; $p = 0.000$) and similar levels of 16:0 were observed at all concentrations ($R^2 = 0.110$; $p = 0.292$). The main product of desaturation of 16:0, *i.e.*16:1n-7, increased with the graded supplementation of the mix 16:0+18:1n-9 ($R^2 = 0.740$; $p = 0.000$).

5.3.2. Effects of SFA and MUFA (lipid content) on the metabolism of LOA

5.3.2.1. Supplementation with LOA in presence of increasing 16:0

Table 5.8 shows the lipid content and the lipid class composition of CHSE-214 cells after being incubated with 20 μM of LOA and graded increased concentrations of 16:0. The cell lipid content decreased with the graded supplementation of 16:0 (R^2 = 0.429; p = 0.021). In the lipid class data, TAG increased with the graded supplementation of the FA (R^2 = 0.685; p = 0.001), the increment in TAG was mainly balanced by decreased cholesterol.

Table 5.8. Lipid contents and class compositions of CHSE-214 cells incubated with 20 μM LOA and increasing concentrations of 16:0

Lipid	20/20 μM	20/40 μM	20/60 μM	20/80 μM	R^2	P-value
LC (μg)	306.7 ± 35.1	250.0 ± 45.8	243.3 ± 37.9	230.0 ± 17.3	0.429	0.021
CC (%)						
PC	13.1 ± 0.4	18.0 ± 1.2	13.3 ± 0.3	13.2 ± 1.0	0.050	0.485
PE	23.7 ± 1.2	19.3 ± 1.7	28.3 ± 0.6	28.4 ± 0.5	0.426	0.021
PS	5.1 ± 0.1	4.1 ± 0.7	4.2 ± 0.6	5.2 ± 0.8	0.001	0.909
PI	4.8 ± 0.6	6.9 ± 0.2	5.9 ± 0.3	5.7 ± 0.0	0.086	0.355
PA/CL	2.3 ± 0.2	2.0 ± 0.2	1.9 ± 0.4	1.9 ± 0.7	0.182	0.167
SM	1.9 ± 0.3	1.6 ± 0.1	2.5 ± 0.2	3.8 ± 0.3	0.705	0.001
TP	50.9 ± 2.1	51.9 ± 1.8	56.1 ± 1.0	58.2 ± 0.6	0.807	0.000
TN	49.1± 2.1	48.1 ± 1.8	43.9 ± 1.0	41.8 ± 0.6	0.807	0.000
TAG	6.6 ± 1.5	6.8 ± 0.3	11.4 ± 1.4	12.5 ± 0.2	0.685	0.001
CHOL	41.3 ± 1.9	39.9 ± 1.8	31.5 ± 0.6	28.3 ± 0.6	0.846	0.000
FFA	1.2 ± 0.2	1.4 ± 0.5	1.0 ± 0.1	1.0 ± 0.2	0.082	0.367

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

Table 5.9 shows the FA profile of CHSE-214 cells after being incubated with 20 μM of LOA and graded increased concentrations of 16:0. The proportions of ARA slightly decreased with the graded supplementation of 16:0, but data were not statistically

significant (R^2 = 0.270; p = 0.083). The proportions of LOA, 18:3n-6 and 20:3n-6 decreased with the graded supplementation of 16:0 (R^2 = 0.819; p = 0.000, R^2 = 0.924; p = 0.000 and R^2 = 0.793; p = 0.000, respectively). Cellular percentages of 16:0 increased with the graded supplementation of the FA itself, some of which was further desaturated to 16:1n-9 (R^2 = 0.876; p = 0.000) and 16:1n-7 (R^2 = 0.972; p = 0.000); both FA increased with the graded supplementation of 16:0. The increment in total SFA was mainly balanced by decreased percentages of n-6 PUFA (R^2 = 0.876; p = 0.000).

Table 5.9. Fatty acid compositions (%) of CHSE-214 cells incubated with 20 µM LOA and increasing concentrations of 16:0

Fatty acid	20/20 µM	20/40 µM	20/60 µM	20/80 µM	R^2	P-value
14:0	0.6 ± 0.1	0.7 ± 0.1	0.9 ± 0.0	0.8 ± 0.1	0.404	0.026
15:0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.000	0.951
16:0	13.1 ± 0.8	15.3 ± 0.4	22.1 ± 0.3	22.3 ± 0.8	0.883	0.000
17:0	0.8 ± 0.0	0.9 ± 0.0	1.2 ± 0.1	1.1 ± 0.0	0.767	0.000
18:0	7.9 ± 0.2	8.0 ± 0.6	6.6 ± 0.6	6.0 ± 0.3	0.723	0.000
22:0	0.4 ± 0.1	0.5 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.286	0.073
Σ SFA	**23.1 ± 1.1**	**25.7 ± 0.7**	**31.4 ± 0.4**	**30.8 ± 1.1**	**0.824**	**0.000**
16:1n-9	2.7 ± 0.2	3.2 ± 0.2	4.0 ± 0.3	4.1 ± 0.0	0.876	0.000
16:1n-7	2.4 ± 0.2	4.5 ± 0.6	7.7 ± 0.1	10.0 ± 0.7	0.972	0.000
18:1n-9	32.5 ± 0.5	30.0 ± 0.8	26.9 ± 0.5	25.9 ± 0.7	0.930	0.000
18:1n-7	2.6 ± 0.2	2.6 ± 0.1	3.1 ± 0.1	2.6 ± 0.0	0.081	0.368
24:1n-9	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	0.5 ± 0.1	0.115	0.281
Σ MUFA	**40.8 ± 1.1**	**40.9 ± 0.6**	**42.3 ± 0.6**	**43.1 ± 0.3**	**0.657**	**0.001**
18:2n-6	16.6 ± 0.7	15.9 ± 0.7	13.8 ± 0.6	12.9 ± 0.9	0.819	0.000
18:3n-6	3.9 ± 0.3	3.3 ± 0.3	2.3 ± 0.1	1.8 ± 0.3	0.924	0.000
20:2n-6*	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.397	0.028
20:3n-6	4.4 ± 0.4	3.5 ± 0.2	2.0 ± 0.2	2.2 ± 0.4	0.793	0.000
20:4n-6	2.0 ± 0.2	1.9 ± 0.2	1.5 ± 0.3	1.6 ± 0.2	0.270	0.083
Σ n-6 PUFA	**27.4 ± 1.9**	**25.1 ± 0.7**	**20.1 ± 0.8**	**19.0 ± 1.1**	**0.876**	**0.000**
20:5n-3	0.7 ± 0.1	0.7 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.417	0.023
22:5n-3	0.7 ± 0.1	0.7 ± 0.0	0.5 ± 0.1	0.7 ± 0.1	0.168	0.186
22:6n-3	1.3 ± 0.2	1.2 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	0.255	0.094
Σ n-3 PUFA	**2.7 ± 0.4**	**2.6 ± 0.1**	**2.0 ± 0.1**	**2.4 ± 0.1**	**0.327**	**0.052**
18:2n-9	3.5 ± 0.3	3.5 ± 0.3	2.8 ± 0.1	3.0 ± 0.3	0.396	0.028
20:2n-9	2.5 ± 0.2	2.2 ± 0.3	1.4 ± 0.2	1.7 ± 0.3	0.554	0.005
Σ n-9 PUFA	**6.0 ± 0.5**	**5.7 ± 0.6**	**4.2 ± 0.3**	**4.7 ± 0.5**	**0.531**	**0.007**
Σ PUFA	**36.1 ± 2.2**	**33.4 ± 0.4**	**26.3 ± 1.0**	**26.1 ± 1.0**	**0.849**	**0.000**

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LOA = linoleic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; *contains 20:3n-9.

5.3.2.2. Supplementation with LOA in presence of increasing 18:1n-9

Table 5.10 shows the lipid content and the lipid class composition of CHSE-214 cells after being incubated with 20 µM of LOA in presence of graded increased concentrations of 18:1n-9. No clear trend was observed in cell lipid content (R^2 = 0.040; p = 0.533). Apart from increased proportions of TAG with the graded supplementation of the 18:1n-9 (R^2 = 0.934; p = 0.000), no other clear trends were observed in the lipid class data. The increment in TAG was balanced by decreasing proportions of cholesterol (R^2 = 0.828; p = 0.000).

Table 5.10. Lipid content and lipid class composition of CHSE-214 cells incubated with 20 µM LOA and increasing concentrations of 18:1n-9

Lipid	20/20 µM	20/40 µM	20/60 µM	20/80 µM	R^2	P-value
LC (µg)	300.0 ± 26.5	280.0 ± 60.8	286.7 ± 49.3	320.0 ± 10.0	0.040	0.533
CC (%)						
PC	19.1 ± 1.2	19.8 ± 1.4	19.2 ± 1.7	18.2 ± 1.0	0.078	0.379
PE	22.1 ± 0.0	20.2 ± 1.2	20.2 ± 0.4	20.1 ± 0.7	0.406	0.026
PS	7.0 ± 0.7	7.0 ± 0.4	7.1 ± 0.2	6.9 ± 0.6	0.007	0.792
PI	8.6 ± 1.1	9.2 ± 0.8	8.5 ± 0.2	8.4 ± 0.7	0.045	0.507
PA/CL	2.4 ± 0.2	1.8 ± 0.1	2.6 ± 0.1	2.8 ± 0.1	0.333	0.050
SM	2.7 ± 0.5	4.6 ± 0.2	4.0 ± 0.1	3.1 ± 0.2	0.008	0.776
TP	61.9 ± 0.4	62.6 ± 1.1	61.6 ± 1.5	59.5 ± 0.8	0.419	0.023
TN	38.1 ± 0.4	37.4 ± 1.1	38.4 ± 1.5	40.5 ± 0.8	0.419	0.023
TAG	4.7 ± 0.5	7.4 ± 0.4	11.6 ± 1.0	13.4 ± 1.4	0.934	0.000
CHOL	32.4 ± 0.9	28.8 ± 1.0	26.1 ± 0.5	26.0 ± 0.7	0.828	0.000
FFA	1.0 ± 0.1	1.2 ± 0.1	0.7 ± 0.0	1.1 ± 0.3	0.016	0.694

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LOA = linoleic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

Table 5.11 shows the FA composition of CHSE-214 cells after being incubated with 20 µM of LOA and graded increased concentrations of 18:1n-9. The percentage of ARA was unaffected with the graded supplementation of 18:1n-9 (R^2 = 0.005; p = 0.829). Similar levels of LOA and 18:3n-6 were recorded in all treatments, without showing any

clear trends (R^2 = 0.130; p = 0.250 and R^2 = 0.201; p = 0.144, respectively), whereas the percentage of 20:3n-6 decreased with graded supplementation of 18:1n-9 (R^2 = 0.782; p = 0.000). The 18:1n-9 itself was incorporated with the graded supplementation of the FA (R^2 = 0.900; p = 0.000), and some was desaturated to 18:2n-9 (R^2 = 0.857; p = 0.001). The increment of total MUFA was primarily balanced by decreased proportions SFA (R^2 = 0.873; p = 0.000).

Table 5.11. Fatty acid compositions (%) of CHSE-214 cells incubated with 20 µM LOA and increasing concentrations of 18:1n-9

Fatty acid	20/20 µM	20/40 µM	20/60 µM	20/80 µM	R^2	P-value
14:0	0.9 ± 0.0	0.9 ± 0.1	0.8 ± 0.0	0.8 ± 0.0	0.577	0.004
15:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.158	0.200
16:0	13.2 ± 0.7	11.2 ± 0.5	8.7 ± 0.5	7.7 ± 0.5	0.934	0.000
17:0	0.7 ± 0.1	0.7 ± 0.1	0.5 ± 0.0	0.6 ± 0.0	0.400	0.027
18:0	7.8 ± 0.6	6.3 ± 0.6	5.1 ± 0.5	4.7 ± 0.5	0.846	0.000
22:0	0.3 ± 0.0	0.8 ± 0.1	0.9 ± 0.1	1.4 ± 0.0	0.884	0.000
Σ SFA	**23.1 ± 1.3**	**20.1 ± 1.2**	**16.2 ± 1.0**	**15.4 ± 0.7**	**0.873**	**0.000**
16:1n-9	3.5 ± 0.1	4.2 ± 0.1	4.4 ± 0.1	4.3 ± 0.4	0.505	0.010
16:1n-7	1.9 ± 0.2	1.6 ± 0.0	1.2 ± 0.1	1.2 ± 0.2	0.828	0.000
18:1n-9	36.2 ± 0.8	41.6 ± 0.4	46.1 ± 0.2	46.7 ± 0.1	0.900	0.000
18:1n-7	2.2 ± 0.2	2.4 ± 0.2	1.9 ± 0.0	1.9 ± 0.2	0.366	0.037
24:1n-9	0.6 ± 0.0	0.7 ± 0.1	0.5 ± 0.0	0.7 ± 0.1	0.114	0.282
Σ MUFA	**44.4 ± 0.8**	**50.5 ± 0.6**	**54.1 ± 0.2**	**54.8 ± 0.3**	**0.873**	**0.000**
18:2n-6	10.4 ± 0.5	9.1 ± 0.3	9.0 ± 0.4	9.6 ± 1.2	0.130	0.250
18:3n-6	3.2 ± 0.2	2.8 ± 0.1	3.1 ± 0.3	2.7 ± 0.5	0.201	0.144
20:2n-6*	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.0	0.5 ± 0.1	0.451	0.017
20:3n-6	4.1 ± 0.2	3.0 ± 0.0	2.6 ± 0.0	2.6 ± 0.2	0.782	0.000
20:4n-6	2.9 ± 0.4	2.4 ± 0.2	2.6 ± 0.1	2.8 ± 0.2	0.005	0.829
22:5n-6	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.440	0.019
Σ n-6 PUFA	**21.4 ± 1.1**	**18.1 ± 0.4**	**18.0 ± 0.8**	**18.4 ± 1.2**	**0.415**	**0.024**
20:5n-3	1.0 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.298	0.066
22:5n-3	0.9 ± 0.0	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.0	0.555	0.005
22:6n-3	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	0.445	0.018
Σ n-3 PUFA	**3.3 ± 0.1**	**2.8 ± 0.3**	**2.9 ± 0.2**	**2.7 ± 0.1**	**0.542**	**0.006**
18:2n-9	4.6 ± 0.1	5.5 ± 0.2	6.0 ± 0.2	6.1 ± 0.2	0.857	0.000
20:2n-9	2.7 ± 0.1	2.6 ± 0.0	2.2 ± 0.1	2.1 ± 0.3	0.719	0.000
22:2n-9	0.5 ± 0.0	0.4 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	0.069	0.410
Σ n-9 PUFA	**7.8 ± 0.2**	**8.5 ± 0.2**	**8.8 ± 0.1**	**8.7 ± 0.5**	**0.546**	**0.006**
Σ PUFA	**32.5 ± 0.8**	**29.4 ± 0.7**	**29.7 ± 1.1**	**29.8 ± 0.7**	**0.391**	**0.030**

Footnotes: Results are expressed as means ± SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LOA = linoleic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; ND = not detected; *contains 20:3n-9.

5.3.2.3. Supplementation with LOA in presence of increasing 16:0 and 18:1n-9

Table 5.12 shows the lipid content and the lipid class composition of CHSE-214 cells after being incubated with 20 µM of LOA and increasing concentrations of 16:0+18:1n-9 mix (1:1). The cell lipid content did not show a clear trend and the results were not statistically significant (R^2 = 0.010; p = 0.754). Lipid class data showed clear trends in the proportions of TAG (R^2 = 0.541; p = 0.006) and PE (R^2 = 0.687; p = 0.001), which both increased with the graded supplementation of 16:0+18:1n-9 mix. These increments were balanced by decreased cholesterol (R^2 = 0.555; p = 0.005) and polar lipids, such as PI (R^2 = 0.688; p = 0.001), and sphingomyelin (R^2 = 0.650; p = 0.002).

Table 5.12. Lipid content and lipid class composition of CHSE-214 cells incubated with 20 µM LOA and increasing concentrations of a 1:1 mix of 16:0 and 18:1n-9 combined

Lipid	20/20 µM	20/40 µM	20/60 µM	20/80 µM	R^2	P-value
LC (µg)	316.7 ± 15.3	320.0 ± 17.3	410.0 ± 80.0	303.3 ± 25.2	0.010	0.754
CC (%)						
PC	18.4 ± 0.3	19.7 ± 1.0	16.6 ± 1.1	17.6 ± 0.8	0.206	0.138
PE	20.9 ± 0.6	21.3 ± 0.8	27.3 ± 0.4	25.9 ± 0.1	0.687	0.001
PS	7.5 ± 0.4	7.1 ± 0.3	6.3 ± 0.3	7.0 ± 0.6	0.159	0.199
PI	8.4 ± 0.4	9.3 ± 0.2	7.5 ± 0.2	4.3 ± 0.2	0.688	0.001
PA/CL	2.5 ± 0.1	1.7 ± 0.1	1.5 ± 0.2	1.3 ± 0.1	0.378	0.033
SM	2.6 ± 0.5	2.2 ± 0.2	2.8 ± 0.1	1.5 ± 0.0	0.650	0.002
TP	60.3 ± 0.9	61.3 ± 1.2	62.0 ± 1.2	57.6 ± 0.7	0.205	0.139
TN	39.7 ± 0.9	38.7 ± 1.2	38.0 ± 1.2	42.4 ± 0.7	0.205	0.139
TAG	10.3 ± 0.5	10.3 ± 0.3	11.3 ± 1.1	16.6 ± 0.6	0.541	0.006
CHOL	28.0 ± 0.5	27.2 ± 0.8	25.3 ± 0.5	24.4 ± 1.0	0.557	0.005
FFA	1.4 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	1.4 ± 0.0	0.079	0.377

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LOA = linoleic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA composition of CHSE-214 cells after being incubated with 20 µM of LOA and graded concentrations of 16:0+18:1n-9 is shown in Table 5.13. Proportions of LOA (R^2 = 0.672; p = 0.001), 18:3n-6 (R^2 = 0.605; p = 0.003), 20:3n-6 (R^2 = 0.967; p = 0.000)

and ARA (R^2 = 0.883; p = 0.000) all decreased with the graded supplementation of the mix 16:0+18:1n-9. There was graded incorporation of 16:0 and 18:1n-9 with the supplementation of the 16:0+18:1n-9 mix (R^2 = 0.420; p = 0.023 and R^2 = 0.796; p = 0.000, respectively). The desaturation products of 16:0, *i.e.* 16:1n-9 and 16:1n-7 (R^2 = 0.899; p = 0.000 and R^2 = 0.912; p = 0.000, respectively) and 18:1n-9, *i.e.* 18:2n-9 (R^2 = 0.714; p = 0.001) increased with the graded supplementation of 16:0+18:1n-9.

Table 5.13. Fatty acid compositions (%) of CHSE-214 cells incubated with 20 µM LOA and increasing concentrations of a 1:1 mix of 16:0 and 18:1n-9 combined

Fatty acid	20/20 µM	20/40 µM	20/60 µM	20/80 µM	R^2	P-value
14:0	1.2 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	0.7 ± 0.0	0.859	0.000
15:0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.145	0.222
16:0	13.6 ± 0.6	14.5 ± 0.8	14.0 ± 0.5	15.6 ± 1.0	0.420	0.023
17:0	0.6 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.0	0.607	0.003
18:0	9.0 ± 0.3	8.5 ± 0.5	7.2 ± 0.2	5.6 ± 0.3	0.906	0.000
22:0	0.8 ± 0.2	1.4 ± 0.1	1.8 ± 0.2	1.7 ± 0.1	0.645	0.002
Σ SFA	**25.5 ± 1.2**	**26.5 ± 1.3**	**25.0 ± 0.4**	**24.7 ± 1.4**	**0.148**	**0.217**
16:1n-9	2.8 ± 0.1	3.5 ± 0.0	3.9 ± 0.1	4.1 ± 0.3	0.899	0.000
16:1n-7	1.5 ± 0.1	1.9 ± 0.0	2.4 ± 0.2	4.1 ± 0.1	0.912	0.000
18:1n-9	24.0 ± 0.3	31.1 ± 0.5	32.9 ± 0.6	33.7 ± 1.1	0.796	0.000
18:1n-7	2.2 ± 0.2	2.1 ± 0.1	2.0 ± 0.1	2.1 ± 0.0	0.293	0.069
24:1n-9	0.6 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.000	0.973
Σ MUFA	**31.1 ± 0.6**	**39.4 ± 0.5**	**42.0 ± 0.3**	**44.6 ± 1.1**	**0.892**	**0.000**
18:2n-6	18.6 ± 1.0	13.9± 1.2	13.5 ± 0.3	13.4 ± 0.1	0.672	0.001
18:3n-6	6.8 ± 0.5	4.1 ± 0.1	4.0 ± 0.1	3.6 ± 0.1	0.605	0.003
20:2n-6*	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	0.019	0.668
20:3n-6	5.9 ± 0.2	4.1 ± 0.0	3.2 ± 0.1	2.5 ± 0.1	0.967	0.000
20:4n-6	3.3 ± 0.2	2.4 ± 0.3	2.0 ± 0.2	1.8 ± 0.1	0.883	0.000
22:5n-6	0.4 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.637	0.002
Σ n-6 PUFA	**35.6 ± 1.8**	**25.3 ± 1.5**	**23.5 ± 0.4**	**22.1 ± 0.2**	**0.791**	**0.000**
20:5n-3	0.6 ± 0.0	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.029	0.595
22:5n-3	0.7 ± 0.0	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	0.116	0.278
22:6n-3	1.2 ± 0.0	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	0.448	0.017
Σ n-3 PUFA	**2.5 ± 0.1**	**2.6 ± 0.3**	**2.3 ± 0.3**	**2.2 ± 0.0**	**0.224**	**0.120**
18:2n-9	3.3 ± 0.1	4.0 ± 0.2	4.7 ± 0.2	4.4 ± 0.2	0.714	0.001
20:2n-9	1.8 ± 0.1	2.1 ± 0.2	2.2 ± 0.1	1.8 ± 0.2	0.000	0.984
22:2n-9	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.157	0.203
Σ n-9 PUFA	**5.3 ± 0.1**	**6.2 ± 0.1**	**7.2 ± 0.2**	**6.4 ± 0.3**	**0.494**	**0.011**
Σ PUFA	**43.4 ± 1.7**	**34.1 ± 1.8**	**33.0 ± 0.5**	**30.7 ± 0.3**	**0.796**	**0.000**

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LOA =linoleic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; *contains 20:3n-9.

5.3.3. Effects of SFA and MUFA (lipid content) on the metabolism of EPA

5.3.3.1. Supplementation with EPA in presence of increasing levels of 16:0

Table 5.14 shows the lipid content and the lipid class composition of CHSE-214 cells after being incubated with 20 µM of EPA and graded increased concentrations of 16:0. The cell lipid content did not show a clear trend (R^2 = 0.101; p = 0.314). In the lipid class data, there was a clear trend of increasing TAG with the supplementation of EPA at 20 µM and graded concentrations of 16:0 (R^2 = 0.670; p = 0.001).

Table 5.14. Lipid contents and class compositions of CHSE-214 cells incubated with 20 µM EPA and increasing concentrations of 16:0

Lipid	20/20 µM	20/40 µM	20/60 µM	20/80 µM	R^2	P-value
LC (µg)	430.0 ± 17.3	330.0 ± 50.0	370.0 ± 50.0	370.0 ± 40.0	0.101	0.314
CC (%)						
PC	25.3 ± 0.9	14.3 ± 0.9	13.0 ± 1.9	20.5 ± 1.5	0.108	0.297
PE	26.9 ± 1.7	32.8 ± 1.0	37.0 ± 3.4	23.6 ± 1.5	0.017	0.684
PS	5.5 ± 0.6	3.5 ± 0.3	3.5 ± 0.3	4.3 ± 0.5	0.189	0.158
PI	8.9 ± 0.6	9.4 ± 1.0	10.7 ± 1.2	6.3 ± 1.2	0.181	0.169
PA/CL	1.5 ± 0.2	2.0 ± 0.4	1.5 ± 0.7	1.2 ± 0.1	0.140	0.231
SM	4.2 ± 1.1	2.9 ± 0.2	2.7 ± 0.5	1.8 ± 0.6	0.658	0.001
TP	72.3 ± 3.2	64.9 ± 1.7	68.4 ± 4.0	57.7 ± 0.1	0.597	0.003
TN	27.7 ± 3.2	35.1 ± 1.7	31.6 ± 4.0	42.3 ± 0.1	0.597	0.003
TAG	3.1 ± 0.2	6.0 ± 0.9	6.7 ± 1.0	8.3 ± 0.4	0.670	0.001
CHOL	23.5 ± 1.6	26.0 ± 0.2	23.2 ± 3.2	33.1 ± 0.5	0.453	0.016
FFA	1.1 ± 0.3	3.1 ± 0.6	1.7 ± 0.2	0.9 ± 0.0	0.076	0.387

Footnotes: Results are expressed as mean ± 1 SD (n=3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* EPA = eicosapentaenoic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids.

The FA composition of CHSE-214 cells incubated with 20 µM of EPA and graded increased concentrations of 16:0 is presented in Table 5.15. FA were in the following rank order: MUFA > PUFA > SFA. The EPA itself was greatly incorporated (R^2 = 0.593; p = 0.003), increasing from concentration 20/20 µM to 20/60 µM, and then decreased with

concentration 20/60 µM to 20/80 µM, whereas percentages of DHA and 22:5n-3 decreased with the graded supplementation of 16:0 (R^2 = 0.620; p = 0.002 and R^2 = 0.849; p = 0.000, respectively). The 16:0 was incorporated with the graded supplementation of the FA itself (R^2 = 0.739; p = 0.000) and some of the 16:0 was desaturated to 16:1n-7 (R^2 = 0.967; p = 0.000).

Table 5.15. Fatty acid compositions (%) of CHSE-214 cells incubated with 20 µM EPA and increasing concentrations of 16:0

Fatty acid	20/20 µM	20/40 µM	20/60 µM	20/80 µM	R^2	P-value
14:0	1.0 ± 0.1	1.0 ± 0.0	1.1 ± 0.0	1.0 ± 0.1	0.005	0.832
15:0	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.248	0.099
16:0	13.9 ± 0.4	14.3 ± 0.4	15.9 ± 0.5	15.7 ± 0.1	0.739	0.000
17:0	0.7 ±0.1	0.3 ± 0.0	0.5 ± 0.1	0.3 ± 0.0	0.343	0.046
18:0	8.8 ± 0.3	8.3 ± 0.4	8.5 ± 0.7	7.3 ± 0.3	0.533	0.007
20:0	ND	ND	ND	ND	-	-
22:0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.097	0.325
Σ SFA	**24.9 ± 0.7**	**24.4 ± 0.8**	**26.7 ± 1.3**	**24.9 ± 0.3**	**0.054**	**0.468**
16:1n-9	2.5 ± 0.1	2.7 ± 0.0	2.4 ± 0.2	2.8 ± 0.3	0.040	0.531
16:1n-7	2.4 ± 0.1	3.5 ± 0.1	4.8 ± 0.6	6.8 ± 0.3	0.967	0.000
18:1n-9	32.1 ± 0.6	31.2 ± 0.7	29.7 ± 1.2	29.4 ± 0.4	0.715	0.001
18:1n-7	2.1 ± 0.0	2.7 ± 0.1	2.6 ± 0.4	2.7 ± 0.1	0.396	0.028
24:1n-9	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.0	0.017	0.685
Σ MUFA	**39.9 ± 0.5**	**40.9 ± 0.6**	**40.3 ± 0.8**	**42.5 ± 0.1**	**0.544**	**0.006**
18:2n-6	2.7 ± 0.1	2.6 ± 0.1	2.4 ± 0.1	2.4 ± 0.0	0.628	0.002
18:3n-6	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.124	0.262
20:2n-6*	0.5 ± 0.1	0.6 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	0.256	0.093
20:3n-6	1.5 ± 0.0	1.4 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	0.339	0.047
20:4n-6	1.6 ± 0.2	1.4 ± 0.1	1.2 ± 0.2	1.5 ± 0.1	0.079	0.375
22:5n-6	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.327	0.052
Σ n-6 PUFA	**6.9 ± 0.1**	**6.6 ±0.2**	**5.9 ± 0.3**	**6.3 ± 0.1**	**0.486**	**0.012**
18:3n-3	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.089	0.346
18:4n-3	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.069	0.409
20:4n-3	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.884	0.000
20:5n-3	16.0 ± 0.4	17.4 ± 0.7	18.1 ± 0.1	17.9 ± 0.4	0.593	0.003
22:5n-3	4.4 ± 0.5	2.8 ± 0.2	1.9 ± 0.3	1.8 ± 0.0	0.849	0.000
22:6n-3	1.2 ± 0.0	1.1 ± 0.0	1.0 ± 0.1	1.0 ± 0.0	0.620	0.002
Σ n-3 PUFA	**22.2 ± 0.5**	**22.0 ± 0.9**	**21.6 ± 0.3**	**21.1 ± 0.3**	**0.430**	**0.021**
18:2n-9	2.9 ± 0.0	2.9 ± 0.0	2.6 ± 0.4	2.7 ± 0.0	0.217	0.127
20:2n-9	3.2 ± 0.2	3.2 ± 0.2	2.9 ± 0.2	2.5 ± 0.1	0.715	0.001
Σ n-9 PUFA	**6.1 ± 0.2**	**6.1 ± 0.1**	**5.5 ± 0.2**	**5.2 ± 0.0**	**0.834**	**0.000**
Σ PUFA	**35.2 ± 0.5**	**34.7 ± 1.0**	**33.2 ± 0.8**	**32.6 ± 0.3**	**0.742**	**0.000**

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* EPA = eicosapentaenoic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; ND = not detected; *contains 20:3n-9.

5.3.3.2. Supplementation with EPA in presence of increasing levels of 18:1n-9

The lipid content and the lipid class composition of CHSE-214 cells after being incubated with 20 µM of EPA and graded increased concentrations of 18:1n-9 are shown in Table 5.16. Cell lipid content was similar at all concentrations of FA supplemented, without showing any clear trend (R^2 = 0.081; p = 0.370). In the lipid class data, there was increased cellular TAG with the graded supplementation of 18:1n-9 (R^2 = 0.794; p = 0.000). The increment in TAG was balanced by decreasing proportions of some polar lipids, *i.e.* PC (R^2 = 0.469; p = 0.014) and PA/CL (R^2 = 0.634; p = 0.002).

Table 5.16. Lipid contents and class compositions of CHSE-214 cells incubated with 20 µM EPA and increasing concentrations of 18:1n-9

Lipid	20/20 µM	20/40 µM	20/60 µM	20/80 µM	R^2	P-value
LC (µg)	256.7 ± 25.2	270.0 ± 26.5	250.0 ± 50.0	290.0 ± 26.5	0.081	0.370
CC (%)						
PC	23.6 ± 0.5	21.9 ± 1.8	22.3 ± 0.5	21.0 ± 0.2	0.469	0.014
PE	18.5 ± 0.6	15.7 ± 0.2	17.6 ± 0.9	17.0 ± 0.8	0.065	0.422
PS	4.6 ± 0.2	3.9 ± 0.0	4.9 ± 0.2	3.2 ± 0.4	0.291	0.070
PI	8.8 ± 0.6	7.6 ± 0.2	8.6 ± 0.1	7.0 ± 0.1	0.381	0.033
PA/CL	2.2 ± 0.4	1.6 ± 0.1	1.4 ± 0.2	1.4 ± 0.1	0.634	0.002
SM	2.6 ± 0.2	4.3 ± 0.4	5.9 ± 0.6	4.6 ± 0.1	0.533	0.007
TP	60.3 ± 1.5	55.0 ± 2.3	60.7 ± 1.8	54.2 ± 0.8	0.185	0.163
TN	39.7 ± 1.5	45.0 ± 2.3	39.3 ± 1.8	45.8 ± 0.8	0.185	0.163
TAG	9.8 ± 1.0	17.0 ± 1.6	17.2 ± 1.0	20.6 ± 0.2	0.794	0.000
CHOL	27.9 ± 0.9	25.4 ± 0.6	19.6 ± 0.6	22.2 ± 0.4	0.628	0.002
FFA	2.0 ± 0.3	2.6 ± 0.5	2.5 ± 0.7	3.0 ± 0.4	0.409	0.025

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* EPA = eicosapentaenoic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids.

Table 5.17 shows the FA composition of CHSE-214 cells after being incubated with 20 µM of EPA and graded increased concentrations of 18:1n-9. The levels of DHA and, to a greater extent 22:5n-3, decreased with the graded supplementation of 18:1n-9 (R^2 = 0.438; p = 0.019 and R^2 = 0.913; p = 0.000, respectively). The supplementation of 18:1n-

9 did not affect the cellular proportions of EPA, that were essentially the same at all concentrations (R^2 = 0.147; p = 0.219). The 18:1n-9 was incorporated with the graded supplementation of the FA itself (R^2 = 0.948; p = 0.000) and the increment in total MUFA was balanced by reduced proportions of total SFA (R^2 = 0.841; p = 0.000) and n-3 PUFA (R^2 = 0.975; p = 0.000).

Table 5.17. Fatty acid compositions (%) of CHSE-214 cells incubated with 20 µM EPA and increasing concentrations of 18:1n-9

Fatty acid	20/20 µM	20/40 µM	20/60 µM	20/80 µM	R^2	P-value
14:0	1.3 ± 0.1	0.9 ± 0.0	0.8 ± 0.0	0.6 ± 0.1	0.897	0.000
15:0	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.382	0.032
16:0	10.1 ± 0.1	6.8 ± 0.3	6.8 ± 0.5	5.8 ± 0.5	0.775	0.000
17:0	0.6 ± 0.1	0.4 ± 0.0	0.4 ± 0.1	0.2 ± 0.0	0.680	0.001
18:0	7.3 ± 0.2	5.8 ± 0.3	5.5 ± 0.2	4.9 ± 0.3	0.861	0.000
20:0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.742	0.000
22:0	0.6 ± 0.1	0.8 ± 0.2	1.0 ± 0.1	0.7 ± 0.0	0.100	0.316
Σ SFA	**20.7 ± 0.3**	**15.4 ± 0.8**	**15.2 ± 0.6**	**12.8 ± 0.6**	**0.841**	**0.000**
16:1n-9	3.3 ± 0.1	3.5 ± 0.0	3.9 ± 0.3	4.3 ± 0.3	0.805	0.000
16:1n-7	1.4 ± 0.2	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.534	0.007
18:1n-9	33.8 ± 0.6	41.2 ± 1.1	43.7 ± 0.1	48.4 ± 1.0	0.948	0.000
18:1n-7	1.6 ± 0.2	1.3 ± 0.0	1.4 ± 0.2	1.2 ± 0.1	0.537	0.007
24:1n-9	0.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.107	0.299
Σ MUFA	**40.8 ± 0.6**	**47.5 ± 1.1**	**50.6 ± 0.2**	**55.4 ± 0.7**	**0.968**	**0.000**
18:2n-6	2.5 ± 0.1	3.0 ± 0.1	2.3 ± 0.2	2.4 ± 0.2	0.191	0.155
18:3n-6	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.447	0.017
20:2n-6*	1.8 ± 0.1	1.8 ± 0.2	1.8 ± 0.2	1.4 ± 0.2	0.134	0.242
20:3n-6	1.3 ± 0.2	1.9 ± 0.2	1.7 ± 0.0	1.2 ± 0.2	0.001	0.914
20:4n-6	1.3 ± 0.1	1.2 ± 0.0	1.1 ± 0.1	1.2 ± 0.1	0.466	0.014
Σ n-6 PUFA	**7.2 ± 0.1**	**8.2 ± 0.4**	**7.3 ± 0.5**	**6.6 ± 0.3**	**0.242**	**0.104**
18:3n-3	0.2 ± 0.0	ND	ND	ND	-	-
18:4n-3	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.256	0.093
20:4n-3	0.5 ± 0.0	0.3 ± 0.0	ND	ND	-	-
20:5n-3	19.5 ± 0.3	20.8 ± 0.2	19.8 ± 0.2	19.1 ± 0.2	0.147	0.219
22:5n-3	6.5 ± 0.9	3.8 ± 0.3	3.1 ± 0.2	2.1 ± 0.2	0.913	0.000
22:6n-3	1.2 ± 0.0	1.0 ± 0.0	1.1 ± 0.1	1.0 ± 0.1	0.438	0.019
Σ n-3 PUFA	**28.2 ± 0.7**	**26.2 ± 0.4**	**24.3 ± 0.3**	**22.5 ± 0.2**	**0.975**	**0.000**
18:2n-9	2.2 ± 0.1	2.2 ± 0.0	2.2 ± 0.0	2.3 ± 0.1	0.210	0.135
20:2n-9	0.9 ± 0.2	0.5 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.618	0.002
Σ n-9 PUFA	**3.1 ± 0.1**	**2.7 ± 0.1**	**2.6 ± 0.1**	**2.7 ± 0.1**	**0.475**	**0.013**
Σ PUFA	**38.5± 0.5**	**37.1 ± 0.3**	**34.2 ± 0.5**	**31.8 ± 0.3**	**0.964**	**0.000**

Footnotes: Results are expressed as mean ± 1SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* EPA = eicosapentaenoic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; ND = not detected; *contains 20:3n-9.

5.3.3.3. Supplementation with EPA in presence of increasing levels of a 1:1 mix of 16:0 and 18:1n-9 combined

Table 5.18 shows the cell lipid content and the lipid class composition of CHSE-214 cells after being incubated with 20 µM of EPA and graded increased concentrations of 16:0+18:1n-9 mix. The cell lipid content did not show a clear trend (R^2 = 0.005; p = 0.831). In the lipid class data, TAG increased with the graded supplementation of 16:0+18:1n-9, but the results were not statistically significant (R^2 = 0.220; p = 0.124); this increment in TAG was balanced by decreasing percentages of PC (R^2 = 0.821; p = 0.000).

Table 5.18. Lipid contents and class compositions of CHSE-214 cells incubated with 20 µM EPA and increasing concentrations of a 1:1 mix of 16:0 and 18:1n-9 combined

Lipid	20/20 µM	20/40 µM	20/60 µM	20/80 µM	R^2	P-value
LC (µg)	293.3 ± 5.8	290.0 ± 10.0	323.3 ± 20.8	286.7 ± 30.6	0.005	0.831
CC (%)						
PC	27.1 ± 2.3	25.2 ± 1.2	19.4 ± 0.9	18.5 ± 1.6	0.821	0.000
PE	22.1 ± 1.4	22.0 ± 0.6	23.2 ± 1.1	18.7 ± 0.1	0.289	0.072
PS	5.9 ± 0.2	6.2 ± 0.4	6.8 ± 0.6	5.8 ± 0.3	0.007	0.803
PI	7.2 ± 0.6	6.1 ± 0.4	8.5 ± 0.6	6.2 ± 1.1	0.008	0.776
PA/CL	1.0 ± 0.1	3.0 ± 0.2	2.9 ± 0.1	2.2 ± 0.2	0.245	0.102
SM	4.2 ± 0.1	5.3 ± 0.5	4.1 ± 0.2	4.8 ± 0.6	0.108	0.296
TP	67.5 ± 1.7	67.8 ± 2.6	64.9 ± 1.3	56.2 ± 1.6	0.663	0.001
TN	32.5 ± 1.7	32.2 ± 2.6	35.1 ± 1.3	43.8 ± 1.6	0.663	0.001
TAG	6.3 ± 0.8	6.5 ± 0.2	9.4 ± 0.6	17.3 ± 0.7	0.220	0.124
CHOL	24.7 ± 1.1	24.2 ± 2.4	24.2 ± 0.5	24.8 ± 1.0	0.532	0.007
FFA	1.5 ± 0.2	1.5 ± 0.3	1.5 ± 0.1	1.7 ± 0.4	0.519	0.008

Footnotes: Results are expressed as mean ± 1 SD (n=3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* EPA = eicosapentaenoic; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids.

The FA profile of CHSE-214 cells incubated with 20 µM EPA and graded increased concentrations of the 16:0+18:1n-9 mix is presented in Table 5.19. In all treatments the proportions of the main FA groups were in the following order: MUFA > PUFA > SFA. The levels of DHA and 22:5n-3 decreased (R^2 = 0.426; p = 0.021 and R^2 =

0.934; p = 0.000) and EPA increased (R^2 = 0.658; p = 0.001) with the graded supplementation of 16:0+18:1n-9. The proportions of 16:0 were the same in all treatments (R^2 = 0.006; p = 0.808), whereas 18:1n-9 slightly increased with the graded supplementation of 16:0+18:1n-9 (R^2 = 0.758; p = 0.000). Some of 16:0 was further desaturated to 16:1n-9 (R^2 = 0.333; p = 0.049) and to a lesser extent to 16:1n-7 (R^2 = 0.867; p = 0.000).

Table 5.19. Fatty acid compositions (%) of CHSE-214 cells incubated with 20 µM EPA and increasing concentrations of a 1:1 mix of 16:0 and 18:1n-9 combined

Fatty acid	20/20 µM	20/40 µM	20/60 µM	20/80 µM	R^2	P-value
14:0	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.0	0.315	0.058
15:0	0.4 ± 0.0	0.5 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.276	0.079
16:0	14.2 ± 0.3	13.9 ± 0.6	14.3 ± 0.3	14.2 ± 0.9	0.006	0.808
17:0	0.8 ± 0.1	0.8 ± 0.0	0.7 ± 0.1	0.8 ± 0.0	0.141	0.230
18:0	8.5 ± 0.5	8.3 ± 0.2	7.7 ± 0.2	6.2 ± 0.2	0.796	0.000
22:0	0.6 ± 0.1	0.6 ± 0.1	0.8 ± 0.0	0.3 ± 0.0	0.159	0.199
Σ SFA	**25.4 ± 0.4**	**25.0 ± 0.7**	**24.7 ± 0.4**	**22.6 ± 1.0**	**0.630**	**0.002**
16:1n-9	2.9 ± 0.0	3.1 ± 0.1	3.4 ± 0.2	3.1 ± 0.2	0.333	0.049
16:1n-7	1.8 ± 0.0	2.3 ± 0.1	2.7 ± 0.1	2.8 ± 0.2	0.867	0.000
18:1n-9	33.3 ± 0.3	32.8 ± 0.1	34.9 ± 0.7	36.2 ± 0.6	0.758	0.000
18:1n-7	2.5 ± 0.1	2.2 ± 0.3	2.3 ± 0.1	2.0 ± 0.3	0.479	0.013
24:1n-9	0.8 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.002	0.902
Σ MUFA	**41.3 ± 0.3**	**41.2 ± 0.5**	**44.2 ± 0.5**	**44.9 ± 1.0**	**0.774**	**0.000**
18:2n-6	3.1 ± 0.1	3.4 ± 0.1	2.9 ± 0.3	2.9 ± 0.3	0.230	0.114
18:3n-6	0.2 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.4 ± 0.1	0.525	0.008
20:2n-6*	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.171	0.182
20:3n-6	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.0	1.3 ± 0.1	0.247	0.100
20:4n-6	1.6 ± 0.1	1.5 ± 0.2	1.4 ± 0.0	1.3 ± 0.0	0.184	0.164
22:5n-6	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.472	0.014
Σ n-6 PUFA	**6.9 ± 0.2**	**7.1 ± 0.5**	**6.7 ± 0.4**	**6.5 ± 0.2**	**0.246**	**0.101**
18:4n-3	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.107	0.300
20:4n-3	0.4 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.786	0.000
20:5n-3	13.9 ± 0.2	16.8 ± 0.4	15.6 ± 0.3	18.1 ± 0.2	0.658	0.001
22:5n-3	5.8 ± 0.2	3.9 ± 0.3	2.8 ± 0.1	2.4 ± 0.0	0.934	0.000
22:6n-3	1.3 ± 0.1	1.2 ± 0.2	1.0 ± 0.0	1.0 ± 0.0	0.426	0.021
Σ n-3 PUFA	**21.6 ± 0.2**	**22.2 ± 0.3**	**19.8 ± 0.4**	**21.9 ± 0.1**	**0.027**	**0.608**
18:2n-9	2.4 ± 0.2	2.4 ± 0.2	2.3 ± 0.0	2.3 ± 0.0	0.115	0.281
20:2n-9	2.2 ± 0.1	1.8 ± 0.1	2.0 ± 0.2	1.6 ± 0.2	0.395	0.029
22:2n-9	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.202	0.143
Σ n-9 PUFA	**4.8 ± 0.2**	**4.5 ± 0.2**	**4.6 ± 0.2**	**4.1 ± 0.3**	**0.456**	**0.016**
Σ PUFA	**33.3 ± 0.3**	**33.8 ± 0.7**	**31.1 ± 0.6**	**32.5 ± 0.4**	**0.240**	**0.106**

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* EPA = eicosapentaenoic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; *contains 20:3n-9.

5.3.4. Effects of SFA and MUFA (lipid) on the metabolism of supplemented LNA and EPA combined

5.3.4.1. Supplementation with LNA and EPA in presence of increasing levels of 16:0

Table 5.20 shows the lipid content and the lipid class composition of CHSE-214 cells after being incubated with LNA+EPA (20/20 μM) and graded concentrations of 16:0. Cell lipid content decreased with the graded supplementation of 16:0, but results were not statistically significant (R^2 = 0.413; p = 0.062). The lipid class data showed increased TAG (R^2 = 0.078; p = 0.468), PE (R^2 = 0.763; p = 0.002), and SM (R^2 = 0.802; p = 0.001) with the graded supplementation of 16:0, although the data for TAG were not statistically significant.

Table 5.20. Lipid contents and class compositions of CHSE-214 cells incubated with 40 μM of LNA and EPA (1:1) and increasing concentrations of 16:0

Lipid	40/20 μM	40/40 μM	40/60 μM	R^2	P-value
LC (μg)	336.7 ± 20.8	330.0 ± 10.0	256.7 ± 37.9	0.413	0.062
CC (%)					
PC	21.4 ± 0.5	21.0 ± 0.6	21.1 ± 0.1	0.056	0.538
PE	20.2 ± 0.4	21.8 ± 0.9	22.5 ± 0.1	0.763	0.002
PS	10.5 ± 0.5	7.1 ± 0.3	6.9 ± 0.3	0.202	0.225
PI	8.6 ± 0.6	8.6 ± 0.1	8.5 ± 0.0	0.418	0.060
PA/CL	1.3 ± 0.2	1.7 ± 0.1	1.1 ± 0.3	0.069	0.496
SM	6.3 ± 0.3	6.9 ± 0.4	7.7 ± 1.3	0.802	0.001
TP	68.3 ± 0.8	67.1 ± 1.5	67.8 ± 1.3	0.685	0.006
TN	31.7 ± 0.8	32.9 ± 1.5	32.2 ± 0.8	0.685	0.006
TAG	8.1 ± 1.9	9.1 ± 1.3	11.7 ± 0.5	0.078	0.468
CHOL	21.3 ± 1.5	21.7 ± 0.3	18.6 ± 0.6	0.492	0.035
FFA	2.3 ± 0.3	2.1 ± 0.2	1.9 ± 0.5	0.174	0.264

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LNA = α-linolenic acid; EPA = eicosapentaenoic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

Table 5.21 shows the FA profile of CHSE-214 cells after being incubated with LNA+EPA (20/20 μM) and graded concentrations of 16:0. The level of DHA was

unaffected with the supplementation of 16:0, being similar in all treatments (R^2 = 0.384; p = 0.075), whereas the percentage of 22:5n-3 decreased with the graded supplementation of 16:0 (R^2 = 0.745; p = 0.003).

Table 5.21. Fatty acid compositions (%) of CHSE-214 cells incubated with 40 µM of LNA and EPA (1:1) and increasing concentrations of 16:0

Fatty acid	40/20 µM	40/40 µM	40/60 µM	R^2	P-value
14:0	1.2 ± 0.1	1.1 ± 0.2	1.2 ± 0.1	0.014	0.758
15:0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.143	0.316
16:0	14.3 ± 0.7	14.3 ± 0.5	14.9 ± 0.3	0.226	0.196
17:0	0.9 ± 0.1	1.1 ± 0.1	1.0 ± 0.0	0.272	0.150
18:0	9.8 ± 0.3	9.7 ± 0.3	9.4 ± 0.8	0.159	0.288
22:0	0.5 ± 0.1	0.4 ± 0.1	0.8 ± 0.1	0.332	0.104
ΣSFA	**27.0 ± 1.2**	**27.0 ± 1.0**	**27.7 ± 0.7**	**0.103**	**0.400**
16:1n-9	2.3 ± 0.2	2.1 ± 0.1	1.7 ± 0.1	0.776	0.002
16:1n-7	2.3 ± 0.3	2.4 ± 0.2	2.8 ± 0.4	0.384	0.075
18:1n-9	25.6 ± 0.8	23.6 ± 0.6	27.9 ± 0.5	0.272	0.150
18:1n-7	2.8 ± 0.2	2.9 ± 0.1	3.1 ± 0.1	0.544	0.023
24:1n-9	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.0	0.001	0.927
ΣMUFA	**33.7 ± 1.0**	**31.7 ± 0.5**	**36.2 ± 0.1**	**0.298**	**0.129**
18:2n-6	3.6 ± 0.1	4.2 ± 0.3	3.5 ± 0.5	0.039	0.611
18:3n-6	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.933	0.000
20:2n-6*	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.078	0.467
20:3n-6	1.5 ± 0.2	1.2 ± 0.1	1.1 ± 0.3	0.476	0.040
20:4n-6	1.6 ± 0.2	1.3 ± 0.2	1.3 ± 0.0	0.419	0.060
Σ n-6 PUFA	**7.3 ± 0.5**	**7.4 ± 0.4**	**6.7 ± 0.4**	**0.276**	**0.276**
18:3n-3	2.9 ± 0.3	3.1 ± 0.3	3.5 ± 0.3	0.255	0.166
18:4n-3	2.0 ± 0.1	1.6 ± 0.1	2.0 ± 0.1	0.000	0.981
20:4n-3	1.5 ± 0.2	1.1 ± 0.1	1.1 ± 0.1	0.473	0.041
20:5n-3	14.7 ± 0.4	17.7 ± 0.1	14.3 ± 0.5	0.012	0.780
22:5n-3	5.4 ± 0.1	5.3 ± 0.4	3.6 ± 0.2	0.745	0.003
22:6n-3	2.1 ± 0.4	1.8 ± 0.3	2.0 ± 0.0	0.384	0.075
Σ n-3 PUFA	**28.6 ± 1.3**	**30.6 ± 0.7**	**26.5 ± 0.5**	**0.217**	**0.207**
18:2n-9	2.2 ± 0.1	2.1 ± 0.2	1.7 ± 0.2	0.628	0.011
20:2n-9	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	0.010	0.799
Σ n-9 PUFA	**3.4 ± 0.2**	**3.3 ± 0.3**	**2.9 ± 0.2**	**0.529**	**0.026**
Σ PUFA	**39.3 ± 1.7**	**41.3 ± 1.5**	**36.1 ± 0.6**	**0.304**	**0.124**

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis ($p < 0.05$). *Abbreviations:* LNA = α-linolenic acid; EPA = eicosapentaenoic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; *contains 20:3n-9.

The incorporation of EPA did not show a clear trend (R^2 = 0.012; p = 0.780) and the incorporation of LNA increased with the graded supplementation of 16:0, but results were not statistically significant (R^2 = 0.255; p = 0.166). There was no effect on

proportions of cellular 16:0 as results were not statistically significant (R^2 = 0.226; p = 0.196). Some of the supplemented 16:0 was desaturated to 16:1n-7 (R^2 = 0.384; p = 0.075).

5.3.4.2. Supplementation with LNA and EPA in presence of increasing levels of 18:1n-9

Table 5.22 shows the lipid content and the lipid class composition of CHSE-214 cells after being incubated with LNA+EPA (20/20 μM) and graded increased concentrations of 18:1n-9. The cell lipid content increased with the graded supplementation of 18:1n-9, although results were not statistically significant (R^2 = 0.072; p = 0.486). In the lipid class data, percentages of TAG (R^2 = 0.936; p = 0.000), PE (R^2 = 0.896; p = 0.000) and PS (R^2 = 0.878; p = 0.000) increased with the graded supplementation of 18:1n-9. The increments in TAG and PE were balanced by decreased proportions of cholesterol (R^2 = 0.991; p = 0.000).

Table 5.22. Lipid contents and class compositions of CHSE-214 cells incubated with 40 μM of LNA and EPA (1:1) and increasing concentrations of 18:1n-9

Lipid	40/20 μM	40/40 μM	40/60 μM	R^2	P-value
LC (μg)	236.7 ± 37.9	243.3 ± 32.1	253.3 ± 15.3	0.072	0.486
CC (%)					
PC	21.6 ± 0.8	21.3 ± 1.1	21.7 ± 0.7	0.000	0.960
PE	23.1 ± 0.5	26.1 ± 0.5	27.7 ± 0.9	0.896	0.000
PS	5.5 ± 0.1	7.5 ± 0.2	8.0 ± 0.2	0.878	0.000
PI	3.8 ± 0.1	4.3 ± 0.2	4.8 ± 0.5	0.729	0.003
PA/CL	2.2 ± 0.1	1.5 ± 0.1	2.0 ± 0.3	0.032	0.644
SM	1.2 ± 0.2	0.9 ± 0.1	1.7 ± 0.2	0.285	0.139
TP	57.4 ± 0.9	61.6 ± 0.5	65.9 ± 0.5	0.977	0.000
TN	42.6 ± 0.9	38.4 ± 0.5	34.1 ± 0.5	0.977	0.000
TAG	3.7 ± 0.4	6.1 ± 0.2	7.5 ± 0.2	0.936	0.000
CHOL	37.2 ± 0.6	30.1 ± 0.8	24.0 ± 0.3	0.991	0.000
FFA	1.7 ± 0.2	2.2 ± 0.2	2.6 ± 0.2	0.800	0.001

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LNA = α-linolenic acid; EPA = eicosapentaenoic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA profile of CHSE-214 cells after being incubated with LNA+EPA (20/20 µM) and graded increased concentrations of 18:1n-9 is shown in Table 5.23. Proportions of DHA (R^2 = 0.823; p = 0.001), 22:5n-3 (R^2 = 0.823; p = 0.001), EPA (R^2 = 0.823; p = 0.001) and LNA (R^2 = 0.823; p = 0.001) all decreased with the graded supplementation of 18:1n-9, as well as 18:4n-3 (R^2 = 0.123; p = 0.356) and 20:4n-3 (R^2 = 0.344; p = 0.097), although the latter two were not statistically significant. The 18:1n-9 was incorporated with the graded supplementation of the FA (R^2 = 0.932; p = 0.000).

Table 5.23. Fatty acid compositions (%) of CHSE-214 cells incubated with 40 µM of LNA and EPA (1:1) and increasing concentrations of 18:1n-9

Fatty acid	40/20 µM	40/40 µM	40/60 µM	R^2	P-value
14:0	0.8 ± 0.1	0.9 ± 0.0	0.8 ± 0.1	0.013	0.772
15:0	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.037	0.619
16:0	10.3 ± 0.7	10.7 ± 0.5	9.6 ± 0.7	0.152	0.300
17:0	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	0.227	0.195
18:0	6.3 ± 0.4	6.5 ± 0.2	5.1 ± 0.1	0.552	0.022
22:0	0.4 ± 0.0	0.8 ± 0.0	1.0 ± 0.1	0.895	0.000
Σ SFA	**18.7 ± 1.0**	**19.8 ± 0.4**	**17.5 ± 0.8**	**0.186**	**0.246**
16:1n-9	3.0 ± 0.2	3.8 ± 0.1	4.0 ± 0.3	0.786	0.001
16:1n-7	1.4 ± 0.0	1.3 ± 0.1	1.4 ± 0.1	0.000	0.999
18:1n-9	29.5 ± 0.8	36.3 ± 0.7	39.3 ± 0.3	0.932	0.000
18:1n-7	2.1 ± 0.1	1.7 ± 0.3	2.0 ± 0.1	0.024	0.692
24:1n-9	0.6 ± 0.1	0.7 ± 0.1	1.2 ± 0.1	0.844	0.000
Σ MUFA	**36.6 ± 0.9**	**43.8 ± 0.9**	**47.9 ± 0.5**	**0.952**	**0.000**
18:2n-6	2.9 ± 0.0	2.2 ± 0.2	2.2 ± 0.3	0.558	0.021
18:3n-6	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.101	0.404
20:2n-6*	0.6 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	0.198	0.230
20:3n-6	1.6 ± 0.1	1.3 ± 0.1	1.3 ± 0.2	0.419	0.059
20:4n-6	1.3 ± 0.0	1.2 ± 0.1	1.1 ± 0.2	0.452	0.047
22:5n-6	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.699	0.005
Σ n-6 PUFA	**7.2 ± 0.1**	**5.8 ± 0.4**	**5.6 ± 0.6**	**0.626**	**0.011**
18:3n-3	4.4 ± 0.1	3.2 ± 0.3	2.7 ± 0.3	0.871	0.000
18:4n-3	1.9 ± 0.4	1.8 ± 0.2	1.6 ± 0.3	0.123	0.356
20:4n-3	2.7 ± 0.5	2.3 ± 0.3	2.1 ± 0.2	0.344	0.097
20:5n-3	15.8 ± 0.5	13.2 ± 0.3	13.6 ± 0.8	0.510	0.031
22:5n-3	6.1 ± 0.5	3.9 ± 0.3	2.7 ± 0.4	0.925	0.000
22:6n-3	1.1 ± 0.0	1.0 ± 0.0	0.8 ± 0.1	0.789	0.001
Σ n-3 PUFA	**32.0 ± 1.5**	**25.4 ± 0.2**	**23.5 ± 0.9**	**0.865**	**0.000**
18:2n-9	2.8 ± 0.2	2.7 ± 0.1	3.0 ± 0.1	0.128	0.345
20:2n-9	2.5 ± 0.4	2.2 ± 0.2	2.2 ± 0.1	0.238	0.183
22:2n-9	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.001	0.939
Σ n-9 PUFA	**5.5 ± 0.6**	**5.2 ± 0.3**	**5.5 ± 0.2**	**0.033**	**0.640**
Σ PUFA	**44.7 ± 0.8**	**36.4 ± 0.6**	**34.6 ± 1.2**	**0.849**	**0.000**

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LNA = α-linolenic acid; EPA = eicosapentaenoic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; *contains 20:3n-9.

5.3.4.3. Supplementation with LNA and EPA in presence of increasing levels of a 1:1 mix of 16:0+18:1n-9 combined

Table 5.24 shows the lipid content and the lipid class composition of CHSE-214 cells after being incubated with LNA+EPA (20/20 µM) and graded increased concentrations of a 1:1 mix of 16:0+18:1n-9. There was no clear trend in the cell lipid content, being essentially the same in all treatments (R^2 = 0.011; p = 0.785). In the lipid class data, TAG showed a clear trend to increase with the graded supplementation of 16:0+18:1n-9 (R^2 = 0.692; p = 0.005).

Table 5.24. Lipid contents and class compositions of CHSE-214 cells incubated with 40 µM of LNA and EPA (1:1) and increasing concentrations of a 1:1 mix of 16:0 and 18:1n-9 combined

Lipid	40/20 µM	40/40 µM	40/60 µM	R^2	P-value
LC (µg)	236.7 ± 41.6	243.3 ± 23.1	243.3 ± 25.2	0.011	0.785
CC (%)					
PC	17.2 ± 0.4	14.1 ± 0.8	15.5 ± 0.7	0.290	0.134
PE	29.5 ± 0.5	27.6 ± 0.5	25.9 ± 1.0	0.867	0.000
PS	6.9 ± 0.5	6.8 ± 0.7	4.7 ± 0.2	0.669	0.007
PI	6.6 ± 0.4	4.8 ± 0.2	4.7 ± 0.1	0.672	0.007
PA/CL	3.5 ± 0.2	2.5 ± 0.3	2.5 ± 0.3	0.650	0.009
SM	6.2 ± 0.2	2.4 ± 0.1	2.0 ± 0.0	0.832	0.001
TP	69.9 ± 0.6	58.2 ± 0.2	55.3 ± 0.4	0.886	0.000
TN	30.1 ± 0.6	41.8 ± 0.2	44.7 ± 0.4	0.886	0.000
TAG	4.7 ± 0.3	5.2 ± 0.4	7.9 ± 1.5	0.692	0.005
CHOL	23.7 ± 0.8	35.0 ± 0.4	34.6 ± 1.1	0.716	0.004
FFA	1.7 ± 0.3	1.6 ± 0.1	2.2 ± 0.3	0.381	0.076

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LNA = α-linolenic acid; EPA = eicosapentaenoic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA profile of CHSE-214 cells after being incubated with LNA+EPA (20/20 µM) and graded increased concentrations of 16:0+18:1n-9 is shown in Table 5.25. The level of DHA was not affected with the graded supplementation of 16:0+18:1n-9, being essentially the same at all concentrations (R^2 = 0.004; p = 0.872). EPA levels increased

with the graded supplementation of 16:0+18:1n-9 (R^2 = 0.775; p = 0.002). The proportion of cellular LNA was similar in all treatments (R^2 = 0.001; p = 0.940) and there was evidence of some desaturation to 18:4n-3, which increased with the graded supplementation of 16:0+18:1n-9 (R^2 = 0.749; p = 0.003). The percentage of 16:0 decreased (R^2 = 0.858; p = 0.000) and 18:1n-9 increased (R^2 = 0.695; p = 0.005) with the graded supplementation of 16:0+18:1n-9.

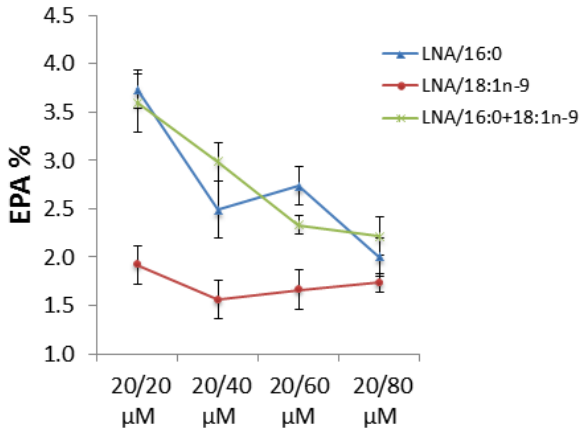
Table 5.25. Fatty acid compositions (%) of CHSE-214 cells incubated with 40 µM of LNA and EPA (1:1) and increasing concentrations of a 1:1 mix of 16:0 and 18:1n-9 combined

Fatty acid	40/20 µM	40/40 µM	40/60 µM	R^2	P-value
14:0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.1	0.017	0.741
15:0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.001	0.954
16:0	17.3 ± 0.5	15.5 ± 0.7	13.9 ± 0.7	0.858	0.000
17:0	0.6 ± 0.0	0.7 ± 0.1	0.7 ± 0.0	0.005	0.852
18:0	9.9 ± 0.0	8.6 ± 0.3	8.5 ± 0.6	0.643	0.009
20:0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.865	0.000
22:0	2.0 ± 0.2	0.7 ± 0.1	0.3 ± 0.0	0.949	0.000
Σ SFA	**31.3 ± 0.4**	**26.9 ± 1.0**	**24.8 ± 1.4**	**0.876**	**0.000**
16:1n-9	3.0 ± 0.1	3.1 ± 0.3	3.4 ± 0.1	0.493	0.035
16:1n-7	1.9 ± 0.1	2.1 ± 0.1	2.2 ± 0.1	0.674	0.007
18:1n-9	29.6 ± 0.2	30.0 ± 1.0	32.1 ± 0.5	0.695	0.005
18:1n-7	2.2 ± 0.1	2.4 ± 0.1	2.2 ± 0.3	0.001	0.944
24:1n-9	0.8 ± 0.1	0.6 ± 0.1	0.7 ± 0.0	0.369	0.083
Σ MUFA	**37.5 ± 0.5**	**38.2 ± 1.4**	**40.6 ± 0.6**	**0.689**	**0.006**
18:2n-6	2.2 ± 0.3	2.6 ± 0.4	2.0 ± 0.1	0.063	0.514
18:3n-6	0.4 ± 0.1	0.5 ± 0.0	0.3 ± 0.0	0.255	0.166
20:2n-6*	0.5 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.199	0.229
20:3n-6	1.3 ± 0.2	1.2 ± 0.0	1.1 ± 0.1	0.396	0.069
20:4n-6	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.2	0.208	0.217
Σ n-6 PUFA	**5.5 ± 0.4**	**6.1 ± 0.5**	**5.2 ± 0.4**	**0.060**	**0.526**
18:3n-3	2.4 ± 0.2	2.4 ± 0.1	2.4 ± 0.3	0.001	0.940
18:4n-3	1.2 ± 0.1	1.5 ± 0.1	1.7 ± 0.2	0.749	0.003
20:4n-3	2.0 ± 0.3	1.9 ± 0.1	1.7 ± 0.3	0.344	0.097
20:5n-3	10.2 ± 0.4	13.2 ± 0.5	13.7 ± 0.9	0.775	0.002
22:5n-3	2.9 ± 0.2	3.0 ± 0.2	2.6 ± 0.2	0.304	0.124
22:6n-3	0.9 ± 0.1	0.9 ± 0.0	0.9 ± 0.1	0.004	0.872
Σ n-3 PUFA	**19.6 ± 0.5**	**22.9 ± 1.0**	**23.0 ± 1.6**	**0.574**	**0.018**
18:2n-9	2.9 ± 0.1	3.0 ± 0.1	3.3 ± 0.2	0.635	0.010
20:2n-9	2.8 ± 0.1	2.6 ± 0.0	2.8 ± 0.0	0.015	0.752
22:2n-9	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.102	0.402
Σ n-9 PUFA	**6.1 ± 0.1**	**5.9 ± 0.1**	**6.4 ± 0.2**	**0.324**	**0.110**
Σ PUFA	**31.2 ± 0.9**	**34.9 ± 0.9**	**34.6 ± 1.7**	**0.504**	**0.032**

Footnotes: Results are present as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical differences were determined by regression analysis (p < 0.05). *Abbreviations:* LNA = α-linolenic acid; EPA = eicosapentaenoic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; *contains 20:3n-9.

The desaturation products of 16:0, *i.e.* 16:1n-9 and 16:1n-7, increased with the graded supplementation of 16:0+18:1n-9 (R^2 = 0.493; p = 0.035 and R^2 = 0.674; p = 0.007, respectively), as well as the desaturation product of 18:1n-9, *i.e.* 18:2n-9 (R^2 = 0.635; p = 0.010).

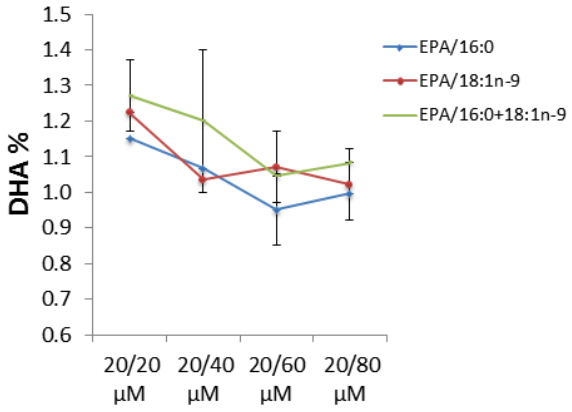
The following figures summarise the results obtained in the current Chapter focusing on LC-PUFA cellular levels following the supplementation of FA combinations. Figure 5.1 shows the EPA levels in CHSE-214 cells incubated with 20 µM of LNA and graded concentrations of 16:0, 18:1n-9, and 16:0+18:1n-9. The highest EPA level was achieved with the supplementation of LNA only, at 20 µM, and the lowest with LNA and 18:1n-9 at 20/40 µM. EPA levels were higher with the supplementation of LNA with 16:0, and 16:0+18:1n-9, compared with the combination of LNA and 18:1n-9. The ANOVA indicates that cellular EPA were affected by the treatment (FA mix supplemented), the concentration and the interaction between them (p < 0.05).



ANOVA		
Fatty acid supplemented	Concentration	Interaction
<0.000	<0.000	<0.000

Figure 5.1. EPA levels of CHSE-214 cells after being incubated for five days with different combinations and concentrations of fatty acids. *Abbreviations:* LNA = α-linolenic acid; EPA = eicosapentaenoic acid. Data were analysed by two-way ANOVA (p < 0.05). The interaction between the two factors (fatty acid supplemented and concentration) was also analysed (inlet table).

Figure 5.2 shows the levels of DHA of CHSE-214 cells incubated with 20 µM of EPA and graded concentrations of 16:0, 18:1n-9, and 16:0+18:1n-9 mix. In all treatments the DHA levels were lower than ~1.3%. The highest level of DHA was recorded with the combination EPA/16:0+18:1n-9 at 20/20 µM, and the lowest with EPA/16:0 at 20/60 µM. Figure 5.3 shows that the highest level of DHA occurred with the combination LNA+EPA/16:0 at 40/20 µM. In the combination LNA+EPA/18:1n-9, levels of DHA decreased with the graded supplementation of 18:1n-9. The supplementation of LNA+EPA/16:0+18:1n-9 at all concentration resulted in levels of DHA lower than 1%. In both figures, the ANOVA indicates that cellular DHA levels were affected by the treatment and the concentration of the FA supplemented ($p < 0.05$) but the effect of treatment-concentration interaction was only observed in Figure 5.3 ($p < 0.05$).

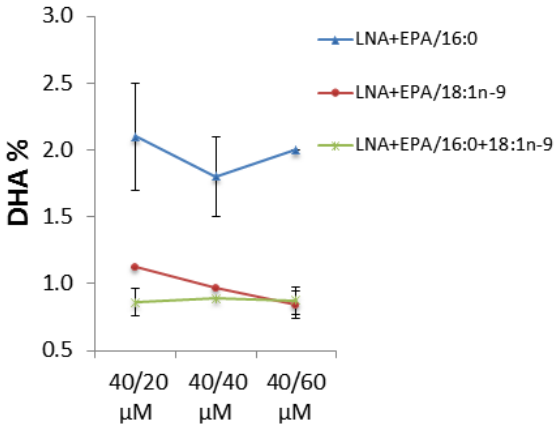


ANOVA		
Fatty acid supplemented	Concentration	Interaction
0.010	<0.000	0.556

Figure 5.2. DHA levels of CHSE-214 cells after being incubated for five days with different combinations and concentrations of fatty acids. *Abbreviations:* EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid. Data were analysed by two-way ANOVA ($p < 0.05$). The interaction between the two factors (fatty acid supplemented and concentration) was also analysed (inlet table).

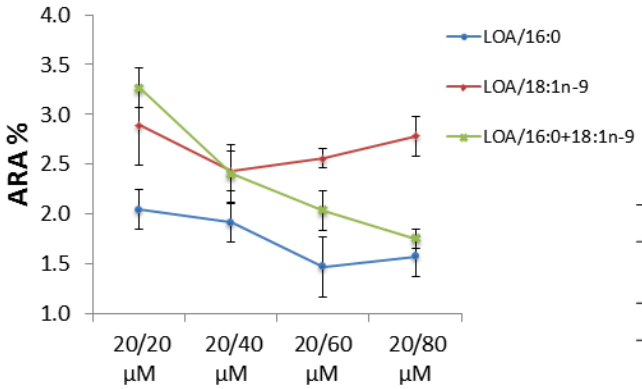
Figure 5.4 shows the ARA levels in CHSE-214 cells incubated with 20 µM of LOA and graded concentrations of 16:0, 18:1n-9, and 16:0+18:1n-9 mix. The highest ARA level was achieved by supplementing LOA/16:0+18:1n-9 at 20/20 µM, whereas the lowest was recorded with LOA/16:0 at 20/60 µM. ARA levels decreased with the graded

supplementation of 16:0+18:1n-9 in presence of 20 µM of LOA. The FA combination supplemented, the concentration and the interaction of these two had an effect on cellular ARA levels ($p < 0.05$)



ANOVA		
Fatty acid supplemented	Concentration	Interaction
<0.000	0.011	<0.000

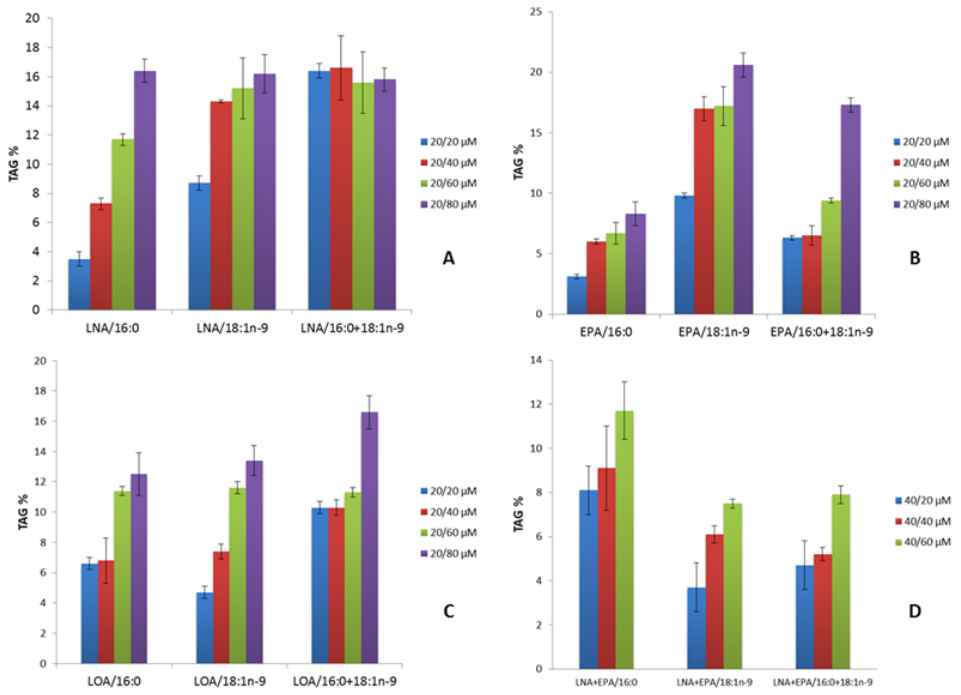
Figure 5.3. DHA levels of CHSE-214 cells after being incubated for five days with different combinations and concentrations of fatty acids. *Abbreviations:* LNA = α-linolenic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid. Data were analysed by two-way ANOVA ($p < 0.05$). The interaction between the two factors (fatty acid supplemented and concentration) was also analysed (inlet table).



ANOVA		
Fatty acid supplementation	Concentration	Interaction
<0.000	<0.000	0.011

Figure 5.4. ARA levels of CHSE-214 cells after being incubated for five days with different combinations and concentrations of fatty acids. *Abbreviations:* LOA = linoleic acid; ARA = arachidonic acid. Data were analysed by two-way ANOVA ($p < 0.05$). The interaction between the two factors (fatty acid supplemented and concentration) was also analysed (inlet table).

Figure 5.5A shows the increment of TAG with the graded supplementation of the FA, except for the combination LNA/16:0+18:1n-9, where TAG levels were about 16% from 20/20 µM to 20/80 µM. The lowest levels of TAG were recorded with the supplementation of EPA/16:0 at 20/20 µM, whereas the highest were recorded with EPA/18:1n-9 at 20/80 µM (Figure 5.5B).



ANOVA		
Fatty acid supplementation	Concentration	Interaction
<0.000	<0.000	<0.000

Figure 5.5 A-D. TAG levels of CHSE-214 cells after being incubated for five days with different combinations and concentrations of fatty acids. *Abbreviations:* TAG = triacylglycerol; LNA = α-linolenic acid; EPA = eicosapentaenoic acid; LOA = linoleic acid. Data analysed by two-way ANOVA ($p < 0.05$). The interaction between the two factors (fatty acid supplemented and concentration) was also analysed (inlet table).

The increment of TAG with the supplementation of 20 µM of LOA and graded concentrations of SFA, MUFA and SFA+MUFA is shown in Figure 5.5C. The supplementation of LNA+EPA combined with 16:0 resulted in higher TAG levels, in

comparison with the combinations LNA+EPA/18:1n-9 and LNA+EPA/18:1n-9+16:0 (Figure 5.5D). The highest level of TAG was observed with LNA+EPA/16:0 at 40/60 µM, while the lowest with LNA+EPA/18:1n-9 at 40/20 µM. The ANOVA suggests that cellular TAG levels were affected by the treatment (FA combination supplemented), the concentration of the FA supplemented and the interaction between these two ($p = 0.05$).

5.4. Discussion

The primary objective of the present study was to investigate the effect of lipid level on the LC-PUFA biosynthetic pathway, using SFA (16:0) and MUFA (18:1n-9) (which do not compete with C_{18} PUFA for the pathway) to represent "dietary" lipid in CHSE-214 cell line. Cells were incubated for 5 d with either LNA or LOA, in presence of graded concentrations of 16:0, 18:1n-9, and the combination of the last two as a mix (1:1 ratio). The effects of the FA supplementation on the FA profile and lipid class composition of CHSE-214 cells were determined by standard techniques.

5.4.1. Effect of the supplementation with SFA and MUFA in combination with PUFA on lipid content of CHSE-214

Since *in vivo* trials in Atlantic salmon have often shown increased tissue lipid contents with increased levels of dietary lipids (Bell *et al.*, 1998; Hemre and Sandnes, 1999; Martinez-Rubio *et al.*, 2013), an increment in cell lipid content with the graded supplementation of SFA, MUFA and SFA+MUFA was expected in the current study. However, this trend was only observed with the supplementation of LNA+EPA/18:1n-9, but results were not statistically significant ($R^2 = 0.072$; $p = 0.486$), whereas in other treatments, such as LOA/16:0 and LNA+EPA/16:0, lipid content decreased with the graded supplementation of 16:0. This may be related to the high levels of supplementation

(100 µM) being toxic, which causes cell death and, therefore, a lower lipid content recovered from the flask harvested.

5.4.2. Effect of supplementation of FA on lipid class composition of CHSE-214

In the lipid class data, the only clear trends were observed in cell TAG content, whilst changes in other lipid classes were probably just the consequences of the changes in TAG. In CHSE-214 cells incubated with C_{18} PUFA and EPA in presence of 16:0, 18:1n-9 and the mix 16:0+18:1n-9, increased TAG with the graded supplementation of SFA, MUFA and SFA+MUFA was observed, except for treatment LNA/16:0+18:1n-9, where TAG was about 15% in all supplemented concentrations. This is in agreement with Martinez-Rubio *et al.* (2013), who reported that TAG levels in liver of Atlantic salmon increased with the graded increased dietary lipid content.

5.4.3. Effect of supplementation of combinations of FA on FA composition of CHSE-214 cell line

Tocher *et al.* (1988) observed that the FA profile of six cell lines, including RTG-2 (rainbow trout gonad), BF-2 (bluegill fry), FHM (fathead minnow), AS (Atlantic salmon), CHSE-214 (Chinook salmon embryo) and TF (turbot fin) reflected the FA composition of the media. In the current study, the FA profile of the CHSE-214 cell line largely reflected the combination of FA supplemented to the cultures. All combinations of FA changed the FA compositions of the cells due partly to the incorporation of the FA supplemented themselves and partly due to endogenous metabolism of the cells, through conversion and the production of intermediate metabolites including desaturated and/or elongated products. Consistent with this, *in vivo* trials have reported that the FA profile of the diet is reflected in the FA profile of fish flesh and tissues (Bell *et al.*, 2003b; Tocher *et al.*, 2003a;

b; Menoyo *et al.*, 2005; Tocher, 2010; Alves Martins *et al.*, 2011; Xu *et al.*, 2014; Betancor *et al.*, 2015).

5.4.3.1. EPA production from LNA mixed with SFA (16:0), MUFA (18:1n-9) and SFA+MUFA (16:0+18:1n-9)

The EPA levels of CHSE-214 cells incubated with LNA in presence of SFA, MUFA and SFA+MUFA mix (1:1) showed a clear trend to decrease with the graded supplementation of the SFA and SFA+MUFA, whereas similar levels of EPA were recorded in cells incubated with LNA/16:0+18:1n-9 at all concentrations, ranging from 1.5–1.8% without a clear trend. The highest EPA level was recorded with the supplementation of LNA/16:0 at 20/20 µM, while the lowest was observed with the supplementation of LNA/18:1n-9 at 20/40 µM. The data obtained in the present study were in agreement with previous *in vivo* trials, where Atlantic salmon fed low lipid diets had higher levels of n-3 LC-PUFA (EPA and DHA) in comparison with fish fed high lipid diets (Martinez-Rubio *et al.*, 2013) and with other studies reporting higher synthesis of LC-PUFA in liver of Atlantic salmon fed low lipid diet, in comparison with salmon fed high lipid diets (Tocher *et al.*, 2003a; b).

5.4.3.2. DHA production from EPA and EPA+LNA mixed with SFA (16:0), MUFA (18:1n-9) and SFA+MUFA (16:0+18:1n-9)

The adverse effect of lipid content (SFA and MUFA) described on the conversion of LNA to EPA was not clearly observed on the conversion of EPA to DHA, which may be due to the low ability of the CHSE-214 cell line to convert EPA to DHA, as has been previously reported in other fish cell lines (Tocher and Sargent, 1990; Gregory *et al.*, 2011).

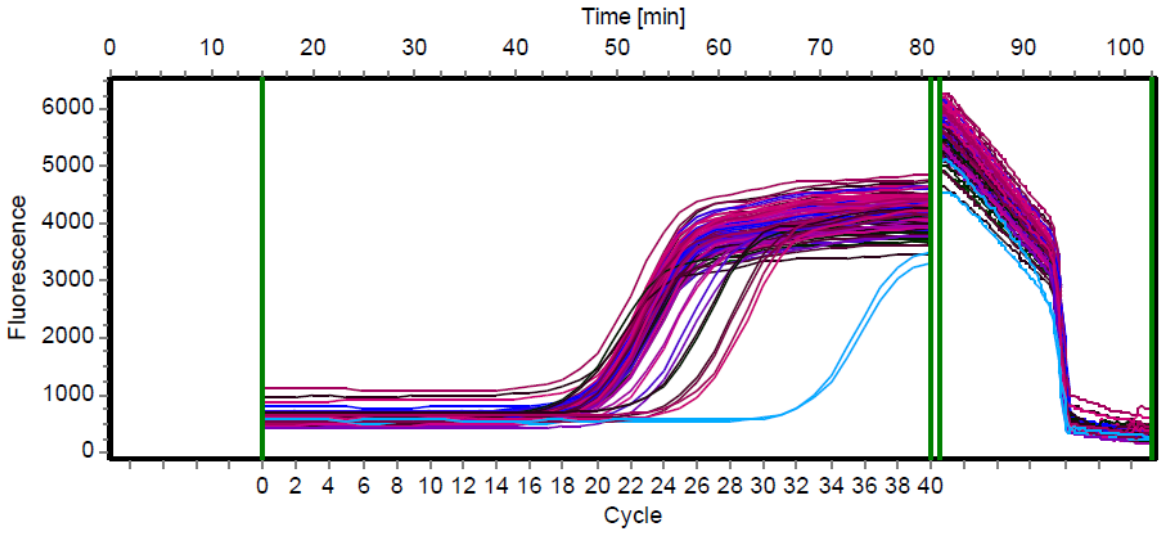
5.4.3.3. ARA production from LOA mixed with SFA (16:0), MUFA (18:1n-9) and SFA+MUFA (16:0+18:1n-9)

There is insufficient evidence supporting the fact that lipid content has a detrimental effect on the conversion of LOA to ARA. The main detrimental effect has been associated with the enzyme competition between C_{18} PUFA *i.e*. LOA and LNA, as it has been reported that the enzymes involved in the pathway of LC-PUFA synthesis prefer the n-3 series over the n-6 series (Bell *et al.*, 2001, 2002; Bell and Sargent, 2003). In the current study, the ARA levels steadily decreased with increasing concentrations of 16:0+18:1n-9 (*i.e.* 3.3% (20/20 μM) →2.4% (20/40 μM) →2.0% (20/60 μM) →1.8% (20/80 μM)), while in the other two treatments, LOA/18:1n-9 and LOA/16:0, the ARA levels remained relatively constant, although a slight fall was observed, without showing any detrimental effect of the supplementation SFA, MUFA and the combination of the last two, on the conversion of LOA to ARA. The highest ARA level was achieved in cells incubated with LOA/16:0+18:1n-9 at 20/20 μM (3.3%), while the lowest with LOA/16:0 at 20/60 μM (1.5%).

To the best of our knowledge, the current *in vitro* study is the first to highlight that lipid content (represented by increased supply of SFA and MUFA) negatively affected the conversion of LNA to EPA in cell culture system. However, there is insufficient evidence to support the hypothesis that the conversion of LOA to ARA was affected by lipid content. The data produced in the present study highlight the importance of lipid content in fish feeds in determining the final LC-PUFA profile in salmonids and that dietary lipid content is a factor for consideration when formulating diets to optimise endogenous production of EPA and DHA in low marine feeds.

Chapter 6

Molecular mechanisms involved in the conversion of α-linolenic acid to eicosapentaenoic acid in CHSE-214 cells



An example of the fluorescence profile of cDNA of CHSE-214 cell line incubated with different fatty acids [original image]

"Learn from yesterday, live for today, hope for tomorrow. The important thing is to not stop questioning."

Albert Einstein

6.1. Introduction

One approach to study the pathways of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis, *i.e.* eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), was through the formulation of experimental diets deficient in LC-PUFA metabolic precursors, and later refined with the use of radiolabeled fatty acids (FA) substrates in *in vitro* cell studies using established cell lines and isolated (including primary) cell cultures (Tocher, 2003). However, the advent of molecular biology techniques and their availability has made it possible to significantly further progress the study of LC-PUFA biosynthesis in fish (Monroig *et al.*, 2011b). Nowadays, it is recognised that essential fatty acid (EFA) requirements of a particular fish species depend on the ability of the fish to synthesise LC-PUFA, supported by the presence of the required genes and consequent enzymatic processes, and regulated by other key genes (Tocher, 2010).

The aquaculture industry and, more specifically, the diet composition of farmed Atlantic salmon (*Salmo salar* L.) has been changing rapidly because of the need to find sustainable alternatives to marine raw materials, particularly fish oil (FO), which is an excellent and virtually unique source of n-3 LC-PUFA. The replacement of marine ingredients, such as FO, by terrestrial ingredients such as vegetable oil (VO), is one of the most prominent changes. Although total replacement of FO by VO barely affects the growth of Atlantic salmon (Torstensen *et al.*, 2005), major effects have been reported on the expression and regulation of genes involved with fatty acid and cholesterol metabolism (Leaver *et al.*, 2008; Morais *et al.*, 2009). These effects were mainly related to the low dietary levels of n-3 LC-PUFA, which are completely absent in VO (Leaver *et al.*, 2008). Salmonids have the capacity to synthesise LC-PUFA from the C_{18} EFA, α-linolenic acid (LNA; 18:3n−3) and linoleic acid (LOA; 18:2n−6) and, therefore, salmonids can always have an indirect ”dietary” supply of LC-PUFA provided at least the C_{18} EFA present in the

diet (Castell *et al.*, 1972; Bell *et al.*, 1993; Tocher *et al.*, 2000). The enzymatic conversion of polyunsaturated fatty acids (PUFA), LNA and LOA, to n-3 and n-6 LC-PUFA, respectively, is carried out by fatty acyl desaturases, specifically the Δ6 and Δ5 fatty acyl desaturases (Fads2d6 and Fads2d5, respectively), catalysing the introduction of a double bond into the acyl chain at the Δ6 and Δ5 positions, and elongases of very long-chain fatty acids, *elovl5* and *elovl2*, that add two carbon atoms to a pre-existing fatty acyl chain (Monroig *et al.*, 2011b). It has been suggested that *elovl2* is primarily involved in the elongation of C_{20} to C_{22}, while *elovl5* participates in the elongation of C_{18} to C_{20} PUFA and, to a lesser extent, in the elongation of C_{20} to C_{22} PUFA (Morais *et al.*, 2009).

Transcription factors, such as sterol regulatory element binding protein (Srebp) 1 and 2, liver X receptor (Lxr), peroxisome proliferator activated receptors (Ppar) α and β, and retinoid X receptors (Rxr) are involved in the regulation of genes of LC-PUFA biosynthesis (Minghetti *et al.*, 2011; Carmona-Antoñanzas *et al.*, 2014). In mammals, three LXR have been identified LXRs, LXRα and LXRβ, these nuclear receptors regulate cholesterol and lipid metabolism. The activation of LXR decreases the synthesis and absorption of cholesterol (Repa *et al.*, 2000; Schultz *et al.*, 2000), increases the expression of genes involved in reverse cholesterol transport and mobilisation of cholesterol (Repa *et al.*, 2002), and stimulates the expression of *SREBP1c* (Schultz *et al.*, 2000), which in turn activates genes involved in lipogenesis and triglyceride metabolism (Jung *et al.*, 2011). In mammals, three PPAR have been identified: PPARα, PPARβ and PPARγ. The activation of PPAR occurs when these bind FA or their oxidised derivatives, and then regulate the expression of genes involved in lipid degradation and biosynthesis (Carmona-Antoñanzas *et al.*, 2014). RXR are involved in the regulation of cell growth, development, survival, cell differentiation and cell death (Dawson and Xia, 2012). When they form heterodimers with other nuclear receptors, they play roles in multiple metabolic systems (Pérez *et al.*, 2012).

In recent years some of these genes have also been successfully cloned and studied in fish, including salmonids and particularly Atlantic salmon (Leaver *et al.*, 2008; Cruz-Garcia *et al.*, 2009; Minghetti *et al.*, 2011; Carmona-Antoñanzas *et al.*, 2014).

Other genes involved with FA metabolism are: fatty acid synthase (*FAS*), carnitine palmitoyl transferase 1 (*CPT1*), acyl coenzyme A oxidase (*ACO*), and fatty acid-binding protein (*FABP*) (Haunerland and Spener, 2004; Tang *et al.*, 2013). FAS is a key enzyme in lipogenesis, using malonyl-CoA to produce palmitate, whereas CPT1 transport FA into the mitochondria and is reported to be a rate-limiting-step in mitochondrial fatty acid β-oxidation (McGarry and Brown, 1997). *ACO* oxidases acyl chains, which is the first step in peroxisomal fatty acid β-oxidation (Lazarow and de Duve, 1976). FABP have different functions such as cellular uptake and transport of FA, regulation of growth and gene expression, and targeting of FA to metabolic pathways (Haunerland and Spener, 2004).

The LC-PUFA play several roles in different fish tissues (Betancor *et al.*, 2014). In Atlantic salmon, the liver is the main organ where LC-PUFA biosynthesis and lipid metabolism take place (Monroig *et al.*, 2010), but the function of other tissues such as brain, retina, head kidney and gills, are influenced by the dietary intake of LC-PUFA (Bell *et al.*, 1992, 1995, 1996b; Waagbø, 1994; Lall, 2000). Moreover, LC-PUFA are precursors of bioactive molecules involved in homeostasis, immune and inflammatory responses, and cell signaling. The adverse effects of long-term deficiency in dietary LC-PUFA have being studied in Atlantic salmon (Martinez-Rubio *et al.*, 2012; Carmona-Antoñanzas *et al.*, 2014), but the information available are still limited compared to mammals. Nutritional studies in C57BL/6 mice reported that diets rich in n-3 LC-PUFA, formulated with FO, decreased serum triglycerides, inhibited hepatic lipogenesis, and stimulated FA oxidation in liver (Rustan *et al.*, 1988; McKenney and Sica, 2007), whereas diets rich in saturated FA

induced hepatic steatosis, non-alcoholic fatty liver disease and non-alcoholic fatty pancreatic disease (Lin *et al.*, 2005; Oosterveer *et al.*, 2009; Fraulob *et al.*, 2010).

The present study aims to develop and utilise a multi-well plate system suitable for investigating the expression and regulation of genes involved in lipid and FA metabolism, with particular focus on LC-PUFA biosynthesis, specifically the conversion of LNA to EPA (20:5n-3), in the cell culture model system, CHSE-214 cell line. The results obtained previously in Chapters 3-5 from the investigation of how fatty acid supplementation affected cellular lipids and fatty acid composition highlighted that the CHSE-214 cell line was able to produce EPA from LNA and also how competing PUFA and SFA/MUFA (as a proxy for dietary lipid content) affected this conversion. Among the many combinations investigated previously a carefully chosen selection of FA treatments (combinations of LNA and other competing FA), including those which showed the highest EPA levels were chosen for the current Chapter. In the present chapter the effects of these combinations of supplemented FA on the expression of a selected range of key genes involved in the regulation of lipid and FA metabolism including, in particular, LC-PUFA biosynthesis were determined. The present study represents the first to specifically explore the molecular mechanisms involved in the conversion of LNA to EPA, and the effects of SFA, MUFA and PUFA supplementation on EPA production in an *in vitro* cell culture model system.

6.2. Materials and Methods

The present study consisted of three experimental phases: 1) a time-course experiment designed to determine the effect of incubation time on gene expression. This was carried out in order to determine the optimal time for supplementation of the CHSE-214 with FA before analysis of gene expression so that changes in the expression of genes

involved in lipid and LC-PUFA metabolism could be detected; 2) an analysis of the effects of the selected FA supplementations and their combinations on expression of genes of lipid and FA metabolism and their regulation. The ultimate aim and predicted output was to use these data to develop a molecular framework or model for the control and regulatory mechanisms of LC-PUFA biosynthesis; and, 3) a series of experiments designed to test the regulatory framework. In order to test the model created in Phase 2, a new, previously untested, set of experiments (particular combinations of FA) were designed in Phase 3 and the results of the analysis in terms of both final outcomes (cellular lipid and FA compositions) and gene expression predicted before testing. Both lipid and FA compositions of the cells and the expression of genes were determined in order to test if the predictions based on the model were accurate and, based on the results, the model revised accordingly if necessary.

6.2.1. Cells and routine culture procedures

Details of the CHSE-214 cell line, the media and the routine culture procedures have been previously described in Chapter 2, sections 2.1.1 and 2.1.2. To provide experimental conditions for gene expression analyses, CHSE-214 were seeded into six-well tissue culture plates (Sarstedt Ltd., Leicester, UK). Cells in each well were maintained in 3 ml of L-15 medium containing 5% FBS and 200 µM L-glutamine. For lipid analyses the experimental conditions were the same as described in Chapter 2, section 2.1.2. In all cases CHSE-214 were incubated at 20ºC.

6.2.2. FA supplementation for gene expression analyses

LNA and LOA (99% purity) were supplemented as complexes bound to BSA (First Link Ltd., Wolverhampton, UK) and suspended in PBS (Spector and Hoak, 1969). Full

details of the procedure are provided in Chapter 2, section 2.2.1. FA concentration was determined as previously described in Chapter 2, section 2.3.3.

6.2.3. Experimental design: Phase 1, Phase 2 and Phase 3

Cells were harvested from the 75 cm^2 tissue culture flasks (see section 2.1.2.2), counted (see section 2.1.2.4) and then seeded into six-well plates at a density of 1.6×10^6 cells per well. FA were supplemented 24 h later, once the cells were attached and at about 80% confluent and cells were incubated at 20ºC. The plates were incubated with the FA for the various times: 24 h, 48 h and 5 d for Phase 1 and 48 h for Phases 2 and 3. Two plates per treatment were required to produce six replicate samples of two pooled wells, as previously suggested by other authors (Minghetti *et al.*, 2011). Each well contained 3 ml of L-15 media. After the incubation time, cells were washed twice with 1 ml DPBS per well, and then harvested by trypsinisation. Cell detachment was stimulated by a hand tap and the detached cells centrifuged and the pellet re-suspended in 0.7 ml of DPBS. Combined cell suspensions from two wells were further washed by collecting in a 1.5 ml micro-centrifuge tube on ice and the tubes centrifuged to form cell pellets. The supernatant DPBS solution was decanted and 1 ml of TRI Reagent® RNA extraction buffer (Sigma-Aldrich® Ltd., Dorset, UK) was added to each sample. Samples were stored at -70ºC for further RNA extraction.

For the experiments in Phase 1 CHSE-214 cell line was seeded into six-well plates and individual wells were supplemented with either LNA or LOA at a final concentration of 20 µM, whilst for the control-wells fatty acid-free bovine serum albumin in phosphate buffer solution (FAF-BSA-PBS) was used. Tables 6.1 and 6.2 list the experiments carried out during Phases 2 and 3, respectively. In both Phases, 20 µM of LNA were used as

control. The set of experiments in Phase 3 was designed to predict the results in terms of cellular lipid and FA compositions, and the expression of genes involved in lipid and FA metabolism, based on the data produced and the model developed in the second phase of this study. The lipids of CHSE-214 were analysed after the supplementation of different concentrations and combinations of FA for 5 d.

Table 6.1. Fatty acid combinations and concentration supplemented to CHSE-214 cell line for gene analysis during the Phase 2 experiments

Fatty acids	Low concentration (µM)	High concentration (µM)
LNA/16:0	A) 20/20	B) 20/80
LNA/18:1n-9	C) 20/20	D) 20/80
LNA/16:0+18:1n-9 (1:1)	E) 20/20	F) 20/80
LNA/LOA	G) 20/5	H) 20/20
LOA/LNA	I) 20/5	H) 20/20
LNA/EPA		J) 20/20
LNA/DHA		K) 20/20

Abbreviations: LNA = α-linolenic acid; LOA = linoleic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid.

Table 6.2. Fatty acid combinations and concentration supplemented to CHSE-214 cell line for gene analysis during the experimental Phase 3

Fatty acids	Concentration (µM)			
LNA+LOA/16:0	L) 20+15/20	M) 20+15/40	N) 20+15/60	
LNA+LOA/18:1n-9	O) 20+15/20	P) 20+15/40	Q) 20+15/60	
LNA/LOA+16:0	R) 20/5+5	S) 20/10+10	T) 20/15+15	U) 20/20+20

Abbreviations: LNA = α-linolenic acid; LOA = linoleic acid.

6.2.4. Gene expression analyses

For Phase 1 the expression of five genes was analysed (*fads2d5, cpt1*, *elovl2, pparα, srebp*), whereas for Phase 2 and 3 the expression of 16 genes including those involved in LC-PUFA biosynthesis (*fads2d5*, *fads2d6, elovl2 and elovl5*), transcription factors (*srebp1*, *srebp2*, *lxr*, *pparα*, *pparβ* and *rxr*), those involved in FA metabolism (*fas*, *cpt1*, *fabp* and *aco*) and two reference genes (*β-act* and *ef1α*) was determined by qPCR analyses. Details of the primers are provided in Table 6.3. Primers designed in the very

close species Atlantic salmon were used for *fads2d5*, *elovl2*, *srebp1*, *srebp2*, *lxr*, *pparα*, *pparβ*, *rxr*, *fas*, *cpt1*, *fabp*, *aco*, *β-act* and *ef1α*.

Table 6.3. Forward (F) and reverse (R) primers used for qPCR analysis of CHSE-214 cell line

Gene	Primer sequence (5'-3')	Fragment (bp)	T°	Source
LC-PUFA BIOSYNTHESIS				
fads2d5	F: GTGAATGGGGATCCATAGCA R: AAACGAACGGACAACCAGA	192	56ºC	Hastings *et al.* (2005)
fads2d6	F: CCATCGCAGATTAACGACCT R: AACACTTTCAGACAATGCGATTA	171	59ºC	current study
elovl2	F: CGGGTACAAAATGTGCTGGT R: TCTGTTTGCCGATAGCCATT	145	60ºC	Morais *et al.* (2009)
elovl5	R: TCAGCTGGCCTTGTGTGA F: ACTCCTACTTTGGTGCCTCC	135	59ºC	current study
TRANSCRIPTION FACTORS				
srebp1	F: GCCATGCGCAGGTTGTTTCTTCA R: TCTGGCCAGGACGCATCTCACACT	151	63ºC	Minghetti *et al.* (2011)
srebp2	F: GACAGGCACAACACAAGGTG R: CAGCAGGGGTAAGGGTAGGT	215	60ºC	Leaver *et al.* (2008)
lxr	F: AGGACCATGAACTGGTGGAG R: CGAAGACCTGCTCAGAGTGG	210	58ºC	current study
pparα	F: TCCTGGTGGCCTACGGATC R: CGTTGAATTTCATGGCGAACT	111	60ºC	Kleveland *et al.* (2006)
pparβ	F: GAGACGGTCAGGGAGCTCAC R: CCAGCAACCCGTCCTTGTT	151	60ºC	Kleveland *et al.* (2006)
rxr	F: CACCAAACTGCAAACAGGAA R: CGGACAAGTTGACAAGCAGA	113	52ºC	current study
FA METABOLISM				
fas	F: CTCTCTCATGCCCAGTCACA R: TTTCCGCCATCTCCAGATAC	189	60ºC	current study
cpt1	F: CCTGTACCGTGGAGACCTGT R: CAGCACCTCTTTGAGGAAGG	212	60ºC	Leaver *et al.* (2008)
aco	F: AAAGCCTTCACCACATGGAC R: TAGGACACGATGCCACTCAG	230	60ºC	Leaver *et al.* (2008)
fabp	F: ACCACCATCATCGAGGTAGC R: CACTTTTGCACGTGAACCAT	176	59ºC	current study
REFERENCE				
β-act	F:TATCCACGAGACCACCTACA R:ATCCAGACGGAGTATTTACG	204	56ºC	Peña *et al.* (2010)
ef1α	F: GTCTACAAAATCGGCGGTAT R: CTTGACGGACACGTTCTTGA	198	56ºC	Peña *et al.* (2010)

Abbreviations: fads2d5 = Δ5 fatty acyl desaturase; *fads2d6* = Δ6 fatty acyl desaturase; *elovl2* = fatty acyl elongase 2; *elovl5* = fatty acyl elongase 5; *srebp1* = sterol regulatory element binding protein 1; *srebp2* = sterol regulatory element binding protein 2; *lxr* = liver X receptor; *pparα* = peroxisome proliferator activated receptor α; *pparβ* = peroxisome proliferator activated receptor β; *rxr* = retinoid X receptor; *fas* = fatty acid synthase; *cpt1* = carnitine palmitoyl transferase 1; *aco* = acyl Co-A oxidase; *fabp* = fatty acid binding protein; *β-act* = β-actin; *ef1α* = elongation factor 1α.

The PCR products obtained were purified (QIAquick, Quiagen, Manchester, U.K.) and consequently sequenced (Sanger ABI3730xl, GATC Biotech, Konstanz, Germany) to check the correspondence with the gene of interest. Sequences corresponding to the open reading frame of *fads2d6* and *elovl5* from several fish species were aligned and primers designed on common conserved regions. After sequencing (Sanger ABI3730xl, GATC Biotech, Konstanz, Germany) the open reading frame, primers for qPCR were designed using Primer3 software (Rozen and Skaletsky, 2000).

6.2.5. RNA extraction

Samples were defrosted and incubated at room temperature for 15 min prior to extraction. The cell pellets were re-suspended in TRI Reagent® and homogenised with a mini beadbeater (Biospec Inc.-Thistle Scientific, Uddingstone, Glasgow,U.K.) for 30 s. Once the pellet was completely disrupted, 100 µl of 1-bromo-3 chloropropan (BCP) were added and samples shaken vigorously by hand for 15 s, and incubated for further 15 min at room temperature. Samples were centrifuged at 14000 g for 15 min at 4ºC and after centrifugation, three layers were visible in the microcentrifuge tubes. Only 450 µl of the upper layer was carefully collected to avoid disturbing the interface, and transferred into a fresh 1.5 ml microcentrifuge tube. A total of 250 µl of cold RNA precipitation solution (1.2 M NaCl and 0.8 M sodium citrate sesquihydrate dissolved in 50 ml of distilled water), followed by 250 µl of cold isopropanol, were added to the samples to aid the precipitation of the RNA. The samples were mixed thoroughly by gently invertion (six times) and then incubated for 10 min at room temperature. The RNA pellet formed on the side/bottom of the tube following centrifugation at 14000 g for 15 min at 4ºC. The supernatant was discarded and RNA pellets washed twice with 1 ml portions of ice-cold 75% ethanol (v/v). Samples were briefly vortexed and centrifuged at 7500 g for 5 min at 4ºC. The 75%

ethanol was carefully removed by pipetting and the samples air dried for 5 min, until all ethanol traces were gone or the pellets became transparent. The RNA pellets were re-suspended in 30 µl of RNAse free water, samples incubated at room temperature for 30 min with a gentle flick every 10 min to aid the re-suspension of the RNA. The quantity of the RNA was measured by determination of the ratio of absorbances at 260/280 and 260/230 nm using a ND-1000 NanoDrop spectrophotometer (Labtech Int., East Sussex, UK), and the RNA quality was then assessed by gel electrophoresis using a 1% agarose gel with 0.5× tris base, acetic acid and EDTA (TAE) buffer and 0.8 µl of ethidium bromide at a concentration of 5 mg ml^{-1}. Gel images were produced using the Ingenius syngene bio imaging system (Cambridge, UK). The RNA samples were stored at -70ºC prior to further analyses.

6.2.6. Reverse transcription

Prior to reverse transcription being carried out, all RNA sample concentrations were re-measured in the NanoDrop. In sterile RNase-free 0.2 ml tubes, 2000 ng of each RNA sample were aliquoted, and RNase free water added in order to make the volume up to a total of 10 µl. Samples were heated at 75ºC for 5 min, and then cooled down on ice for 5 min for preventing secondary structures in RNA molecules. Once the samples were cold, the RT master mix was added, which included the following recipe: 2 µl of 10× RT buffer, 0.8 µl of 25× dNTP mix (100 mM), 1.5 µl of 10× RT random primers, 0.5 µl of Oligo DT primers, 1 µl of reverse transcriptase, and 4.2 µl of RNAse free water. The final solution contained, therefore, 10 µl of RNA and 10 µl of the RT master mix. Samples were vortexed and centrifuged at 460 g for 1 min. For every six experimental samples, two RT- (reverse transcription negative) tubes were prepared, as above, but without the enzyme multitranscribe reverse transcriptase, and adding 5.2 µl of RNAse free water instead of 4.2

µl. All tubes were transferred to the thermocycler (Biometra® Tgradient, Goettingen, Germany) and incubated at 40ºC for 10 min, followed by 50 min at 48ºC. The final step was inactivation of the RT enzyme by heating the samples at 70ºC for 15 min. The cDNA produced was diluted in 1:20 with RNase free water, and stored at - 20ºC prior to qPCR analysis.

6.2.7. Quantitative PCR (qPCR)

Initially the amplification efficiency of each primer pair was assessed by serial dilutions of the pooled cDNA (Morais *et al.*, 2011a). The PCR technique was used to determine the relative expression of genes involved in fatty acid metabolism and its regulation, using a Mastercycler® ep gradient S (Eppendorf AG, Hamburg, Germany). Samples in duplicates were loaded in 96-well plates in 20 µL reaction volumes, including 5 µL of cDNA (from the dilution 1:20), 1 µL of each primer (reverse and forward), 3 µL of milliQ water and 10 µL of SYBR Green RT-PCR Master Mix (Applied Biosystems, Paisley, UK). For the analysis of *β-act* and *ef1α*, 2 µL of cDNA were used and 6 µL of milliQ water, SYBR Green and primers were added in the same amount as previously mentioned. All amplifications were carried out with a negative control, which did not contain cDNA and the plates for reference genes also included 2 RT- samples. Plates were sealed using a Techne™ heat sealer (Bibby Scientific® Ltd, Stone, UK), mixed and centrifuged for 1 min at 460 g. Plates were placed into the qPCR machine Mastercycler® ep gradient S (Eppendorf AG) with the following program: 15 min at 95ºC, 40 cycles of 15 s at 95ºC, 30 s at the annealing temperature of the primers being used (see Table 2.2), and 30 s at 72ºC. Finally, a single cycle of 15 s at 95ºC, 15 s at 60ºC, 20 min for the melting curve, and 15 s at 95ºC, were performed. Data were registered and analysed using Eppendorf Mastercycler® ep *Realplex* software ver. 2.2.

6.2.8. Lipid class and FA composition

For lipid class and FA composition analyses of Phase 3, a parallel set of incubations to those described above in Table 6.2 were set up in 75 cm^2 tissue culture flasks as more experimental material was required for these analyses than easily provided by six-well plates. The specifications of cells and media, and preparation of the FA complexes were essentially as described in Chapter 2 section 2.1.2 and 2.2.1, respectively. The time-course was longer than for the gene expression studies as the predicted lipid and FA compositions were based on the experiments in Chapters 3-5 that were over time scale.

Cells were harvested followed by total lipid extraction according to Folch *et al.* (1957), using chloroform/methanol (C/M, 2:1, by volume) containing 0.05% of BHT. Full details of all these procedures are provided in Chapter 2, 2.1.2.2 and 2.3.1, respectively. Lipid class analyses were carried out as described in Chapter 2, section 2.3.2 according to Henderson and Tocher (1992). Fatty acid methyl esters (FAME) were prepared by acid-catalysed transmethylation (Christie, 2003), FAME purified by thin-layer chromatography (TLC), and quantified using a Fisons GC 8160 gas chromatograph (Fisons Ltd., Crawley, UK). Further details are provided in Chapter 2, section 2.3.3.

6.2.9. Statistical analysis

Results from gene expression analysis are presented as means ± SEM (standard error of the mean) with n = 6. The data were normalised expression ratios of the expression of the genes in CHSE-214 cell line incubated with the different FA, in relation to the CHSE-214 incubated with FAF-BSA-PBS (control) in Phase 1 and 20 µM of LNA in Phases 2 and 3. Graphics were created with Microsoft® Excel® and, statistical differences were determined by one-way analysis of variance (ANOVA), with post hoc multiple comparisons applied using Tukey's test, performed with IBM SPSS Statistics for

Windows® software, ver. 19.0 (Armonk, NY: IBM Corp.). In addition to the statistical analyses described above, for Phases 2 and 3 Cluster 3.0 was used to apply a supervised hierarchical clustering of the relative gene expression ratio for each gene, taking into consideration the PCR efficiency reaction and the Ct value of every sample compared to the control (Pfaffl, 2001). A heat map with the treatments and the gene expression was generated using TreeView software ver. 3.0 (Page, 1996).

6.3. Results

6.3.1. Phase 1: Time course experiment

Figure 6.1 shows the expression of genes for *fads2d5*, *elovl2*, *cpt1*, *pparα*, and *srebp1* in CHSE-214 cells individually incubated with LOA and LNA at 20 µM, and the control (FAF-BSA-PBS alone), over 24 h, 48 h, and 5 d. No significant differences were observed in the expression of the aforementioned genes between control and FA supplemented cultures after 24 h and 5 d of incubation ($p > 0.05$). However, significant differences were observed between the expression of the five genes determined in cells incubated with LNA and LOA in comparison with the control at 48 h ($p < 0.05$). Therefore, based on these data, all the gene expression studies in the following experiments in Phases two and three were carried after 48 h incubation with FA.

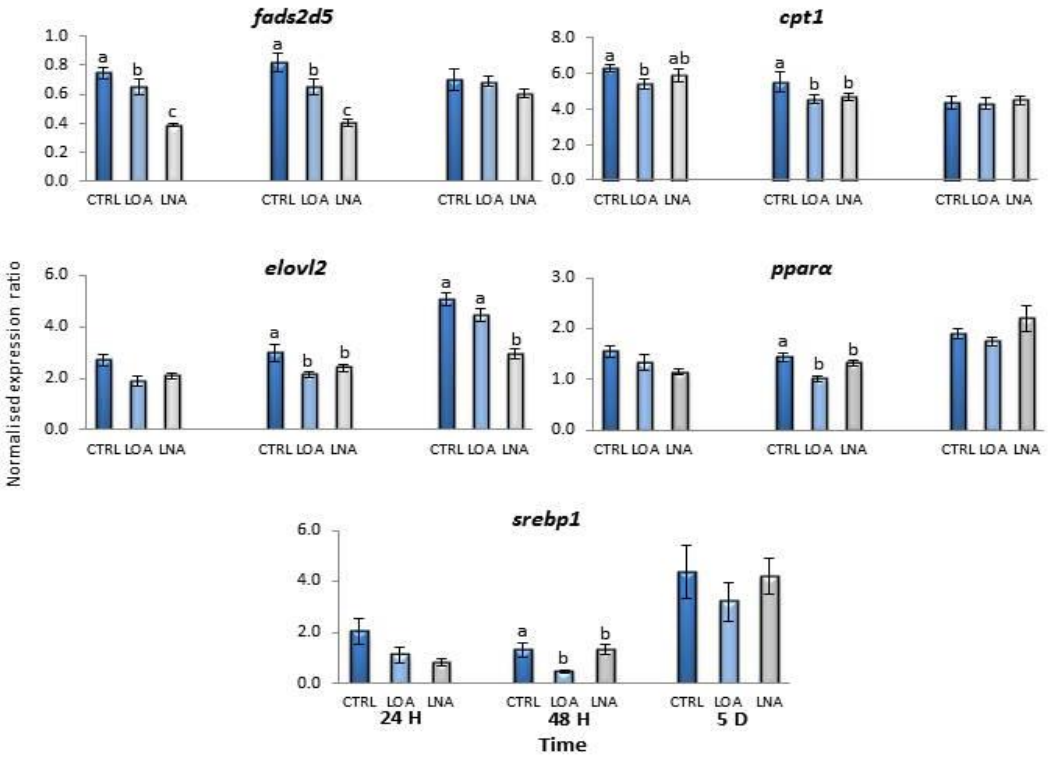


Figure 6.1. Expression of Δ5 fatty acyl desaturase (*fads2d5*), fatty acyl elongase 2 (*elovl2*), carnitine palmitoyl transferase 1 (*cpt1*), peroxisome proliferator activated receptor α (*pparα*) and, sterol regulatory element binding protein 1 (*srebp1*) in CHSE-214 cells incubated with 20 µM of α-linolenic acid (LNA), 20 µM of linoleic acid (LOA) and control (CTRL), for 24 h, 48 h and 5 days. Gene expression was measured by qPCR and results are presented normalised expression ratios (mean ± SEM, n = 6) of the expression of these genes in CHSE-214 incubated with LNA and LOA in relation to CHSE-214 incubated with FAF-BSA-PBS (CTRL). Superscript letters indicate significant differences ($p < 0.05$; one-way ANOVA, Tukey's test).

6.3.2. Phase 2: Gene expression analysis

Heat maps were chosen as a graphical approach to present and easily compare expression data for a reasonably large number of genes and treatments. Thus, the heat map in Figure 6.2 shows how the expression of genes involved in lipid and FA metabolism was affected by the supplementation of different combinations and concentrations of FA to CHSE-214 cells. The expression of all genes was up-regulated in CHSE-214 cells incubated with treatments D (18:3n-3/18:1n-9 at 20/80 µM) and E (18:3n-3/16:0+18:1n-9,

20/20 µM). Treatments B (18:3n-3/16:0, 20/80 µM) and F (18:3n-3/16:0+18:1n-9, 20/80 µM) led to up-regulation of all genes, except for *pparα*, which did not show any change.

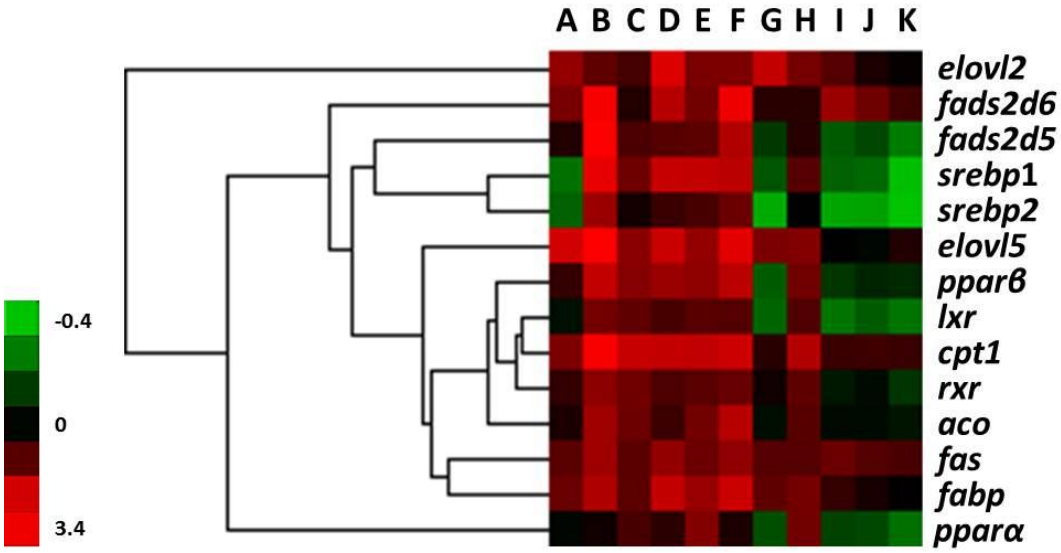


Figure 6.2. Heat map of the fourteen target genes analysed based on qPCR gene expression data. Columns represent mean relative expression data for each gene in CHSE-214 cells incubated with the different combinations and concentrations of fatty acid. Each row represents the effects of the different treatments on the expression of each individual gene. The expression level of each gene was squared-root normalised in relation to a control sample (20 µM of α-linolenic acid). Means are depicted by a colour scale, indicating low (green), neutral (black) or high (red) relative expression levels, as indicated by the colour bar on the left. Treatments: A = 18:3n-3/16:0 20/20 µM; B = 18:3n-3/16:0 20/80 µM; C = 18:3n-3/18:1n-9 20/20 µM; D = 18:3n-3/18:1n-9 20/80 µM; E = 18:3n-3/16:0+18:1n-9 20/20 µM; F = 18:3n-3/16:0+18:1n-9 20/80 µM; G = 18:3n-3/18:2n-6 20/5 µM; H = 18:3n-3/18:2n-6 20/20 µM; I = 18:2n-6/18:3n-3 20/5 µM; J = 18:3n-3/20:5n-3 20/20 µM; K = 18:3n-3/22:6n-3 20/20 µM. *Abbreviations*: *elovl2* = fatty acyl elongase 2; *fads2d6* = Δ6 fatty acyl desaturase; *fads2d5* = Δ5 fatty acyl desaturase; *srebp1* = sterol regulatory element binding protein 1; *srebp2* = sterol regulatory element binding protein 2; *elovl5* = fatty acyl elongase 5; *pparβ* = peroxisome proliferator activated receptor β; *lxr* = liver X receptor; *cpt1* = carnitine palmitoyl transferase 1; *rxr* = retinoid X receptor; *aco* = acyl Co-A oxidase; *fas* = fatty acid synthase; *fabp* = fatty acid binding protein and; *pparα* = peroxisome proliferator activated receptor α.

The most stable expression was observed in cells incubated with treatment J (18:3n-3/20:5n-3 20/20 µM), showing no change in eight out of 14 genes. Down-

regulation of the expression of *fads2d6*, *srebp1*, *srebp2*, *pparβ*, *lxr*, and *pparα* was observed in treatments G (18:3n-3/18:2n-6, 20/5 µM), I (18:2n-6/18:3n-3, 20/5 µM), J (18:3n-3/20:5n-3, 20/20 µM) and K (18:3n-3/22:6n-3, 20/20 µM). The detailed effects of the 11 different treatment conditions on the expression of each individual gene are shown in Figures 6.3 (genes of LC-PUFA biosynthesis), 6.4 (transcription factors) and 6.5 (other FA metabolism).

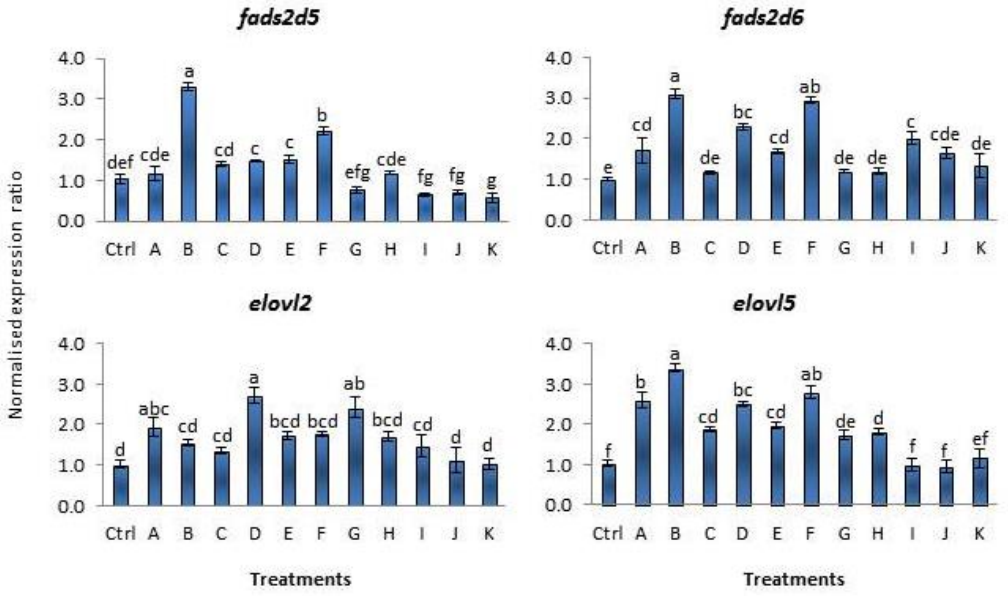


Figure 6.3. Expression of Δ5 fatty acyl desaturase (*fads2d5*), Δ6 fatty acyl desaturase (*fads2d6*), fatty acyl elongase 2 (*elovl2*) and fatty acyl elongase 5 (*elovl5*) in CHSE-214 cells incubated for 48 h with the following treatments: A = 18:3n-3/16:0 20/20 µM; B = 18:3n-3/16:0 20/80 µM; C = 18:3n-3/18:1n-9 20/20 µM; D = 18:3n-3/18:1n-9 20/80 µM; E = 18:3n-3/16:0+18:1n-9 20/20 µM; F = 18:3n-3/16:0+18:1n-9 20/80 µM; G = 18:3n-3/18:2n-6 20/5 µM; H = 18:3n-3/18:2n-6 20/20 µM; I = 18:2n-6/18:3n-3 20/5 µM; J = 18:3n-3/20:5n-3 20/20 µM; K = 18:3n-3/22:6n-3 20/20 µM, measured by qPCR. Results are normalised expression ratios (mean ± SEM, n = 6) of the expression of these genes in CHSE-214 cells incubated with different treatments in relation to cells incubated with 20 µM α-linolenic acid (Ctrl = control). Superscript letters indicate significant differences ($p < 0.05$; one-way ANOVA, Tukey's test).

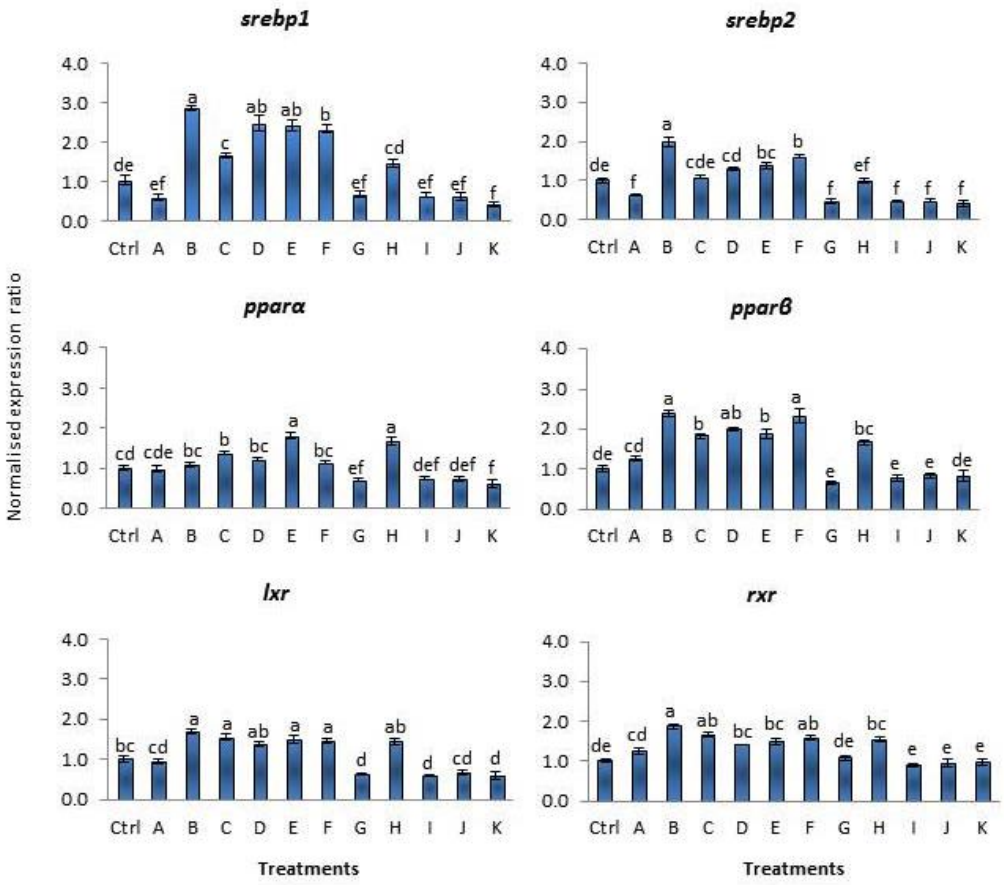


Figure 6.4. Expression of sterol regulatory element binding protein 1 (*srebp1*), sterol regulatory element binding protein 2 (*srebp2*), peroxisome proliferator activated receptor α (*pparα*), peroxisome proliferator activated receptor β (*pparβ*), liver X receptor (*lxr*) and retinoid X receptor (*rxr*) in CHSE-214 cells incubated for 48 h with the following treatments: A = 18:3n-3/16:0 20/20 µM; B = 18:3n-3/16:0 20/80 µM; C = 18:3n-3/18:1n-9 20/20 µM; D = 18:3n-3/18:1n-9 20/80 µM; E = 18:3n-3/16:0+18:1n-9 20/20 µM; F = 18:3n-3/16:0+18:1n-9 20/80 µM; G = 18:3n-3/18:2n-6 20/5 µM; H = 18:3n-3/18:2n-6 20/20 µM; I = 18:2n-6/18:3n-3 20/5 µM; J = 18:3n-3/20:5n-3 20/20 µM; K = 18:3n-3/22:6n-3 20/20 µM, measured by qPCR. Results are normalised expression ratios (mean ± SEM, n = 6) of the expression of these genes in CHSE-214 cells incubated with different treatments in relation to cells incubated with 20 µM α-linolenic acid (Ctrl = control). Superscript letters indicate significant differences ($p < 0.05$; one-way ANOVA, Tukey's test).

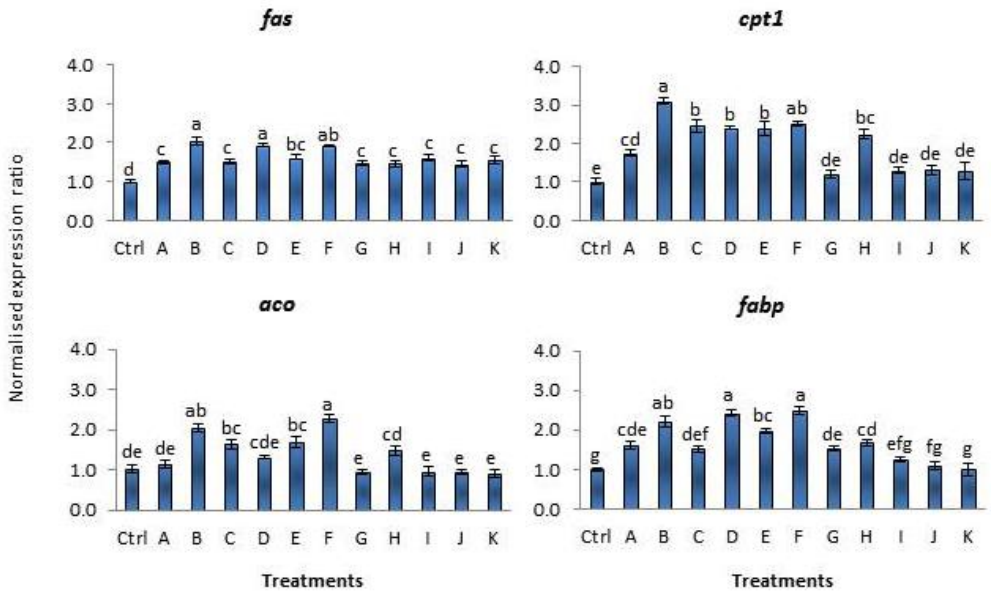


Figure 6.5. Expression of fatty acid synthase (*fas*), carnitine palmitoyl transferase 1 (*cpt1*) acyl Co-A oxidase (*aco*) and fatty acid binding protein (*fabp*) in CHSE-214 cells incubated for 48 h with the following treatments: A = 18:3n-3/16:0 20/20 µM; B = 18:3n-3/16:0 20/80 µM; C = 18:3n-3/18:1n-9 20/20 µM; D = 18:3n-3/18:1n-9 20/80 µM; E = 18:3n-3/16:0+18:1n-9 20/20 µM; F = 18:3n-3/16:0+18:1n-9 20/80 µM; G = 18:3n-3/18:2n-6 20/5 µM; H = 18:3n-3/18:2n-6 20/20 µM; I = 18:2n-6/18:3n-3 20/5 µM; J = 18:3n-3/20:5n-3 20/20 µM; K = 18:3n-3/22:6n-3 20/20 µM, measured by qPCR. Results are normalised expression ratios (mean ± SEM, n = 6) of the expression of these genes in CHSE-214 cells incubated with different combinations and concentrations of fatty acids in relation to cells incubated with 20 µM α-linolenic acid (Ctrl = control). Superscript letters indicate significant differences ($p < 0.05$; one-way ANOVA, Tukey's test).

6.3.3. Phase 3: Prediction experiments

6.3.3.1. Gene expression analysis

The data obtained in Phase 2 was used to develop a model framework (Figure 6.6) to describe the effects of different supplementations on gene expression and LC-PUFA biosynthesis. Based on this model framework, the effects of untested FA combinations on LC-PUFA biosynthesis were tested and the actual results compared with predicted from the model

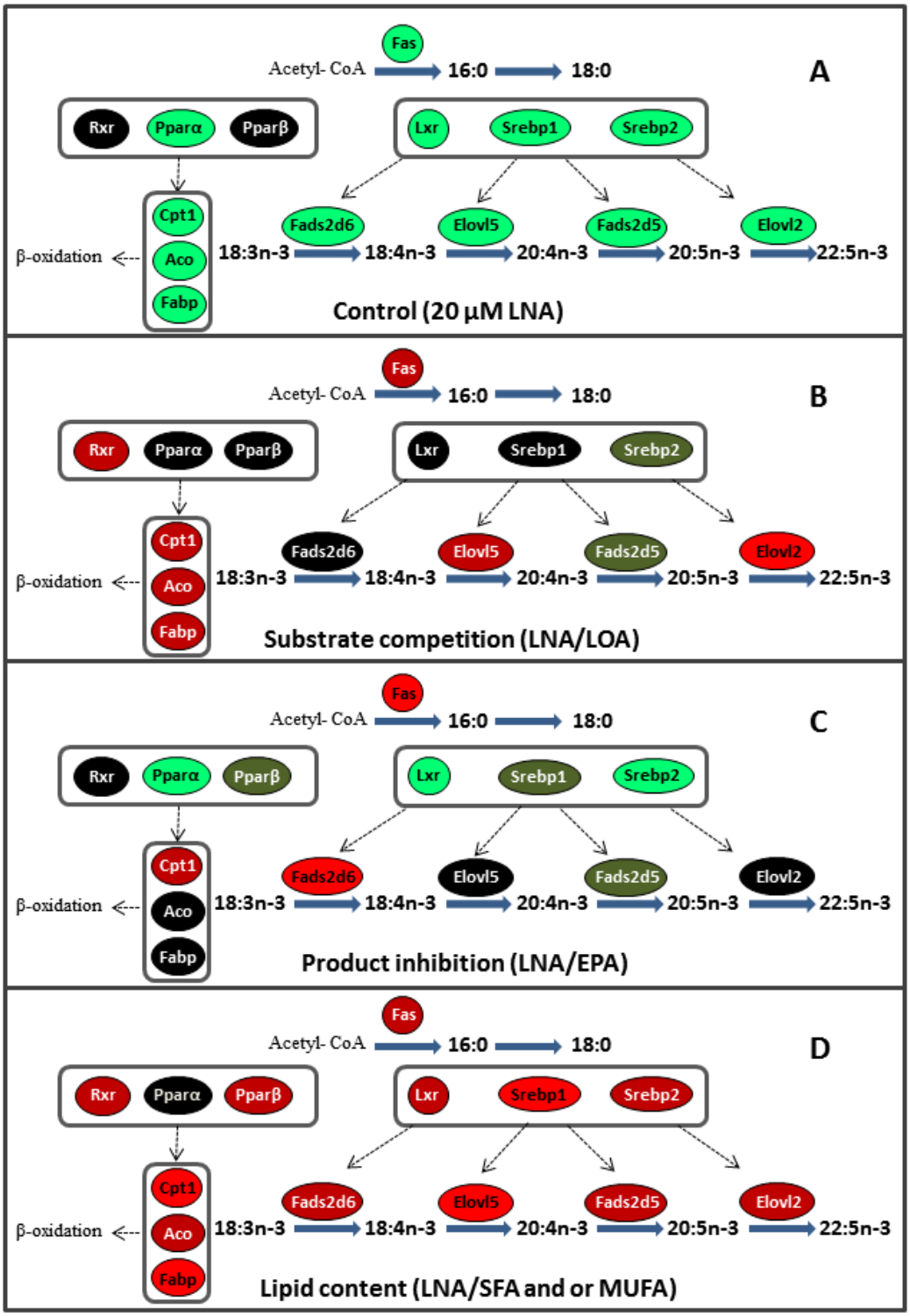


Figure 6.6. Model framework integrating the effects of different FA supplementations and gene expression and LC-PUFA biosynthesis in CHSE-214 cell line. The expression levels are indicated by colours as follows: low (green), slightly low (light green), neutral (black), high (red), slightly high (light red).

. Figure 6.7 shows the predicted patterns of expression of the 14 genes studied after incubation of CHSE-214 cells with the selected untested combinations of FA.

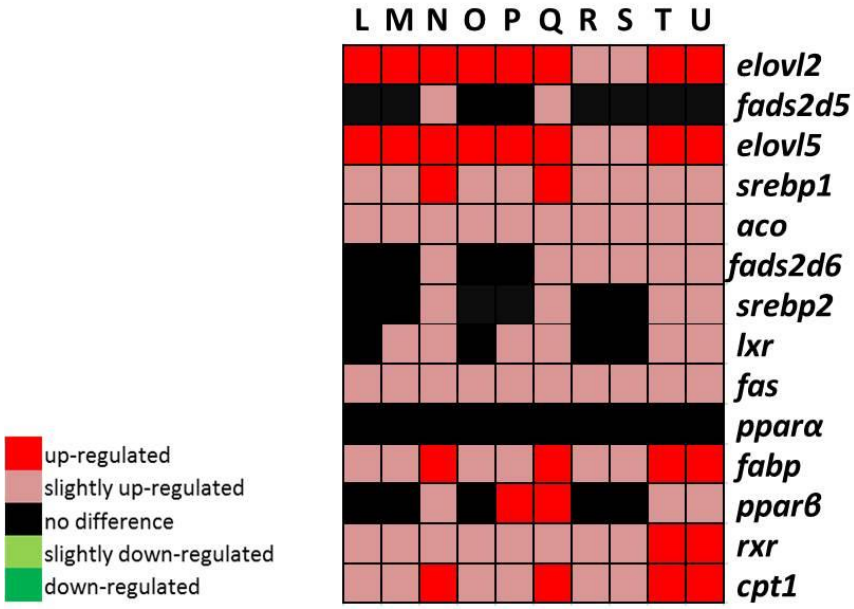


Figure 6.7. A heat map representation of the predicted expression patterns of the fourteen target genes, based on the results of Phase 2 and the model framework. As in Figure 6.2, columns represent the different combinations and concentrations of fatty acid supplemented to CHSE-214 cells, and rows represent expression of the individual genes. The predicted expression levels are indicated by the colour bar on the left, low (green), slightly low (light green), neutral (black), high (red), slightly high (light red). Treatments: L = 18:3n-3+18:2n-6/16:0 20+15/20 µM; M = 18:3n-3+18:2n-6/16:0 20+15/40 µM; N = 18:3n-3+18:2n-6/16:0 20+15/60 µM; O = 18:3n-3+18:2n-6/18:1n-9 20+15/20 µM; P = 18:3n-3+18:2n-6/18:1n-9 20+15/40 µM; Q = 18:3n-3+18:2n-6/18:1n-9 20+15/60 µM; R = 18:3n-3/18:2n-6+16:0 20/5+5 µM; S = 18:3n-3/18:2n-6+16:0 20/10+10 µM; T = 18:3n-3/18:2n-6+16:0 20/15+15 µM; U = 18:3n-3/18:2n-6+16:0 20/20+20 µM. *Abbreviations: elovl2* = fatty acyl elongase 2; *fads2d5* = Δ5 fatty acyl desaturase; *elovl5* = fatty acyl elongase 5; *srebp1* = sterol regulatory element binding protein 1; *aco* = acyl Co-A oxidase; *fads2d6* = Δ6 fatty acyl desaturase; *srebp2* = sterol regulatory element binding protein 2; ; *lxr* = liver X receptor; *fas* = fatty acid synthase; *pparα* = peroxisome proliferator activated receptor α; *fabp* = fatty acid binding protein; *pparβ* = peroxisome proliferator activated receptor β; *rxr* = retinoid X receptor; and *cpt1* = carnitine palmitoyl transferase 1.

Up-regulation of essentially all genes was predicted if CHSE-214 were incubated with high concentrations (approaching 100 µM) of total FA, such as treatments N (18:3n-3+18:2n-6/16:0, 20+15/60 µM) and Q (18:3n-3+18:2n-6/18:1n-9, 20+15/60 µM).The most stable (unaffected) expression pattern was predicted to be observed in cells incubated with treatment R (18:3n-3/18:2n-6+16:0, 20/5+5 µM), with likely no changes in expression of *fads2d5*, *aco*, and *srebp1*. As LC-PUFA (*i.e.* EPA) were not supplemented, it was predicted that most of the transcription factors *i.e. srbp1*, *srbp2*, *pparα*, *pparβ*, and *lxr*, and genes involved with the LC-PUFA biosynthesis were going to be up-regulated in relation to the control (20 µM LNA) in treatments L-U.

The heat map in Figure 6.8 shows the actual experimentally-derived gene expression data based on qPCR analysis. The heat map therefore shows how the actual expression patterns of the genes were affected by the supplementation with different combinations and concentrations of FA to the CHSE-214 cells. The expression of all genes analysed was changed in CHSE-214 cells incubated with treatment N (18:3n-3+18:2n-6/16:0, 20+15/60 µM). In treatment M (18:3n-3+18:2n-6/16:0, 20+15/40 µM), the expression of 13 out of the 14 genes changed, and 12 out of 14 in treatments L (18:3n-3+18:2n-6/16:0, 20+15/20 µM) and Q (18:3n-3+18:2n-6/18:1n-9, 20+15/60 µM). The most stable gene expression was observed in CHSE-214 incubated with treatments S (18:3n-3/18:2n-6+16:0 20/10+10 µM) and T (18:3n-3/18:2n-6+16:0 20/15+15 µM), where expression of 8 out of the 14 genes did not show any change. The detailed effects of the 10 new treatment conditions on the expression of each individual gene are shown in Figures 6.10 (genes of LC-PUFA biosynthesis), 6.11 (transcription factors) and 6.12 (other FA metabolism).

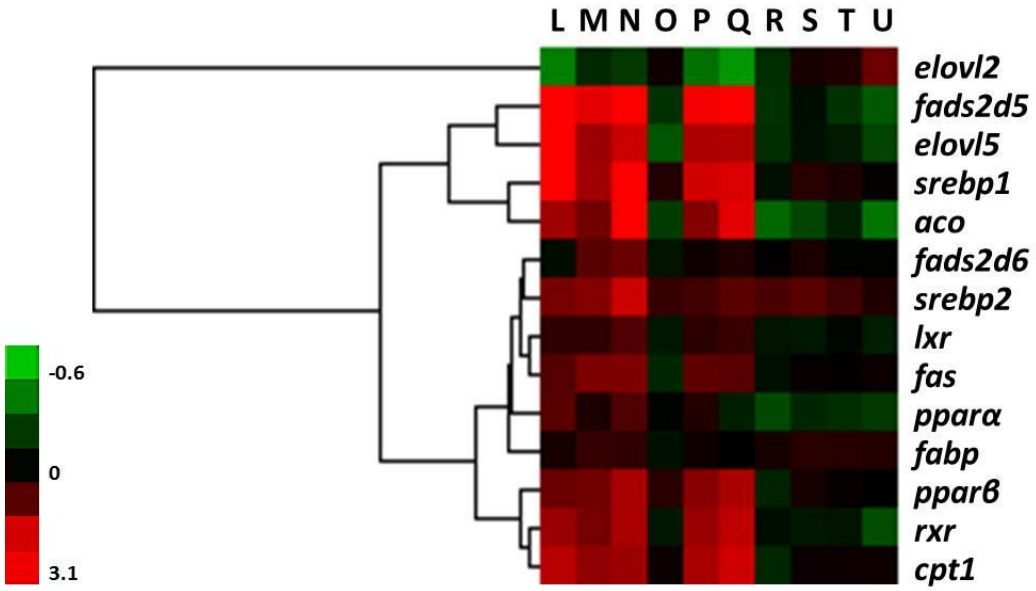


Figure 6.8. Heat map showing the actual experimentally-derived expression patterns of the fourteen target genes based on qPCR data. Columns represent mean relative expression data for each gene in CHSE-214 cells incubated with the different combinations and concentrations of fatty acid. Each row represents the effects of the different treatments on the expression of each individual gene. Expression level of each gene was squared-root normalised in relation to the control sample (20 µM α-linolenic acid). Means are depicted by a colour scale, indicating low (green), neutral (black) or high (red) relative expression levels, as indicated by the colour bar on the left. Treatments: L = 18:3n-3+18:2n-6/16:0 20+15/20 µM; M = 18:3n-3+18:2n-6/16:0 20+15/40 µM; N = 18:3n-3+18:2n-6/16:0 20+15/60 µM; O = 18:3n-3+18:2n-6/18:1n-9 20+15/20 µM; P = 18:3n-3+18:2n-6/18:1n-9 20+15/40 µM; Q = 18:3n-3+18:2n-6/18:1n-9 20+15/60 µM; R = 18:3n-3/18:2n-6+16:0 20/5+5 µM; S = 18:3n-3/18:2n-6+16:0 20/10+10 µM; T = 18:3n-3/18:2n-6+16:0 20/15+15 µM; U = 18:3n-3/18:2n-6+16:0 20/20+20 µM. Abbreviations: *elovl2* = fatty acyl elongase 2; *fads2d5* = Δ5 fatty acyl desaturase; *elovl5* = fatty acyl elongase 5; *srebp1* = sterol regulatory element binding protein 1; *aco* = acyl Co-A oxidase; *fads2d6* = Δ6 fatty acyl desaturase; *srebp2* = sterol regulatory element binding protein 2; *lxr* = liver X receptor; *fas* = fatty acid synthase; *pparα* = peroxisome proliferator activated receptor α; *fabp* = fatty acid binding protein; *pparβ* = peroxisome proliferator activated receptor β; *rxr* = retinoid X receptor and; *cpt1* = carnitine palmitoyl transferase 1.

The heat map in Figure 6.9 shows the accuracy of the predictions made for experiments L-U, in comparison with the actual results. The predictions were scored with the following criteria: red = same colour in predicted and actual result (accurate), green =

different colour in predicted and actual result (inaccurate) and pink = the colours of the predicted and the actual result were not the opposite (*i.e.* when prediction was green or red and the actual result was black, meaning that results were not identical but were not completely opposite either). The majority of the predicted results were slightly accurate, 57 pink squares out of 140, 49 predictions were accurate and 34 predictions were inaccurate.

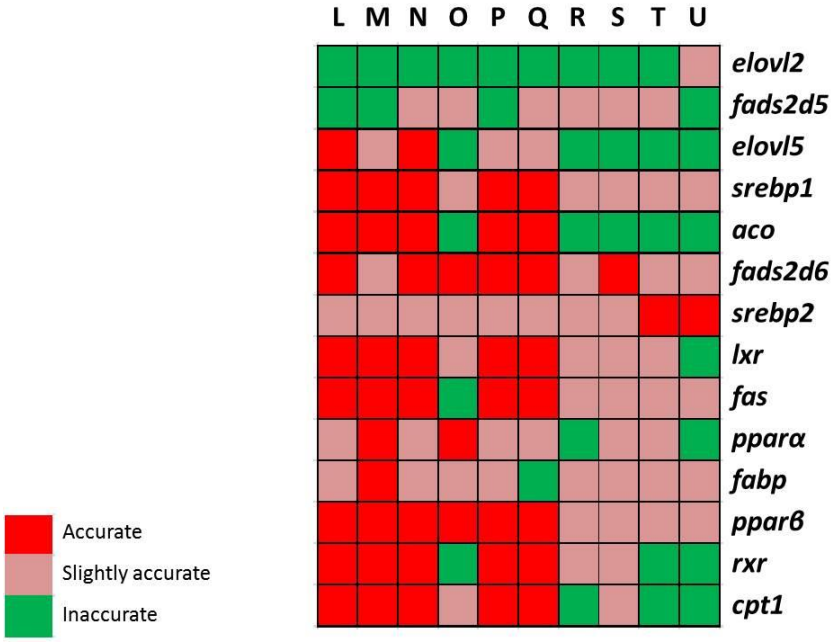


Figure 6.9. Heat map showing the accuracy of the expression patterns predicted of the fourteen target genes based on qPCR data. The accuracy is depicted by a colour scale, indicating accurate (red),slightly accurate (pink) or innacurate (green). Treatments: L = 18:3n-3+18:2n-6/16:0 20+15/20 µM; M = 18:3n-3+18:2n-6/16:0 20+15/40 µM; N = 18:3n-3+18:2n-6/16:0 20+15/60 µM; O = 18:3n-3+18:2n-6/18:1n-9 20+15/20 µM; P = 18:3n-3+18:2n-6/18:1n-9 20+15/40 µM; Q = 18:3n-3+18:2n-6/18:1n-9 20+15/60 µM; R = 18:3n-3/18:2n-6+16:0 20/5+5 µM; S = 18:3n-3/18:2n-6+16:0 20/10+10 µM; T = 18:3n-3/18:2n-6+16:0 20/15+15 µM; U = 18:3n-3/18:2n-6+16:0 20/20+20 µM. *Abbreviations*: *elovl2* = fatty acyl elongase 2; *fads2d5* = Δ5 fatty acyl desaturase; *elovl5* = fatty acyl elongase 5; *srebp1* = sterol regulatory element binding protein 1; *aco* = acyl Co-A oxidase; *fads2d6* = Δ6 fatty acyl desaturase; *srebp2* = sterol regulatory element binding protein 2; *lxr* = liver X receptor; *fas* = fatty acid synthase; *pparα* = peroxisome proliferator activated receptor α; *fabp* = fatty acid binding protein; *pparβ* = peroxisome proliferator activated receptor β; *rxr* = retinoid X receptor and; *cpt1* = carnitine palmitoyl transferase 1.

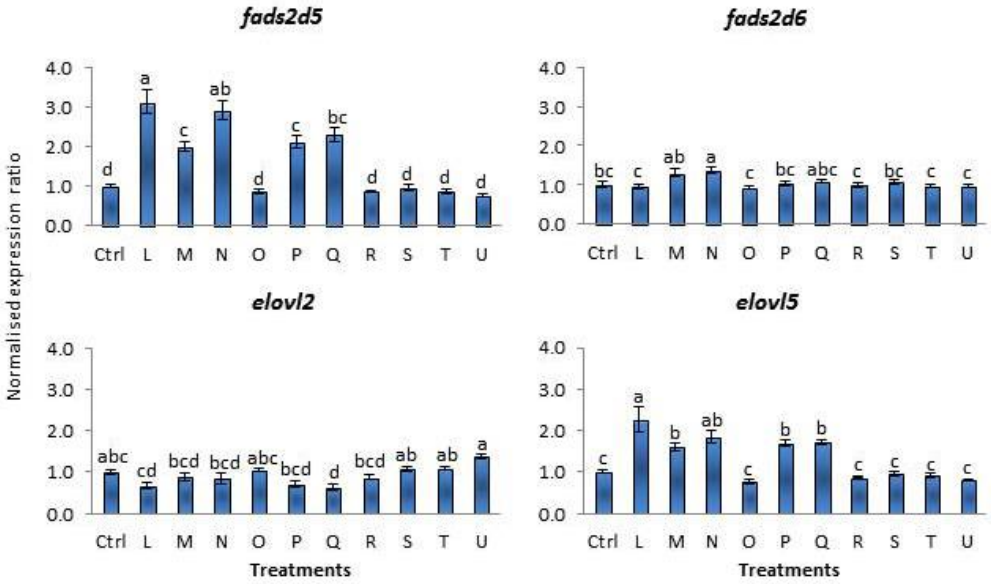


Figure 6.10. Expression of Δ5 fatty acyl desaturase (*fads2d5*), Δ6 fatty acyl desaturase (*fads2d6*), fatty acyl elongase 2 (*elovl2*) and fatty acyl elongase 5 (*elovl5*) in CHSE-214 cells incubated for 48 h with the following treatments: L = 18:3n-3+18:2n-6/16:0, 20+15/20 µM; M = 18:3n-3+18:2n-6/16:0, 20+15/40 µM; N = 18:3n-3+18:2n-6/16:0, 20+15/60 µM; O = 18:3n-3+18:2n-6/18:1n-9, 20+15/20 µM; P = 18:3n-3+18:2n-6/18:1n-9, 20+15/40 µM; Q = 18:3n-3+18:2n-6/18:1n-9, 20+15/60 µM; R = 18:3n-3/18:2n-6+16:0, 20/5+5 µM; S = 18:3n-3/18:2n-6+16:0, 20/10+10 µM; T = 18:3n-3/18:2n-6+16:0, 20/15+15 µM; U = 18:3n-3/18:2n-6+16:0, 20/20+20 µM, measured by qPCR. Results are normalised expression ratios (mean ± SEM, n = 6) of the expression of these genes in CHSE-214 cells incubated with different combinations and concentrations of fatty acids in relation to cells incubated with 20 µM α-linolenic acid (Ctrl = control). Superscript letters indicate significant differences ($p < 0.05$; one-way ANOVA, Tukey's test).

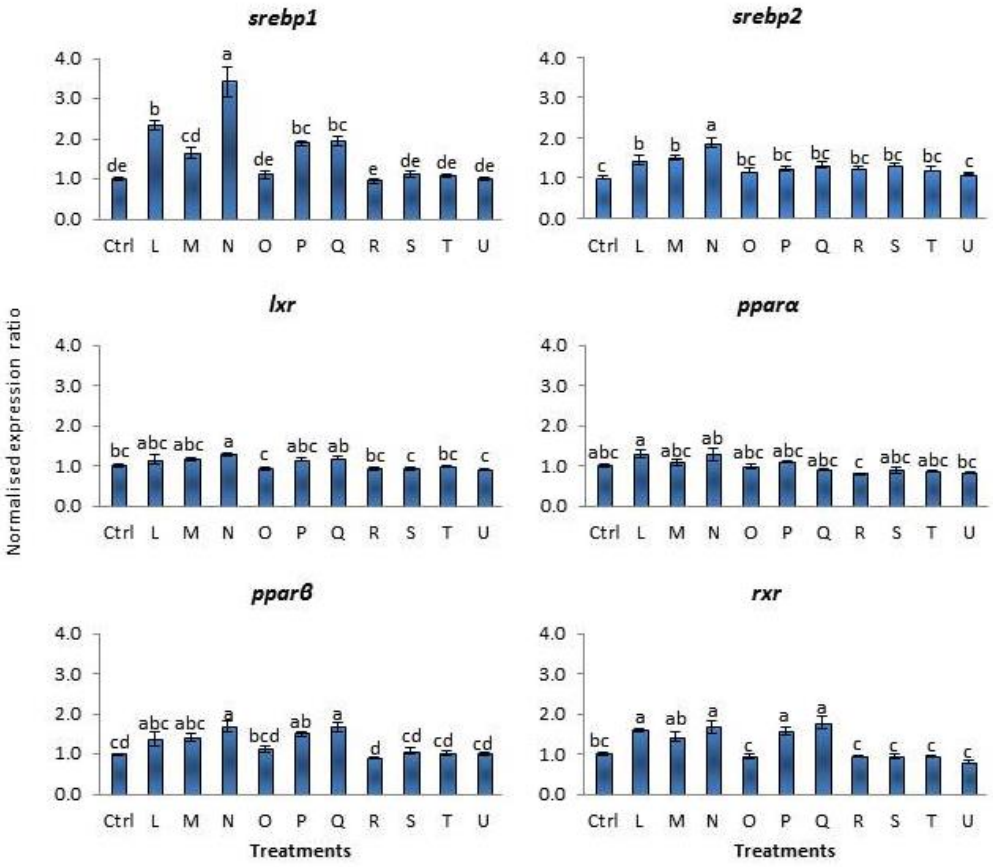


Figure 6.11. Expression of sterol regulatory element binding protein 1 (*srebp1*), sterol regulatory element binding protein 2 (*srebp2*), peroxisome proliferator activated receptor α (*pparα*), peroxisome proliferator activated receptor β (*pparβ*), liver X receptor (*lxr*) and retinoid X receptor (*rxr*) in CHSE-214 cells incubated for 48 h with the following treatments: L = 18:3n-3+18:2n-6/16:0 20+15/20 µM; M = 18:3n-3+18:2n-6/16:0 20+15/40 µM; N = 18:3n-3+18:2n-6/16:0 20+15/60 µM; O = 18:3n-3+18:2n-6/18:1n-9 20+15/20 µM; P = 18:3n-3+18:2n-6/18:1n-9 20+15/40 µM; Q = 18:3n-3+18:2n-6/18:1n-9 20+15/60 µM; R = 18:3n-3/18:2n-6+16:0 20/5+5 µM; S = 18:3n-3/18:2n-6+16:0 20/10+10 µM; T = 18:3n-3/18:2n-6+16:0 20/15+15 µM; U = 18:3n-3/18:2n-6+16:0 20/20+20 µM, measured by qPCR. Results are normalised expression ratios (mean ± SEM, n = 6) of the expression of these genes in CHSE-214 cells incubated with different combinations and concentrations of fatty acids in relation to cells incubated with 20 µM α-linolenic acid (Ctrl = control). Superscript letters indicate significant differences ($p < 0.05$; one-way ANOVA, Tukey's test).

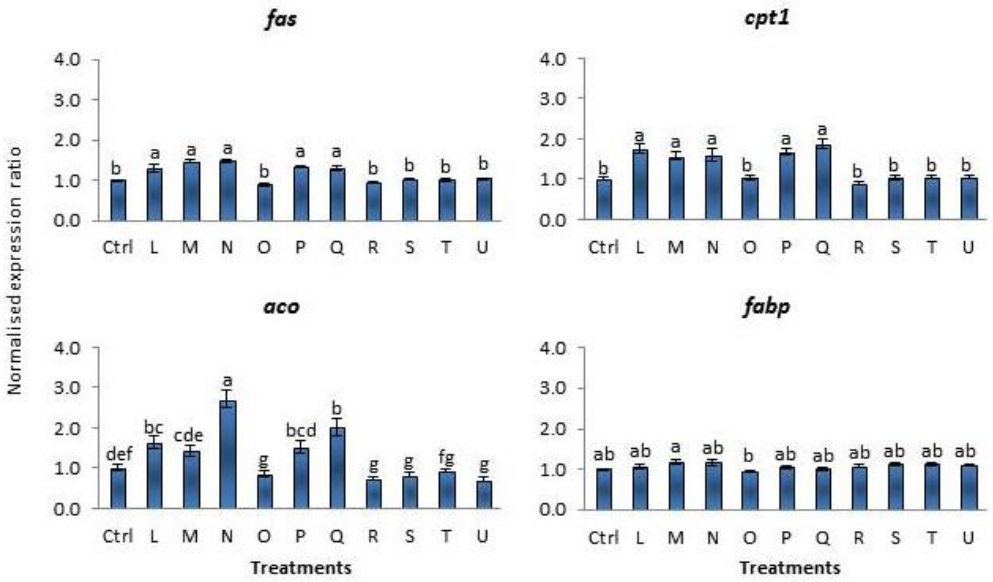


Figure 6.12. Expression of fatty acid synthase (*fas*), carnitine palmitoyl transferase 1 (*cpt1*), acyl Co-A oxidase (*aco*) and fatty acid binding protein (*fabp*) in CHSE-214 cells incubated for 48 h with the following treatments: L = 18:3n-3+18:2n-6/16:0 20+15/20 µM; M = 18:3n-3+18:2n-6/16:0 20+15/40 µM; N = 18:3n-3+18:2n-6/16:0 20+15/60 µM; O = 18:3n-3+18:2n-6/18:1n-9 20+15/20 µM; P = 18:3n-3+18:2n-6/18:1n-9 20+15/40 µM; Q = 18:3n-3+18:2n-6/18:1n-9 20+15/60 µM; R = 18:3n-3/18:2n-6+16:0 20/5+5 µM; S = 18:3n-3/18:2n-6+16:0 20/10+10 µM; T = 18:3n-3/18:2n-6+16:0 20/15+15 µM; U = 18:3n-3/18:2n-6+16:0 20/20+20 µM, measured by qPCR. Results are normalised expression ratios (mean ± SEM, n = 6) of the expression of these genes in CHSE-214 cells incubated with different combinations and concentrations of fatty acids in relation to cells incubated with 20 µM α-linolenic acid (Ctrl = control). Superscript letters indicate significant differences ($p < 0.05$; one-way ANOVA, Tukey's test).

6.3.3.2 Lipid class and fatty acid composition

Figure 6.13 shows the prediction made for EPA levels in the cells incubated with different combinations and concentrations of FA (treatments L-U). The highest EPA level was expected with the supplementation of 18:3n-3/18:2n-6+16:0 at 20/5+5 µM (treatment R), as previous analyses showed that LNA supplemented with low concentrations of LOA (Chapter 4) and SFA (Chapter 5) possibly enhanced the conversion of LNA to EPA. Negative effects on the EPA levels were expected supplementing higher concentration of

MUFA (P = 18:3n-3+18:2n-6/18:1n-9 at 20+15/40 µM; and Q = 18:3n-3+18:2n-6/18:1n-9 at 20+15/60 µM). However, the EPA levels obtained in cells incubated with the treatments L-U (Figure 6.14) were abundantly lower compared with the values predicted. The order of the treatments from high to low showed a moderate accuracy.

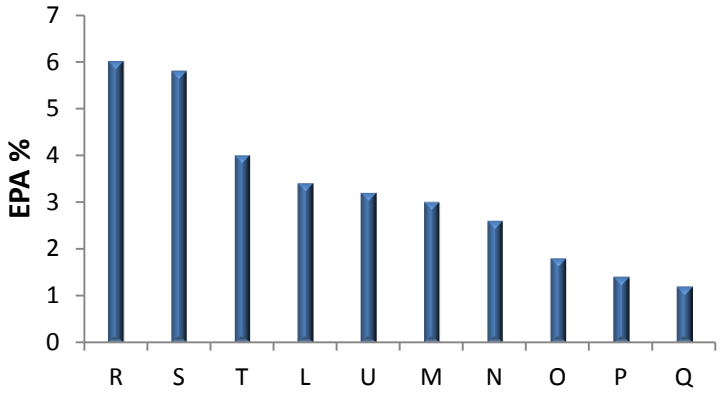


Figure 6.13. Prediction of EPA (eicosapentaenoic acid) levels of treatments: R = 18:3n-3/18:2n-6+16:0 20/5+5 µM; S = 18:3n-3/18:2n-6+16:0 20/10+10 µM; T = 18:3n-3/18:2n-6+16:0 20/15+15 µM; L = 18:3n-3+18:2n-6/16:0 20+15/20 µM; U = 18:3n-3/18:2n-6+16:0 20/20+20 µM; M = 18:3n-3+18:2n-6/16:0 20+15/40 µM; N = 18:3n-3+18:2n-6/16:0 20+15/60 µM; O = 18:3n-3+18:2n-6/18:1n-9 20+15/20 µM; P = 18:3n-3+18:2n-6/18:1n-9 20+15/40 µM; Q = 18:3n-3+18:2n-6/18:1n-9 20+15/60 µM.

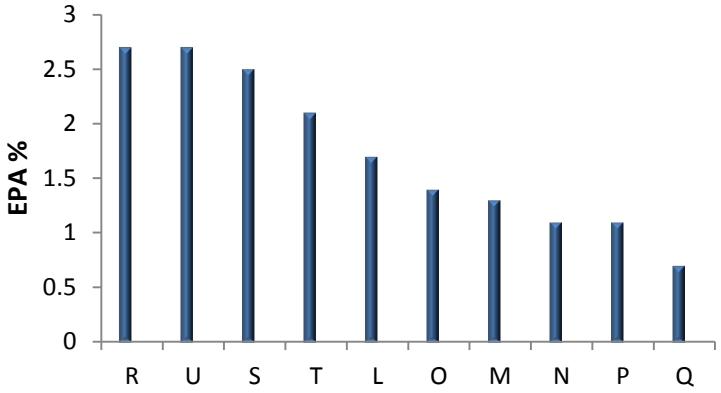


Figure 6.14. EPA (eicosapentaenoic acid) levels of treatments: R = 18:3n-3/18:2n-6+16:0 20/5+5 µM; S = 18:3n-3/18:2n-6+16:0 20/10+10 µM; T = 18:3n-3/18:2n-6+16:0 20/15+15 µM; L = 18:3n-3+18:2n-6/16:0 20+15/20 µM; U = 18:3n-3/18:2n-6+16:0 20/20+20 µM; M = 18:3n-3+18:2n-6/16:0 20+15/40 µM; N = 18:3n-3+18:2n-6/16:0 20+15/60 µM; O = 18:3n-3+18:2n-6/18:1n-9 20+15/20 µM; P = 18:3n-3+18:2n-6/18:1n-9 20+15/40 µM; Q = 18:3n-3+18:2n-6/18:1n-9 20+15/60 µM.

Table 6.4 shows the lipid content and class composition of CHSE-214 cells incubated with 20 µM LNA and 15 µM LOA in the presence of increasing concentrations of 16:0. No consistent trend was observed in the cell lipid content, and no significant differences were found amongst treatments (R^2 = 0.028; p = 0.665). In the lipid class data no clear trends were observed, apart from the increasing total neutral lipids, and consequently decreasing total polar lipids, with the graded supplementation of 16:0. However, no significant differences were found amongst treatments (R^2 = 0.285; p = 0.139).

Table 6.4. Lipid content and lipid class composition of CHSE-214 cells incubated with 20 µM LNA, 15 µM LOA and increasing concentrations of 16:0 (Treatments L, M and N)

Lipid	20+15/20 µM	20+15/40 µM	20+15/60 µM	R^2	P-value
LC (µg)	280.0 ± 17.3	320.0 ± 20.0	296.7 ± 73.7	0.028	0.665
CC (%)					
PC	24.7 ± 1.0	23.5 ± 0.5	25.2 ± 1.0	0.051	0.557
PE	18.9 ± 0.3	19.5 ± 1.0	16.9 ± 0.4	0.447	0.049
PS	7.3 ± 0.6	7.3 ± 0.1	6.3 ± 0.7	0.430	0.055
PI	8.6 ± 0.5	8.4 ± 0.9	8.6 ± 0.4	0.001	0.947
PA/CL	ND	ND	ND	-	-
SM	5.5 ± 0.7	5.1 ± 0.2	5.3 ± 0.6	0.037	0.620
TP	65.0 ± 2.5	63.8 ± 0.5	62.3 ± 2.5	0.285	0.139
TN	35.0 ± 2.5	36.2 ± 0.5	37.7 ± 2.5	0.285	0.139
TAG	11.3 ± 1.3	12.1 ± 1.1	11.8 ± 1.7	0.026	0.678
CHOL	20.2 ± 1.1	20.0 ± 0.5	21.9 ± 0.6	0.416	0.061
FFA	3.5 ± 0.2	4.1 ± 0.5	4.0 ± 0.9	0.125	0.350

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical significance of differences was determined by regression analysis (p < 0.05). *Abbreviations:* LNA = α-linolenic acid; LOA = linoleic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

The FA composition of CHSE-214 cells incubated with 20 µM LNA and 15 µM LOA, in the presence of increasing concentrations of 16:0 is shown in Table 6.5. EPA levels decreased with the graded supplementation of 16:0 (R^2 = 0.823; p = 0.001), but the percentages of 22:5n-3 and DHA were not affected by 16:0 supplementation, being

essentially the same at all concentrations (R^2 = 0.459; p = 0.045 and R^2 = 0.079; p = 0.464, respectively).

Table 6.5. Fatty acid composition (%) of CHSE-214 cells incubated with 20 µM LNA, 15 µM LOA and increasing concentrations of 16:0 (Treatments L, M and N)

Fatty acid	20+15/20 µM	20+15/40 µM	20+15/60 µM	R^2	P-value
14:0	1.1 ± 0.1	1.0 ± 0.2	1.1 ± 0.0	0.010	0.794
15:0	0.4 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.275	0.147
16:0	13.3 ± 0.6	14.8 ± 0.5	16.9 ± 0.9	0.872	0.000
17:0	0.6 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.161	0.285
18:0	8.6 ± 0.5	8.4 ± 0.4	8.0 ± 0.6	0.295	0.131
Σ SFA	24.0 ± 0.9	25.1 ± 0.9	26.7 ± 1.4	0.623	0.011
16:1n-9	2.0 ± 0.0	2.1 ± 0.0	2.3 ± 0.2	0.525	0.027
16:1n-7	2.9 ± 0.1	4.0 ± 0.2	6.0 ± 0.2	0.966	0.000
18:1n-9	29.5 ± 0.3	29.0 ± 0.7	29.7 ± 1.0	0.018	0.730
18:1n-7	2.5 ± 0.0	2.6 ± 0.1	2.9 ± 0.3	0.504	0.032
24:1n-9	0.5 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	0.258	0.163
Σ MUFA	37.4 ± 0.2	38.2 ± 0.6	41.3 ± 0.9	0.818	0.001
18:2n-6	11.0 ± 0.4	10.6 ± 0.2	9.0 ± 1.1	0.627	0.011
18:3n-6	0.7 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.764	0.002
20:2n-6*	1.3 ± 0.0	1.4 ± 0.2	1.1 ± 0.1	0.288	0.136
20:3n-6	1.4 ± 0.1	1.6 ± 0.2	1.3 ± 0.1	0.089	0.436
20:4n-6	1.7 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	0.532	0.026
Σ n-6 PUFA	16.2 ± 0.3	15.8 ± 0.3	13.5 ± 1.3	0.670	0.007
18:3n-3	10.4 ± 0.3	9.7 ± 0.3	7.9 ± 0.3	0.892	0.000
18:4n-3	2.0 ± 0.1	2.1 ± 0.2	1.9 ± 0.2	0.080	0.460
20:4n-3	1.5 ± 0.0	1.2 ± 0.1	0.8 ± 0.1	0.921	0.000
20:5n-3	1.7 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	0.823	0.001
22:5n-3	1.1 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.459	0.045
22:6n-3	1.7 ± 0.1	1.7 ± 0.2	1.6 ± 0.1	0.079	0.464
Σ n-3 PUFA	18.4 ± 0.3	17.1 ± 1.0	14.3 ± 0.4	0.879	0.000
18:2n-9	2.5 ± 0.2	2.5 ± 0.1	2.9 ± 0.2	0.436	0.053
20:2n-9	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.1	0.514	0.030
22:2n-9	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.086	0.444
Σ n-9 PUFA	4.0 ± 0.2	3.9 ± 0.2	4.2 ± 0.3	0.159	0.288
Σ PUFA	39.6 ± 0.7	36.7 ± 1.4	32.0 ± 1.9	0.798	0.001

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical significance of differences was determined by regression analysis (p < 0.05). *Abbreviations:* LNA = α-linolenic acid; LOA = linoleic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; *contains 20:3n-9.

The levels of LNA and LOA decreased with the graded supplementation of 16:0 (R^2 = 0.892; p = 0.000 and R^2 = 0.627; p = 0.011, respectively). Small amounts of the C18 PUFA were converted into the intermediate metabolites. There was graded accumulation

of 16:0 with the supplementation of the FA itself (R^2 = 0.872; p = 0.000), leading to an increment of the total SFA (R^2 = 0.623; p = 0.011), and total MUFA (R^2 = 0.818; p = 0.001) for the increment of desaturation product 16:1n-7 (R^2 = 0.966; p = 0.000), whereas total PUFA decreased (R^2 = 0.798; p = 0.001).

Table 6.6. shows the lipid content and class composition of CHSE-214 cells incubated with 20 µM LNA and 15 µM LOA in the presence of increasing concentrations of 18:1n-9. Cell lipid content increased with the graded supplementation of 18:1n-9 but, no significant differences were found amongst treatments (R^2 = 0.170; p = 0.270). In the lipid class data TAG, and total neutral lipids increased with the graded supplementation of 18:1n-9 although the differences were not significant (R^2 = 0.411; p = 0.063 and R^2 = 0.244; p = 0.177).

Table 6.6. Lipid content and lipid class composition of CHSE-214 cells incubated with 20 µM LNA, 15 µM LOA and increasing concentrations of 18:1n-9 (Treatments O, P and Q)

Lipid	20+15/20 µM	20+15/40 µM	20+15/60 µM	R^2	P-value
LC (µg)	193.3 ± 58.6	226.7 ± 49.3	243.3 ± 56.9	0.170	0.270
CC (%)					
PC	25.0 ± 1.2	24.9 ± 1.1	24.2 ± 0.8	0.116	0.370
PE	14.3 ± 0.3	14.4 ± 0.5	13.3 ± 1.2	0.257	0.164
PS	5.1 ± 1.1	4.7 ± 0.1	4.1 ± 0.7	0.341	0.099
PI	6.5 ± 1.1	6.8 ± 0.3	5.9 ± 1.0	0.103	0.400
PA/CL	ND	ND	ND	-	-
SM	5.2 ± 0.4	5.1 ± 0.5	5.2 ± 0.1	0.001	0.937
TP	56.1 ± 2.9	55.9 ± 2.0	52.6 ± 3.8	0.244	0.177
TN	43.9 ± 2.9	44.1 ± 2.0	47.4 ± 3.8	0.244	0.177
TAG	17.3 ± 2.0	18.6 ± 2.6	21.9 ± 3.4	0.411	0.063
CHOL	22.4 ± 1.2	21.5 ± 0.9	22.4 ± 0.2	0.000	0.982
FFA	4.3 ± 0.3	4.0 ± 0.7	3.2 ± 0.3	0.566	0.019

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical significance of differences was determined by regression analysis (p < 0.05). *Abbreviations:* LNA = α-linolenic acid; LOA = linoleic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids; ND = not detected.

Table 6.7 shows the FA composition of CHSE-214 cells incubated with 20 µM of LNA and 15 µM of LOA, in the presence of increasing concentrations of 18:1n-9. The proportions of EPA (R^2 = 0.861; p = 0.000), 22:5n-3 (R^2 = 0.775; p = 0.002), and DHA (R^2 = 0.743; p = 0.003) all decreased with the graded supplementation of 18:1n-9, but 18:4n-3 levels were unaffected (R^2 = 0.002; p = 0.917).

Table 6.7. Fatty acid composition (%) of CHSE-214 cells incubated with 20 µM LNA, 15 µM LOA and increasing concentrations of 18:1n-9 (Treatments O, P and Q)

Fatty acid	20+15/20 µM	20+15/40 µM	20+15/60 µM	R^2	P-value
14:0	0.7 ± 0.0	0.7 ± 0.1	0.5 ± 0.0	0.472	0.041
15:0	0.2 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.663	0.008
16:0	6.0 ± 0.2	5.8 ± 0.3	5.3 ± 0.4	0.512	0.030
17:0	0.4 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.574	0.018
18:0	5.5 ± 0.2	5.2 ± 0.4	4.3 ± 0.1	0.765	0.002
Σ SFA	12.7 ± 0.4	12.4 ±0.8	10.8 ± 0.4	0.667	0.007
16:1n-9	2.2 ± 0.1	2.6 ± 0.2	2.8 ± 0.3	0.610	0.013
16:1n-7	1.3 ± 0.2	1.3 ± 0.1	1.2 ± 0.2	0.106	0.392
18:1n-9	31.2 ± 0.1	39.5 ± 0.3	50.3 ± 0.2	0.995	0.000
18:1n-7	1.9 ± 0.1	1.6 ± 0.0	1.6 ± 0.2	0.534	0.025
24:1n-9	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.595	0.015
Σ MUFA	36.9 ± 0.3	45.3 ± 0.4	56.0 ± 0.5	0.994	0.000
18:2n-6	24.7 ± 0.2	17.9 ± 0.9	11.0 ± 0.4	0.991	0.000
18:3n-6	2.0 ± 0.2	1.5 ± 0.1	1.0 ± 0.1	0.938	0.000
20:2n-6*	1.1 ± 0.0	1.0 ± 0.1	0.9 ± 0.0	0.689	0.006
20:3n-6	1.6 ± 0.1	1.6 ± 0.2	1.6 ± 0.2	0.008	0.816
20:4n-6	2.1 ± 0.0	1.9 ± 0.1	1.6 ± 0.1	0.890	0.000
Σ n-6 PUFA	31.5 ± 0.5	23.9 ± 0.8	16.2 ± 0.2	0.994	0.000
18:3n-3	6.8 ± 0.2	6.7 ± 0.7	6.4 ± 0.3	0.123	0.355
18:4n-3	2.5 ± 0.3	2.4 ± 0.3	2.4 ± 0.1	0.002	0.917
20:4n-3	0.8 ± 0.1	0.7 ± 0.1	0.3 ± 0.0	0.830	0.001
20:5n-3	1.4 ± 0.0	1.1 ± 0.2	0.7 ± 0.0	0.861	0.000
22:5n-3	1.1 ± 0.0	1.0 ± 0.1	0.8 ± 0.0	0.775	0.002
22:6n-3	1.8 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	0.743	0.003
Σ n-3 PUFA	14.3 ± 0.6	13.6 ± 0.8	12.1 ± 0.1	0.758	0.002
18:2n-9	2.9 ± 0.1	3.1 ± 0.2	3.3 ± 0.0	0.775	0.002
20:2n-9	1.7 ± 0.1	1.7 ± 0.2	1.6 ± 0.0	0.200	0.228
Σ n-9 PUFA	4.6 ± 0.1	4.8 ± 0.4	4.9 ± 0.0	0.309	0.120
Σ PUFA	50.3 ± 0.8	42.2 ± 0.9	33.2 ± 0.2	0.992	0.000

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical significance of differences was determined by regression analysis (p < 0.05). *Abbreviations:* LNA = α-linolenic acid; LOA = linoleic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; *contains 20:3n-9.

The cellular percentage of LNA was not affected by 18:1n-9 supplementation (R^2 = 0.123; p = 0.355). Levels of LOA (R^2 = 0.991; p = 0.000), and the metabolites *i.e.*18:3n-6

(R^2 = 0.938; p = 0.000) and ARA (R^2 = 0.890; p = 0.000) all decreased with graded supplementation of 18:1n-9, with no changes observed in 20:3n-6 levels (R^2 = 0.008; p = 0.816). There was graded incorporation of 18:1n-9 with the supplementation of the FA itself (R^2 = 0.995; p = 0.000), leading to an increment of total MUFA (R^2 = 0.994; p = 0.000), which was balanced by reducing total n-3 PUFA (R^2 = 0.994; p = 0.000), n-6 PUFA (R^2 = 0.758; p = 0.002), and therefore total PUFA (R^2 = 0.992; p = 0.000).

Table 6.8 shows the lipid content and class composition of CHSE-214 cells incubated with 20 µM LNA in the presence of increasing concentrations of LOA+16:0 (1:1). Cell lipid content decreased with the graded supplementation of LOA+16:0 from 20/5+5 to 20/15+15 (R^2 = 0.351; p = 0.042).

Table 6.8. Lipid content and lipid class composition of CHSE-214 cells incubated with 20 µM LNA and increasing concentrations of LOA+16:0 (1:1) (Treatments R, S, T and U)

Lipid	20 + 5/5 µM	20 + 10/10 µM	20 + 15/15 µM	20 + 20/20 µM	R^2	P-value
LC (µg)	340.0 ± 52.0	286.7 ± 80.8	233.3 ± 41.6	253.3 ± 15.3	0.351	0.042
CC (%)						
PC	24.6 ± 0.7	24.3 ± 0.3	24.8 ± 0.5	24.7 ± 1.0	0.031	0.587
PE	20.0 ± 0.2	18.3 ± 0.3	16.9 ± 0.6	17.7 ± 0.7	0.581	0.004
PS	6.8 ± 0.3	6.3 ± 0.2	5.0 ± 0.3	4.8 ± 0.6	0.816	0.000
PI	10.9 ± 0.6	9.4 ± 0.4	8.7 ± 0.1	9.6 ± 0.5	0.330	0.051
SM	4.8 ± 0.5	3.9 ± 0.2	3.1 ± 0.4	5.3 ± 0.6	0.004	0.841
TP	67.2 ± 1.2	62.2 ± 0.4	58.6 ± 0.7	62.1 ± 1.4	0.438	0.019
TN	32.8 ± 1.2	37.8 ± 0.4	41.4 ± 0.7	37.9 ± 1.4	0.438	0.019
TAG	4.9 ± 0.2	4.4 ± 0.6	11.2 ± 0.9	6.9 ± 0.3	0.308	0.061
CHOL	26.5 ± 1.4	31.7 ± 0.4	28.8 ± 0.3	28.9 ± 1.5	0.052	0.477
FFA	1.4 ± 0.3	1.7 ± 0.2	1.5 ± 0.1	2.1 ± 0.3	0.378	0.033

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Lipid class composition is given as a percentage of the total lipid content. Statistical significance of differences was determined by regression analysis (p < 0.05). *Abbreviations:* LNA = α-linolenic acid; LOA = linoleic acid; LC = lipid content; CC = class composition; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PA/CL = phosphatidic acid/cardiolipin; SM = sphingomyelin; TP = total polar; TN = total neutral; TAG = triacylglycerol; CHOL = cholesterol; FFA = free fatty acids.

There were no clear trends observed in cellular lipid class compositions, apart from total neutral lipids, which increased with the graded supplementation of LOA+16:0 from 20/5+5 to 20/15+15 (R^2 = 0.438; p = 0.019), this increase balanced mainly by decreased

PE (R^2 = 0.581; p = 0.004), PS (R^2 = 0.816; p = 0.000), and therefore total polar lipids (R^2 = 0.438; p = 0.019).

The FA composition of CHSE-214 cells incubated with 20 µM of LNA in the presence of increasing concentrations of LOA+16:0 is shown in Table 6.9.

Table 6.9. Fatty acid composition (%) of CHSE-214 cells incubated with 20 µM LNA and increasing concentrations of LOA+16:0 (Treatments R, S, T and U)

Fatty acid	20/5+5 µM	20/10+10 µM	20/15+15 µM	20/20+20 µM	R^2	P-value
14:0	1.4 ± 0.2	1.2 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	0.143	0.226
15:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.379	0.033
16:0	12.1 ± 0.2	12.4 ± 0.4	12.1 ± 0.4	11.5 ± 0.2	0.257	0.093
17:0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.572	0.004
18:0	9.9 ± 0.2	9.6 ± 0.1	9.0 ± 0.4	9.4 ± 0.5	0.271	0.083
Σ SFA	23.9 ± 0.5	23.6 ± 0.3	22.6 ± 0.8	22.6 ± 0.7	0.578	0.004
16:1n-9	2.3 ± 0.20	2.1 ± 0.1	2.0 ± 0.1	2.4 ± 0.1	0.015	0.706
16:1n-7	1.8 ± 0.0	1.8 ± 0.1	2.0 ± 0.1	2.3 ± 0.3	0.550	0.006
18:1n-9	41.1 ± 0.4	39.1 ± 0.2	30.2 ± 0.8	31.8 ± 0.7	0.772	0.000
18:1n-7	1.5 ± 0.3	1.8 ± 0.4	2.1 ± 0.1	2.2 ± 0.2	0.588	0.004
24:1n-9	0.7 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.343	0.045
Σ MUFA	47.6 ± 0.3	45.4 ± 0.2	36.8 ± 0.7	39.3 ± 1.1	0.704	0.001
18:2n-6	4.5 ± 0.4	7.5 ± 0.3	12.0 ± 0.4	11.0 ± 0.5	0.820	0.000
18:3n-6	0.4 ± 0.1	0.3 ± 0.0	0.9 ± 0.1	0.7 ± 0.1	0.539	0.007
20:2n-6*	1.6 ± 0.2	1.6 ± 0.1	0.9 ± 0.0	1.1 ± 0.2	0.520	0.008
20:3n-6	1.1 ± 0.1	1.0 ± 0.0	2.5 ± 0.1	2.6 ± 0.2	0.768	0.000
20:4n-6	1.3 ± 0.3	1.4 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	0.134	0.242
Σ n-6 PUFA	8.9 ± 0.4	11.8 ± 0.1	17.8 ± 0.7	16.9 ± 0.5	0.843	0.000
18:3n-3	5.9 ± 0.3	6.7 ± 0.3	8.6 ± 0.1	6.0 ± 0.1	0.056	0.458
18:4n-3	1.3 ± 0.0	1.7 ± 0.1	2.5 ± 0.2	1.7 ± 0.1	0.295	0.068
20:4n-3	2.1 ± 0.0	1.7 ± 0.3	1.7 ± 0.2	1.8 ± 0.2	0.233	0.112
20:5n-3	2.7 ± 0.1	2.5 ± 0.3	2.1 ± 0.2	2.7 ± 0.4	0.028	0.604
22:5n-3	1.0 ± 0.1	1.0 ± 0.0	1.1 ± 0.1	1.0 ± 0.0	0.000	0.989
22:6n-3	1.5 ± 0.1	1.4 ± 0.2	1.5 ± 0.1	1.3 ± 0.2	0.223	0.121
Σ n-3 PUFA	14.6 ± 0.2	15.0 ± 0.4	17.4 ± 0.8	14.5 ± 0.2	0.039	0.538
18:2n-9	3.1 ± 0.2	2.8 ± 0.1	2.8 ± 0.2	3.1 ± 0.1	0.000	0.962
20:2n-9	1.5 ± 0.1	1.1 ± 0.0	2.3 ± 0.0	3.2 ± 0.0	0.722	0.000
22:2n-9	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.022	0.648
Σ n-9 PUFA	5.0 ± 0.3	4.1 ± 0.1	5.4 ± 0.2	6.6 ± 0.2	0.579	0.004
Σ PUFA	28.5 ± 0.5	31.0 ± 0.5	40.6 ± 1.5	38.1 ± 0.4	0.741	0.000

Footnotes: Results are expressed as mean ± 1 SD (n = 3). Fatty acid composition is given as a percentage of the total fatty acid content. Statistical significance of differences was determined by regression analysis (p < 0.05). *Abbreviations:* LNA = α-linolenic acid; LOA = linoleic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; *contains 20:3n-9.

The graded supplementation of LOA+16:0 did not significantly affect the levels of EPA (R^2 = 0.028; p = 0.604), 22:5n-3 (R^2 = 0.000; p = 0.989), and DHA (R^2 = 0.223; p = 0.121). Levels of LNA increased from 20/5+5 µM to 20/15+15 µM, and some was converted to 18:4n-3, but these differences were not statistically significant (R^2 = 0.056; p = 0.458, R^2 = 0.295; p = 0.068). There was incorporation of LOA with the graded supplementation of the FA itself (R^2 = 0.820; p = 0.000), except from 20/20+20 µM. Some of the supplemented LOA was converted to 18:3n-6 (R^2 = 0.539; p = 0.007), elongated to 20:3n-6 (R^2 = 0.768; p = 0.000) and desaturated to ARA (R^2 = 0.134; p = 0.242). Similar levels of 16:0 were detected at all concentrations (R^2 = 0.257; p = 0.093); a small amount was further converted to 16:1n-7 (R^2 = 0.550; p = 0.006).

6.4. Discussion

The present study describes the use of the CHSE-214 cell line as a model to study the molecular regulation of the synthesis of EPA from LNA in salmon. The study consisted of three experimental phases. In Phase 1, a multi-well plate system was developed, determining seeding density and incubation time with the FA treatments, in order to effectively analyse the effects of FA treatments on the expression of 14 target genes. In Phase 2, changes in gene expression were evaluated in CHSE-214 cells supplemented with 20 µM of LNA in the presence of competing PUFA (LOA) or MUFA and SFA as surrogate or proxy for dietary lipid content. Based on these data a model framework was developed to describe the effects of different supplementations on gene expression and, ultimately, LC-PUFA biosynthesis. In Phase 3 the effects of untested FA combinations on LC-PUFA biosynthesis were predicted based on the model created in Phase 2. These predictions were then tested by actual experiments measuring the effects of the FA supplementation on gene expression and lipid and fatty acid compositions. Most of the

available information regarding molecular mechanisms underpinning the control and regulation lipid and FA metabolism has been carried out in mammals (Sekiya *et al.*, 2003; Fraulob *et al.*, 2010; Jung *et al.*, 2011; Weiss *et al.*, 2011). In contrast, the information available in fish is generally relatively limited although there is increasing data becoming available in a few species including Atlantic salmon. In particular, molecular studies in this field are required particularly in order to optimise diets for farmed fish species to preserve farmed fish as the prime sources of n-3 LC-PUFA for humans despite reduced levels of these FA being included in fish feeds (Betancor *et al.*, 2015). In this context, special attention should be paid to alterations in the molecular control mechanisms that may result from the replacement of dietary FO with VO in feeds for farmed fish. Previous studies have shown that this change in dietary composition does not affect the growth, survival, health and welfare of Atlantic salmon (Bell *et al.*, 2001, 2002; Torstensen *et al.*, 2005; Betancor *et al.*, 2015). However, this dietary change can drastically and significantly affect FA metabolism by altering the expression of genes involved with FA and lipid metabolism, including the genes directly involved in LC-PUFA biosynthesis, elongases and desaturases, and associated transcription factors (Zheng *et al.*, 2005; Leaver *et al.*, 2008; Taggart *et al.*, 2008). This effect has been associated with the increased proportions of dietary C_{18} PUFA (LNA and LOA), and with the much reduced levels of LC-PUFA (EPA and DHA) (Tocher *et al.*, 1997, 2002, 2003b). Therefore, there is a pressing need to understand the molecular control mechanisms that determine endogenous LC-PUFA biosynthesis that could contribute to maintain levels of EPA and DHA in salmon when fed diets with low levels of these FA in the diet.

6.4.1. Phase 1

Significant differences in gene expression were observed after 48 h of incubation, rather than 24 h and 5 days. This incubation time was therefore chosen for the following two phases. Related studies using the Atlantic salmon SHK-1 cell line, and carried out in six-well plates, reported incubation of cells with supplements for 24 h (Carmona-Antoñanzas *et al.*, 2014), and 24 h and 72 h (Minghetti *et al.*, 2011), which may be related to the growth rate of the SHK-1 cell line and the tissue they were originated from.

6.4.2. Phase 2

6.4.2.1. Expression of genes involved in LC-PUFA biosynthesis

In vivo trials have reported reduced expression of *fads2d6* in Atlantic salmon fed diets rich in EPA and DHA, in comparison to Atlantic salmon fed diets lacking these LC-PUFA (Zheng *et al.*, 2005; Leaver *et al.*, 2008; Taggart *et al.*, 2008; Morais *et al.*, 2009). Morais *et al*. (2011b) reported up-regulation of both *fads2d5* and *fads2d6*, and increased expression of *elovl2* was also observed, as a result of the replacement of FO with VO, which was associated to the low levels of dietary n-3 LC-PUFA. In the current study, *fads2d6* was up-regulated in treatments involving supplementation of LNA in presence of 16:0, 18:1n-9 or both 16:0+18:1n-9 (treatments A, B, D, E, F and I), also in treatment LOA/LNA at 20/5 µM and in those supplementing 20 µM LNA in presence of EPA (treatment J), and DHA (treatment K). These latter results are in agreement with those reported by Zheng *et al.* (2009b), who used AS cell line, and observed inhibition of desaturation and elongation of LNA when EPA and DHA were also added in the media, by reducing *fads2d6*. In the current study, *fads2d5* was up-regulated when cells were incubated with LNA in presence of SFA, MUFA or both (treatments B, C, D, E, F and H), and down-regulated when cells were incubated with LNA in presence of EPA, and DHA

(treatments J and K, respectively). *Elovl2* and *elovl5* were up-regulated in all treatments of Phase 2, except for those supplementing LNA in presence of LC-PUFA (treatments J and K), where no statistically significant changes were observed.

6.4.2.2. Expression of transcription factors

Some of the beneficial effects of LC-PUFA stem from their ability to lower elevated serum triglycerides by down-regulating *SREBP1*, which in turn inhibits lipogenesis and stimulates FA oxidation in the liver in mammals (Davidson, 2006; McKenney and Sica, 2007; Kaur *et al.*, 2011; Jung *et al.*, 2011). In Atlantic salmon, similar responses in *srebp1* have been described *in vivo* dietary trials (Morais *et al.*, 2011b; Betancor *et al.*, 2014) and *in vitro* cell culture studies (Minghetti *et al.*, 2011). In agreement with Minghetti *et al*. (2011), who observed up-regulation of *srebp1* when the SHK-1 cell line was supplemented with cholesterol, and down-regulation when supplementing EPA and DHA, in the current study *srebp1* was down-regulated when cells were supplemented with LNA in presence of EPA and DHA (treatments J and K), and up-regulated when EPA and DHA were not present. Minghetti *et al.* (2011) reported increased expression of *lxr* and *srebp2* when the SHK-1 cell line was supplemented with cholesterol, and decreased expression of *srebp2* when cells were supplemented with EPA and DHA. In the current study both transcription factors, *lxr* and *srebp2*, were down-regulated in CHSE-214 supplemented with LNA in presence of EPA and DHA, and up-regulated in CHSE-214 supplemented with LNA in presence of SFA, MUFA or both, particularly in treatments B, C, D, E, and F. The expression of *pparα* did not change in CHSE-214 incubated with LNA in presence of SFA and MUFA (particularly in treatments A, B, D, and F) but, in *pparβ* increased expression was observed in treatments B, C, D, E, and F. *pparα* and *pparβ* were down-regulated in CHSE-214 incubated with LNA in presence of LC-PUFA (treatments J and K, respectively).

6.4.2.3. Expression of genes involved in FA metabolism

In mammals, it has been reported that LC-PUFA have a hypotriglyceridemic effect, increasing the expression of *CPT1* and *ACO*, both genes involved in fatty acid β-oxidation, and decreasing the expression of *SREBP1c* which would result in decreased lipogenesis (Jump and Clarke, 1999; Clarke, 2001). Effects on these pathways were not clear in the present study, but most of the genes involved in FA metabolism, *i.e.cpt1*, *aco*, and *fabp* were down-regulated when CHSE-214 cells were incubated with LNA in presence of n-3 LC-PUFA, and up-regulated when CHSE-214 were incubated with LNA in presence of SFA and MUFA, whereas *fas* was up-regulated in all treatments, including those with supplementation of LC-PUFA. In *in vivo* trials up-regulation of *fas* has been reported in Atlantic salmon fed diets formulated with high levels of VO (Morais *et al.*, 2011b) and high-fat diets (Martinez-Rubio *et al.*, 2013)

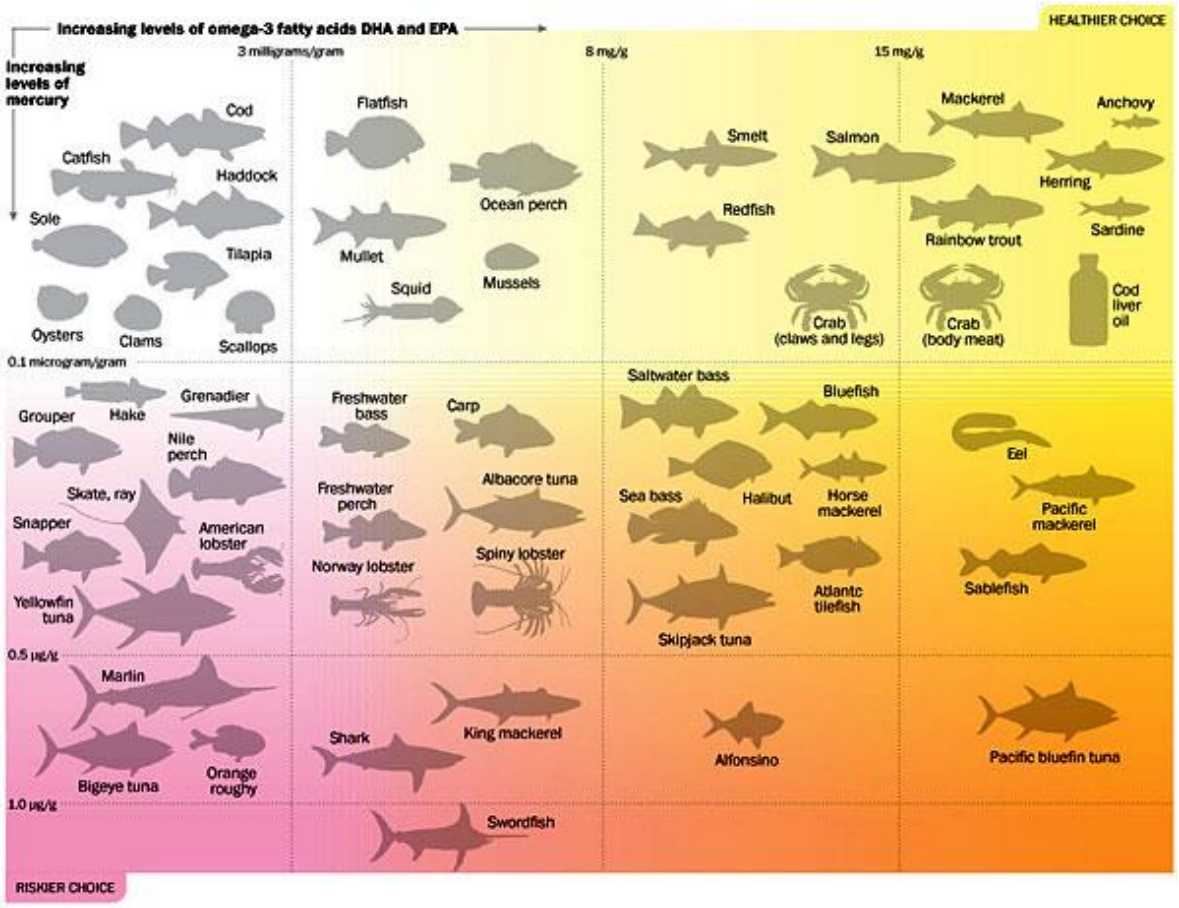
6.4.3. Phase 3

The prediction of the results showed moderate accuracy when compared with the actual results of the new set of experiments. To obtain a more accurate prediction it is therefore necessary to "instruct" the model, as every common neural network, through the performance of several new combination experiments. Having a model that accurately estimates the level of gene expression, without performing the actual analysis, may allow further applications at a lower cost. In conclusion, the expression of genes involved in LC-PUFA and lipid biosynthesis, as well as transcription factors, was affected by the supplementation of FA to the CHSE-214 cell line. CHSE-214 cell line is suitable for the study of molecular mechanisms involved in the conversion of LNA to EPA, however, further studies are required in other salmon cell lines, in order to find a cell line able to synthesise both LC-PUFA, EPA and DHA, from their metabolic precursor LNA. The generation of knowledge in this field, will allow the optimisation of endogenous LC-PUFA

synthesis, and will enable the efficient and effective use of alternative sustainable diets, while maintaining the nutritional quality of farmed fish for consumer.

Chapter 7

Discussion, conclusions and future perspectives



Levels of n-3 LC-PUFA and mercury in different aquatic species [taken from slowplates.blogspot.com]

"It always seems impossible until it's done."

Nelson Mandela

The overall objective of the current research study was to develop and utilise an *in vitro* cell culture model to enable an integrated approach to study the biochemical and molecular regulation of lipid metabolism in Atlantic salmon (*Salmo salar* L.). Since Atlantic salmon is one of the most intensively cultured finfish species worldwide and represents an excellent source of n-3 LC-PUFA in the human diet, the current study focused on this species. The original plan was to develop a cell culture model for Atlantic salmon, using cells derived from the same species. However, Atlantic salmon (AS) cell line previously used in other studies (Tocher and Dick, 1990; Tocher and Sargent, 1990; Ghioni *et al.*, 1999; Zheng *et al.*, 2009b) was not available and SHK-1 demonstrated low growth rates, and difficulties to culture (Minghetti *et al.*, 2011; Carmona-Antoñanzas *et al.*, 2014). For these reasons and for the limited availability of alternative salmon cell lines the present study used the Chinook salmon embryo 214 (CHSE-214) cell line, as it grows fast, and is easy to subculture, therefore has been widely used in other studies (Jensen *et al.*, 2002; McLoughlin and Graham, 2007; Jørgensen *et al.*, 2007; Herath *et al.*, 2009).

The first experimental chapter (Chapter 3) consisted of 11 experiments, aiming to explore the effect of FA supplemented individually at different concentrations (*i.e.* 0, 20, 50, and 100 µM) on total lipid content, lipid class, and FA composition of CHSE-214 cell line. Cells were supplemented with the FA and incubated for 5 d, then lipids were extracted and lipid class and FA analyses were carried out. *In vivo* studies reported higher lipid levels in the tissues of fish fed high lipid diets (Tocher *et al.*, 2003a; b; Martinez-Rubio *et al.*, 2013), therefore, an increment in lipid content with the graded supplementation of FA was expected in the current study; however, the data obtained did not show a clear trend in most of the experiments. During the experiments, many cells died with the highest supplementation of FA, and less lipid content was obtained when cells were harvested. These results were later confirmed with the methyl thiazolyl tetrazolium

(MTT) assay, showing that supplementing FA at 100 µM had a toxic effect that varied with FA, and that the most toxic FA were the ones with a higher number of double bonds, *i.e.* 22:5n-3 and DHA. Low toxic effects were observed at 20 and 40 µM in the current study. Previous studies using human cell lines (Colquhoun and Schumacher, 2001; Bianchi *et al.*, 2004; Shirota *et al.*, 2005; Li *et al.*, 2006; Toit-Kohn *et al.*, 2009; Di Nunzio *et al.*, 2011) and fish cell lines (Tocher *et al.*, 1989; Tocher, 1990; Tocher and Dick, 1990), reported that supplementation of LC-PUFA had a detrimental effect on the survival of the cells. The reason may be that FA with a higher number of double bonds are more susceptible to the attack of reaction oxygen species (ROS), causing cellular damage in the first instance and cellular death thereafter (Mourente *et al.*, 2007; Siddiqui *et al.*, 2008; Di Nunzio *et al.*, 2011). In the cell lipid class compositions, the only clear trend was observed on the levels of TAG, which increased with the graded supplementation of FA. Interesting results were observed at 20 µM supplementation of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), both showing a TAG lowering effect, consistent with previous studies (Manickam *et al.*, 2010; Kaur *et al.*, 2011; Kajikawa *et al.*, 2011). Supplementation of 20 µM arachidonic acid (ARA, 20:4n-6), on the other hand, increased TAG by more than two-fold in comparison with the control (unsupplemented cells). The cellular fatty acid composition data confirmed the ability of CHSE-214 cell line to synthesise EPA from its precursor α-linolenic acid (LNA, 18:3n-3), with the highest EPA level recorded at 20 µM LNA and, therefore, this concentration was used in the subsequent Chapters 4–6. Consistent with EPA-synthesising activity, when the CHSE-214 cell line was supplemented with linoleic acid (LOA, 18:2n-6), synthesis of ARA was also observed. However, the CHSE-214 cell line was not able to synthesise significant levels of DHA from either LNA or EPA. The supplementation of graded concentrations of individual FA showed that 20–40 µM were enough to alter the FA composition of the

cells, without producing a toxic effect, or affecting the cell growth, and without increasing TAG levels. Moreover, at these concentrations no lipid droplets deposited in the cytoplasm were observed. For all the aforementioned, 20 µM was chosen as the concentration of polyunsaturated fatty acids (PUFA) and LC-PUFA supplementation for the experiments carried out in Chapters 4–6. The ideal concentration of FA supplementation determined in this study is in agreement with Tocher *et al*. (1989), and other studies that have suggested 20–25 µM as the optimal concentration for PUFA supplementation to cells in culture to affect membrane FA compositions without affecting lipid deposition (Geyer, 1967; Moskowitz, 1967; Rosenthal, 1981; Stubbs and Smith, 1984; Tocher and Dick, 1990).

No vertebrate, including Atlantic salmon, can synthesise C_{18} PUFA *de novo*, *i.e.* LNA and LOA, therefore they must be provided in the diet, and further converted into n-3 LC-PUFA (EPA and DHA), and n-6 LC-PUFA (ARA), respectively, by enzymatic processes (Tocher, 2003). The pathways involved in the n-3 and n-6 LC-PUFA synthesis in salmonids are well known, and it has been suggested that there is competition between FA substrates by the same enzymes (*e.g.* desaturases and elongases) along the biosynthetic pathway. For the aforementioned, the primary aim of Chapter 4 was to characterise the uptake, incorporation, and metabolism of combinations of n-3 and n-6 PUFA supplemented to the CHSE-214 cell line. Moreover, experiments were designed in order to investigate how competing PUFA (*i.e.* 18:2n-6), via substrate competition, or the pathway end products (*i.e.* EPA, DHA and ARA), via feedback inhibition, affected the production of EPA from LNA. Cells were incubated for 5 d after supplementation with combinations of PUFA and LC-PUFA, and then lipids were extracted, followed by lipid class and FA analyses. Increased cellular lipid content with the graded supplementation of the PUFA combinations was expected; however, this trend was only observed with treatment LOA plus graded ARA. Lower lipid content was recorded in cells supplemented with FA

combinations of the n-3 series, such as LNA/EPA, EPA/DHA and LNA/EPA+DHA in comparison with supplementation of FA of the n-6 series. Data may suggest that higher concentrations of supplemented n-3 PUFA caused increased oxidative stress, resulting in cellular damage and ultimately cell death due to increased lipid radicals (Mourente *et al.*, 2007; Siddiqui *et al.*, 2008; Di Nunzio *et al.*, 2011). As cells were dying less cellular lipid was recovered from the flasks. Consistent with this, Gregory *et al*. (2011) reported more peroxidation in the FHM (fathead minnow, *Pimephales promelas* Rafinesque) cell line supplemented with n-3 LC-PUFA in comparison with cells supplemented with LNA or SFA, and in studies with human cell lines, cell viability was affected by supplementation with LC-PUFA (Colquhoun and Schumacher, 2001; Bianchi *et al.*, 2004; Shirota *et al.*, 2005; Li *et al.*, 2006; Toit-Kohn *et al.*, 2009; Di Nunzio *et al.*, 2011). Supplementation with FA showed no clear effects on cellular lipid content because this was measured in absolute terms on a per flask basis and this was severely affected by cell numbers and cell death. Lipid class composition was reported as relative percentages and this was not affected by the cell number, therefore the data obtained were more consistent, showing the effects of increasing lipid (FA) supplementation to cells giving increased cellular lipid in form of increased deposition of TAG. The increment in cellular TAG was generally balanced by decreasing proportions of polar lipids and/or cholesterol. The graded supplementation of EPA, DHA and the mix EPA+DHA (1:1) lowered TAG levels possibly by down-regulating the transcription factors, which in turn inhibited the lipogenesis, whereas the graded supplementation of the n-6 PUFA (*i.e.* LOA and ARA) increased TAG levels. The increment in TAG with the supplementation of n-6 FA may be due to incorporation and/or deposition of the FA. Previous studies associated the increase in cellular TAG levels with supplementation of ARA (Collier and Collier, 1993; Whelan *et al.*, 1995; Whelan, 1996) and the lowering TAG effect of n-3 LC-PUFA have been

reported *in vivo* (Kajikawa *et al.*, 2011) and *in vitro* (Manickam *et al.*, 2010), suggesting that EPA down-regulates the sterol regulating element binding protein (SREBP), which in turn reduces the expression of genes involved in lipogenesis and, likely, TAG synthesis (Kaur *et al.*, 2011). Regarding FA analysis, all PUFA combinations changed the FA composition of CHSE-214 cells, due to both incorporation and, in some cases, conversion of the FA supplemented by elongation and/or desaturation. The conversion of LNA to EPA was negatively affected with the graded supplementation of LOA and ARA and, in addition, the conversion of LOA to ARA was detrimentally affected with the graded supplementation of n-3 PUFA. The EPA levels of cells incubated with LNA in combination with low levels of LOA were higher (5.6–4.8%) in comparison to cells incubated with LNA alone at 20 µM (4%), and were lower when the cells were supplemented with LNA in combination with all concentrations of ARA (3.9–3.3%). Enzyme competition between C_{18} PUFA was confirmed when LNA and LOA were supplemented at equal concentrations (20/20 µM), when LOA was mainly incorporated without showing further conversion, whereas the conversion of LNA to EPA was still clear, suggesting that enzymes involved in LC-PUFA synthesis have a preference for the n-3 series, as previous studies suggested (Stubbs and Smith, 1984; Tocher *et al.*, 1989; Gregory *et al.*, 2011). The increasing EPA levels in cells incubated with LNA/DHA, may suggest retro-conversion of DHA to EPA (Grønn *et al.*, 1991). When CHSE-214 cells were incubated with EPA in the presence of increasing concentrations of LNA, 22:5n-3 increased with the graded supplementation of LNA; however, no further conversion to DHA was observed.

The LC-PUFA biosynthesis pathways (*i.e.* the elongation and desaturation of C_{18} PUFA) are negatively affected by dietary lipid content (Martinez-Rubio *et al.*, 2013). Therefore, the primary objective of Chapter 5 was to investigate the effect of lipid level on

the LC-PUFA biosynthetic pathway, using SFA (16:0) and MUFA (18:1n-9) (which do not compete with LNA or LOA in the LC-PUFA biosynthesis pathway) to represent "dietary" lipid. Cells were incubated for 5 d with LNA and LOA in presence of graded concentrations of 16:0, 18:1n-9, and the combination of 16:0 and 18:1n-9 as a mix (1:1 ratio), followed by lipid and FA analyses. Increased cell lipid content was expected with the supplementation of C_{18} PUFA (20 µM) and the graded increased concentrations of SFA and MUFA, since *in vivo* trials in Atlantic salmon reported higher lipid contents in tissues in fish fed higher dietary lipid levels (Bell *et al.*, 1998; Hemre and Sandnes, 1999; Martinez-Rubio *et al.*, 2013). However, this trend was only observed in cells supplemented with LNA+EPA plus increasing concentrations of 18:1n-9. The other experiments in this study showed no clear trends in cell lipid content, which may be explained by the fact that higher concentrations of FA supplemented had toxic effects, as mentioned above. Similar to the results obtained in Chapters 3 and 4, the main clear trend observed in the lipid class composition data was increased cellular TAG associated with the graded supplementation of SFA, MUFA and the combination of the two. Other changes in lipid class composition were mainly a consequence on the increased TAG generally reduced proportions of the other lipid classes. In agreement with this, Martinez-Rubio *et al.* (2013) reported increased TAG in the liver of Atlantic salmon, with graded increments of dietary lipid content.

Tocher *et al.* (1988) observed that the FA profile of six fish cell lines reflected the FA profile of the media and *in vivo* trials showed that the FA profile of the diet is reflected in fish flesh (Bell *et al.*, 2003b; Tocher *et al.*, 2003a; c; Menoyo *et al.*, 2005; Tocher, 2010; Alves Martins *et al.*, 2011; Xu *et al.*, 2014; Betancor *et al.*, 2015). According to this, all the combinations of FA supplemented in Chapter 5 changed the FA composition of the cell line, due to incorporation of the FA and, to some extent, conversion to other intermediate metabolites. The EPA levels of CHSE-214 cells incubated with LNA plus graded

increasing concentrations of 16:0, 18:1n-9 or 16:0+18:1n-9 were negatively affected with the graded supplementation of SFA and MUFA. Only low levels of EPA were recorded in all these experiments (1.5–1.8%). Martinez-Rubio *et al*. (2013) reported that liver of Atlantic salmon fed high dietary lipid contained lower n-3 LC-PUFA in comparison with fish fed low dietary lipid. Tocher *et al.* (2003a; b) observed higher synthesis of LC-PUFA in the liver of fish fed low dietary lipid, in comparison with fish fed high lipid diet. In red seabream and yellowtail, the dietary requirements for LC-PUFA increased with the increment of dietary lipid, suggesting that dietary lipid negatively affect the expression of genes involved with LC-PUFA biosynthesis (Takeuchi *et al.*, 1992). The levels of DHA were not clearly affected by the two combinations LNA+EPA plus increasing concentrations of 18:1n-9 and LNA+EPA plus increasing 16:0+18:1n-9, being similar at all concentrations. However, the levels of DHA in cells incubated with LNA+EPA plus 16:0 were two-fold higher in comparison with cells incubated with LNA+EPA plus 18:1n-9 and LNA+EPA plus 16:0+18:1n-9. In cells incubated with LOA plus 16:0+18:1n-9, ARA levels steadily decreased with increasing concentrations of 16:0+18:1n-9 (3.3–1.8%), while cells supplemented with LOA and increasing concentrations of 18:1n-9 or 16:0, similar ARA levels were recorded, without showing any clear detrimental effect of the supplementation of SFA, MUFA and the combination of the last two, on the conversion of LOA to ARA.

Chapter 6 consisted of three phases. In Phase 1 the "ideal" incubation time of CHSE-214 cells with the supplemented FA in order to observe changes in expression of lipid and FA genes was determined. CHSE-214 cells were seeded in six-well plates and then supplemented with 20 µM LNA, LOA and the control (cells incubated with no FA). Cells were incubated for 24 h, 48 h, and 5 d and the expression of five genes (*elovl2*, *fads2d5*, *srebp*, *ppar*, and *lxr*) was analysed. Significant differences were observed at 48 h

for all genes, therefore this incubation time was used for experiments in phases two and three. For Phase 2, key experiments from Chapters 4 and 5 were repeated in order to analyse the expression of a suite of 14 genes involved in lipid and FA metabolism. Chapters 4 and 5 described how the supplementation of PUFA, SFA and MUFA affected the conversion of LNA to EPA and the gene expression analyses allowed the association of the changes in lipid and FA composition with the changes occurring at the gene expression level. From the genes involved with LC-PUFA biosynthesis, the expression of *elovl2* and *elovl5* did not change with the supplementation of LNA in presence of n-3 LC-PUFA, whereas the expression of both genes increased with the supplementation of LNA in presence of 16:0, 18:1n-9 and the mix 16:0+18:1n-9. In the treatments LNA/EPA and LNA/DHA, *fads2d5* was down-regulated, while *fads2d6* was slightly up-regulated in cells supplemented with LNA/EPA, and no changes were observed in cells supplemented with LNA/DHA. However, when LNA was supplemented in presence of 16:0, 18:1n-9 and the mix 16:0+18:1n-9, both *fads2d5* and *fads2d6* were up-regulated. The results from the current study are in agreement with an *in vitro* study using AS cell line (Zheng *et al.*, 2009b), showing inhibition of desaturation of LNA when n-3 LC-PUFA were supplemented in the media, by reducing *fads2d6*, and also with *in vivo* studies in Atlantic salmon, where increased expression of *fads2d6* was observed in fish fed diets lacking of n-3 LC-PUFA, in comparison with fish fed diets rich in EPA and DHA (Zheng *et al.*, 2005; Leaver *et al.*, 2008; Taggart *et al.*, 2008; Morais *et al.*, 2009). Moreover, up-regulation of *fads2d5*, *fads2d6* and *elovl2* was observed in fish fed vegetable oil (VO) based diets, lacking LC-PUFA, in comparison with fish fed fish oil (FO) based diets containing LC-PUFA (Morais *et al.*, 2011b). All transcription factors analysed in the present study were down-regulated when cells were supplemented with LNA in presence of EPA and DHA, and up-regulated when cells were incubated with LNA in presence of SFA, MUFA and the

mix SFA+MUFA, except for treatment LNA plus 16:0 (20/20 µM). In previous Chapters of the current thesis a TAG lowering effect was observed with the supplementation of n-3 LC-PUFA. The analyses of the gene expression suggested that EPA and DHA down-regulated the expression of transcription factors, which in turn inhibited lipogenesis and stimulated FA oxidation, therefore, reducing TAG levels. Similar responses have been described in mammals (Davidson, 2006; McKenney and Sica, 2007; Kaur *et al.*, 2011; Jung *et al.*, 2011), in fish *in vivo* (Morais *et al.*, 2011b; Betancor *et al.*, 2014), and *in vitro* fish cell culture studies (Minghetti *et al.*, 2011). Another way in which n-3 LC-PUFA lower TAG levels in mammals has been through increasing the expression of genes involved in FA β-oxidation, such as carnitine palmitoyl transferase 1 (*CPT1*) and acyl-CoA oxidase (*ACO*), and decreasing the expression of *SREBP1c* which in turn decreases lipogenesis (Jump and Clarke, 1999; Clarke, 2001). However, this pattern was not very clear in the current study, only *fas* and *cpt1* were slightly up-regulated, while no changes were observed in the expression of *aco* and fatty acid binding protein (*fabp*) when cells were incubated with LNA in the presence of EPA and DHA; however, these four genes were up-regulated when cells were supplemented with LNA in presence of SFA, MUFA and the mix SFA+MUFA. *In vivo* trials reported up-regulation of fatty acid synthase (*fas*) in Atlantic salmon fed diets formulated with high levels of VO (Morais *et al.*, 2011b) and high-fat diets (Martinez-Rubio *et al.*, 2013). Based on the data produced in Phase 2, a model framework was created to describe the effects of different FA combinations on gene expression and, ultimately, LC-PUFA biosynthesis. These predictions were then tested by actual experiments, measuring the effects of the FA supplementation on gene expression and lipid and fatty acid compositions. The predicted results showed reasonable accuracy when compared with the actual results of the new set of experiments. However, the model created could be improved with the performance of new FA combinations, where also the

concentration supplemented would be taken into consideration. The creation of a reliable model would allow the estimation of gene expression, without performing the actual analysis, saving time and money. The results produced in the present study indicated that the CHSE-214 cell line could represent a useful system for the study of lipid and FA metabolism in salmon species. Particularly, this cell line can be used for future studies related with the conversion of LNA to EPA and LOA to ARA, but unfortunately it is not suitable for studies focusing on the conversion of either LNA or EPA to DHA in salmon.

The original aim of the current study was that research into lipid and FA metabolism in Atlantic salmon would be facilitated enormously by the availability of a cell model system, as *in vivo* trials are expensive and it takes a long time to obtain the results. The aim was partly achieved, as the CHSE-214 cell line facilitated the study of the pathways of LC-PUFA biosynthesis in a deeper, faster and cheaper way. The information generated from this study might contribute to the optimisation of diets for Atlantic salmon, by taking into consideration the lipid content and the FA profile of the alternative oils used for the formulation of aquafeeds, in order to enhance the endogenous biosynthesis of n-3 LC-PUFA, assuring culture of Atlantic salmon in a sustainable way, while maintaining the nutritional quality of the product for human consumption.

7.1. Summary of results and conclusions

The use of cell lines has greatly facilitated the elucidation of the LC-PUFA biosynthesis pathways in several fish species (Tocher *et al.*, 1988, 1989, 1998; Tocher, 1990; Tocher and Dick, 1990; Tocher and Sargent, 1990; Furth *et al.*, 1992; Ghioni *et al.*, 1999; Minghetti *et al.*, 2011; Carmona-Antoñanzas *et al.*, 2014). It is vital that a specifically developed model cell line derived from salmon is available for undertaking similar research and expanding the lipid and FA biochemical analyses to molecular studies.

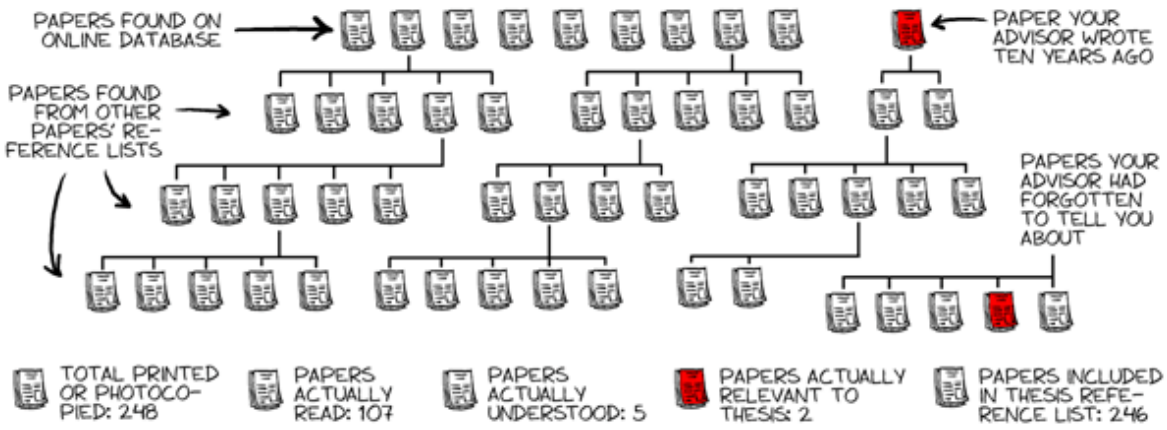
In the current study, the CHSE-214 cell line showed a fast growth rate and was relatively easy to maintain under laboratory conditions, as demonstrated by the large number of experiments that were able to be carried out in the present thesis (see Chapters 3–6). In summary, the results of the present study indicated that:

a) The CHSE-214 cell line represents an important tool for the research of LC-PUFA biosynthesis, particularly EPA and ARA from the C_{18} PUFA, LNA and LOA.
b) There was competition between C_{18} PUFA (*i.e.* LNA and LOA) for the enzymes of the LC-PUFA pathway, and the enzymes showed a preference for the n-3 series FA.
c) A combination of LNA in the presence of low concentrations of LOA (5 µM) enhanced EPA production but higher concentrations (20 µM) of LOA showed enzyme competition, negatively affecting the production of EPA.
d) Dietary lipid represented by the supplementation of SFA and MUFA, had a detrimental effect on LC-PUFA biosynthesis, particularly in the conversion of LNA to EPA.
e) Supplemenation of the n-3 LC-PUFA (*i.e.* EPA and DHA) to the CHSE-214 cell line showed a TAG lowering effect, probably by down-regulating the transcriptional expression of lipogenic genes and therefore inhibiting lipogenesis.
f) Supplementation of SFA and MUFA to CHSE-214 cells up-regulated most of the genes analysed in this study and also increased cellular TAG levels.
g) The patterns observed in the expression of transcription factors involved in the control and regulation of FA metabolism were used to create an integrated model for LC-PUFA biosynthesis, which allowed the prediction of untested experiments with moderate accuracy. However, further studies are required in order to improve the model.

7.2. Future perspectives

In order to improve the integrated model created in this study, more experiments in this field are required. Moreover, it would be very interesting to carry out more untested experiments using the final products, such as EPA, DHA and ARA, to elucidate the gene response as a result of product (feedback) inhibition. The next step after this would be to set up *in vivo* feeding trials, using diets formulated with combinations of dietary oils, including various VO, with a specific FA profile or with different levels of lipid content and compare the results with the data produced with the *in vitro* cell culture model utilised in the present study.

References



The importance of the reference list [taken from www.phdcomics.com]

"If you want something you've never had, you've got to do something you've never done."

Anonymous

Ackman, R. G. (1980). Fish lipids, part 1. In: *Advances in Fish Science and Technology* (Ed. Connell, J. J.), pp. 86–103. Fishing News Books Ltd., Farnham, Surrey (UK).

Adams, R. L. P. (1980). *Cell culture for biochemistry*. 2nd edition. Elsevier/North-Holland Biomedical Press, Amsterdam (The Netherlands), pp. 292.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (2002). *Molecular Biology of the Cell*. 4th edition. Garland Science, New York (USA), pp. 1616.

Alves Martins, D., Valente, L. M. P. and Lall, S. P. (2011). Partial replacement of fish oil by flaxseed oil in Atlantic halibut (*Hippoglossus hippoglossus*) diets: effects on growth, nutritional and sensory quality. *Aquaculture Nutrition* **17**, 671–684.

Amaro, R. and Sánchez, L. (1997). Chromosomal analysis of two established salmonid cell lines: CHSE-214 (*Oncorhynchus tschawytscha*) and RTG-2 (*Oncorhynchus mykiss*). *In Vitro Cellular & Developmental Biology* **33**, 662–664.

Bahich, H. and Borenfreund, E. (1991). Cytotoxicity and genotoxicity assays with cultured fish cells: a review. *Toxicology in Vitro* **5**, 91–100.

Bailey, J. M. and Dunbar, L. M. (1973). Essential fatty acid requirements of cells in tissue culture. *Experimental and Molecular Pathology* **18**, 142–161.

Bell, J. G. and Koppe, W. (2011). Lipids in Aquafeeds. In: *Fish oil replacement and alternative lipid sources in aquaculture feeds* (Ed. Turchini, G. M., Ng, W.-K. and Tocher, D. R.), pp. 21–59. Taylor and Francis group, Boca Raton (USA).

Bell, J. G. and Sargent, J. R. (2003). Arachidonic acid in aquaculture feeds: current status and future opportunities. *Aquaculture* **218**, 491–499.

Bell, J. G., McVicar, A. H., Park, M. T. and Sargent, J. R. (1991). High dietary linoleic acid affects the fatty acid compositions of individual phospholipids from tissues of Atlantic salmon (*Salmo salar*): association with stress susceptibility and cardiac lesion. *The Journal of Nutrition* **121**, 1163–1172.

Bell, J. G., Sargent, J. R. and Raynard, R. S. (1992). Effects of increasing dietary linoleic acid on phospholipid fatty acid composition and eicosanoid production in leucocytes and gill cells of Atlantic salmon (*Salmo salar*). *Prostaglandins, Leukotrienes and Essential Fatty Acids* **45**, 197–206.

Bell, J. G., Dick, J. R., McVicar, A. H., Sargent, J. R. and Thompson, K. D. (1993). Dietary sunflower, linseed and fish oils affect phospholipid fatty acid composition, development of cardiac lesions, phospholipase activity and eicosanoid production in Atlantic salmon (*Salmo salar*). *Prostaglandins Leukotrienes and Essential Fatty Acids* **49**, 665–673.

Bell, J. G., Tocher, D. R. and Sargent, J. R. (1994). Effect of supplementation with 20:3(n-6), 20:4(n-6) and 20:5(n-3) on the production of prostaglandines E and F of the 1-, 2- and 3-series in turbot (*Scophthalmus maximus*) brain astroglial cells in primary culture. *Biochimica et Biophysica Acta* **1211**, 335–342.

Bell, M. V., Batty, R. S., Dick, J. R., Fretwell, K., Navarro, J. C. and Sargent, J. R. (1995). Dietary deficiency of docosahexaenoic acid impairs vision at low light intensities in juvenile herring (*Clupea harengus* L.). *Lipids* **30**, 443–449.

Bell, J. G., Ashton, I., Secombes, C. J., Weitzel, B. R., Dick, J. R. and Sargent, J. R. (1996a). Dietary lipid affects phospholipid fatty acid compositions, eicosanoid production and immune function in Atlantic salmon (*Salmo salar*). *Prostaglandins Leukotrienes and Essential Fatty Acids* **54**, 173–182.

Bell, J. G., Farndale, B. M., Dick, J. R. and Sargent, J. R. (1996b). Modification of membrane fatty acid composition, eicosanoid production, and phospholipase a activity in Atlantic salmon (*Salmo salar*) gill and kidney by dietary lipid. *Lipids* **31**, 1163–1171.

Bell, J. G., McEvoy, J., Webster, J. L., McGhee, F., Millar, R. M. and Sargent, J. R. (1998). Flesh lipid and carotenoid composition of Scottish farmed Atlantic salmon (*Salmo salar*). *Journal of Agricultural and Food Chemistry* **46**, 119–127.

Bell, J. G., McEvoy, J., Tocher, D. R., McGhee, F., Campbell, P. J. and Sargent, J. R. (2001). Replacement of fish oil with rapeseed oil in diets of Atlantic salmon (*Salmo salar*) affects tissue lipid compositions and hepatocyte fatty acid metabolism. *Journal of Nutrition* **131**, 1535–1543.

Bell, J. G., Henderson, R. J., Tocher, D. R., McGhee, F., Dick, J. R., Porter, A., Smullen, R. P. and Sargent, J. R. (2002). Substituting fish oil with crude palm oil in the diet of Atlantic salmon (*Salmo salar*) affects muscle fatty acid composition and hepatic fatty acid metabolism. *The Journal of Nutrition* **132**, 222–230.

Bell, J. G., McGhee, F., Campbell, P. J. and Sargent, J. R. (2003a). Rapeseed oil as an alternative to marine fish oil in diets of post-smolt Atlantic salmon (*Salmo salar*): changes in flesh fatty acid composition and effectiveness of subsequent fish oil "wash out." *Aquaculture* **218**, 515–528.

Bell, J. G., Tocher, D. R., Henderson, R. J., Dick, J. R. and Crampton, V. O. (2003b). Altered fatty acid compositions in Atlantic salmon (*Salmo salar*) fed diets containing linseed and rapeseed oils can be partially restored by a subsequent fish oil finishing diet. *Journal of Nutrition* **133**, 2793–2801.

Bendiksen, E. Å., Berg, O. K., Jobling, M., Arnesen, A. M. and Måsøval, K. (2003). Digestibility, growth, and nutrient utilisation of Atlantic salmon (*Salmo salar* L.) in relation to 447 temperature, feed fat content and oil source. *Aquaculture* **224**, 283–299.

Bendiksen, E. Å., Johnsen, C. A., Olsen, H. J. and Jobling, M. (2011). Sustainable aquafeeds: progress towards reduced reliance upon marine ingredients in diets for farmed Atlantic salmon (*Salmo salar* L.). *Aquaculture* **314**, 132–139.

Benedito-Palos, L., Navarro, J. C., Bermejo-Nogales, A., Saera-Vila, A., Kaushik, S. and Perez-Sanchez, J. (2009). The time course of fish oil wash-out follows a simple dilution model in gilthead sea bream (Sparus aurata L.) fed graded levels of vegetable oils. *Aquaculture* **288**, 98–105.

Berg, J. M., Tymoczko, J. L. and Stryer, L. (2002). *Biochemistry*. 5th edition. Freeman W.H. & Co. Ltd., New York (USA), pp. 1050.

Betancor, M. B., Howarth, F. J. E., Glencross, B. D. and Tocher, D. R. (2014). Influence of dietary docosahexaenoic acid in combination with other long-chain polyunsaturated fatty acids on expression of biosynthesis genes and phospholipid fatty acid compositions in tissues of post-smolt Atlantic salmon (*Salmo salar*). *Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology* **172-173**, 74–89.

Betancor, M. B., Sprague, M., Usher, S., Sayanova, O., Campbell, P. J., Napier, J. A. and Tocher, D. R. (2015). A nutritionally-enhanced oil from transgenic *Camelina sativa* effectively replaces fish oil as a source of eicosapentaenoic acid. *Scientific Reports* **5,** 8104.

Bianchi, A., Dewailly, E., Gautier, H., Merlin, J. L., Slomianny, C., Dauça, M. and Bécuwe, P. (2004). Decrease of human hepatoma cell growth by arachidonic acid is associated with an accumulation of derived products from lipid peroxidation. *Biochimie* **86**, 633–642.

Bols, N. C. and Lee, L. E. J. (1991). Technology and uses of cell culture from tissues and organs of bony fish. *Cytotechnology* **6**, 163–187.

Bols, N. C., Brubacher, J. L., Ganassin, R. C. and Lee, L. E. J. (2001). Ecotoxicology and innate immunity in fish. *Developmental & Comparative Immunology* **25**, 853–873.

Boukouvala, E., Antonopoulou, E., Favre-Krey, L., Diez, A., Bautista, J. M., Leaver, M. J., Tocher, D. R. and Krey, G. (2004). Molecular characterization of three peroxisome proliferator-activated receptors from the sea bass (*Dicentrarchus labrax*). *Lipids* **39**, 1085–1092.

Bransden, M. P., Carter, C. G. and Nichols, P. D. (2003). Replacement of fish oil with sunflower oil in feeds for Atlantic salmon (*Salmo salar* L.): effect on growth performance, tissue fatty acid composition and disease resistance. *Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology* **135**, 611–625.

Calder, P. C. (2001). Polyunsaturated fatty acids, inflammation, and immunity. *Lipids* **36**, 1007–1024.

Carmona-Antoñanzas, G., Monroig, O., Dick, J. R., Davie, A. and Tocher, D. R. (2011). Biosynthesis of very long-chain fatty acids (C>24) in Atlantic salmon: cloning, functional characterisation, and tissue distribution of an Elovl4 elongase. *Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology* **159**, 122–9.

Carmona-Antoñanzas, G., Tocher, D. R., Martinez-Rubio, L. and Leaver, M. J. (2014). Conservation of lipid metabolic gene transcriptional regulatory networks in fish and mammals. *Gene* **534**, 1–9.

Carrel, M. (1912). Pure cultures of cells. *The Journal of Experimental Medicine* **16**, 165–168.

Carter, C. G., Bransden, M. P., Lewis, T. E. and Nichols, P. D. (2003). Potential of thraustochytrids to partially replace fish oil in Atlantic salmon feeds. *Marine biotechnology (New York, N.Y.)* **5**, 480–92.

Castell, J. D., Lee, D. J. and Sinnhuber, R. O. (1972). Essential fatty acids in the diet of rainbow trout (*Salmo gairdneri*): lipid metabolism and fatty acid composition. *Journal of Nutrition* **102**, 93–100.

Catal, A. (2009). Lipid peroxidation of membrane phospholipids generates hydroxyalkenals and oxidized phospholipids active in physiological and/or pathological conditions. *Chemistry and Physics of Lipids* **157**, 1–11.

Christie, W. W. (2003). *Lipid analysis.* 2nd edition. The Oily Press, Bridgwater (UK), pp. 448.

Clarke, S. D. (2001). Polyunsaturated fatty acid regulation of gene transcription: a molecular mechanism to improve the metabolic syndrome *The Journal of Nutrition* **131**, 1129–1132.

Collier, F. M. and Collier, G. R. (1993). Effect of feeding arachidonic acid to streptozotocin diabetic rats. *Annals of the New York Academy of Sciences* **683**, 365–366.

Colquhoun, A. and Schumacher, R. I. (2001). Gamma-linolenic acid and eicosapentaenoic acid induce modifications in mitochondrial metabolism, reactive oxygen species generation, lipid peroxidation and apoptosis in Walker 256 rat carcinosarcoma cells. *Biochimica et Biophysica Acta* **1533**, 207–219.

Cowey, C. B. and Sargent, J. R. (1979). Nutrition. In: *Fish physiology, Vol. VIII* (Ed. Hoar, W. S., Randall, D. J. and Brett, J. R.), pp. 1–69. Academic Press, New York (USA).

Cruz-Garcia, L., Minghetti, M., Navarro, I. and Tocher, D. R. (2009). Molecular cloning, tissue expression and regulation of liver X receptor (LXR) transcription factors of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology* **153**, 81–88.

Davidson, M. H. (2006). Mechanisms for the hypotriglyceridemic effect of marine omega-3 fatty acids. *American Journal of Cardiology* **98**, 27–33.

Davoren, M., Ní Shúilleabháin, S., Hartl, M. G. J., Sheehan, D., O'Brien, N. M., O'Halloran, J., Van Pelt, F. N. A. M. and Mothersill, C. (2005). Assessing the potential of fish cell lines as tools for the cytotoxicity testing of estuarine sediment aqueous elutriates. *Toxicology in Vitro* **19**, 421–431.

Dawson, M. I. and Xia, Z. (2012). The retinoid X receptors and their ligands. *Biochimica et Biophysica Acta* **1821**, 21–56.

De Pablo, M. A. and Alvarez de Cienfuegos, G. (2000). Modulatory effects of dietary lipids on immune system functions. *Immunology & Cell Biology* **78**, 31–39.

Desvergne, B. A., Michalik, L. and Wahli, W. (2006). Transcriptional regulation of metabolism. *Physiological Reviews* **86**, 465–514.

Di Nunzio, M., Valli, V. and Bordoni, A. (2011). Pro- and anti-oxidant effects of polyunsaturated fatty acid supplementation in HepG2 cells. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **85**, 121–127.

Diez, A., Menoyo, D., Pérez-Benavente, S., Calduch-Giner, J. A., Vega-Rubin de Celis, S., Obach, A., Favre-Krey, L., Boukouvala, E., Leaver, M. J., Tocher, D. R., Pérez-Sanchez, J., Krey, G. and Bautista, J. M. (2007). Conjugated linoleic acid affects lipid composition, metabolism, and gene expression in gilthead sea bream (*Sparus aurata* L.). *The Journal of Nutrition* **137**, 1363–1369.

Duplus, E., Glorian, M. and Forest, C. (2000). Fatty acid regulation of gene transcription. *The Journal of Biological Chemistry* **275**, 30749–30752.

Fahy, E., Subramaniam, S., Brown, H. A., Glass, C. K., Merrill, A. H., Murphy, R. C., Raetz, C. R. H., Russell, D. W., Seyama, Y., Shaw, W., Shimizu, T., Spener, F., van Meer, G., VanNieuwenhze, M. S., White, S. H., Witztum, J. L. and Dennis, E. A. (2005). A comprehensive classification system for lipids. *Journal of Lipid Research* **46**, 839–861.

Fänge, R. and Grove, D. (1979). Digestion. In: *Fish Physiology, Vol. VIII* (Ed. Hoar, W. S., Randall, D. J. and Brett, J. R.), pp. 161–260. Academic Press, New York (USA).

FAO (2014). The state of world fisheries and aquaculture 2014. Rome (Italy), pp. 223.

FIN: Fishmeal Information Network (2008). FIN dossier - Annual Review of the feed grade fish stocks used to produce fishmeal and fish oil for the UK market. London (UK), 77 pp.

Finn, R. N., Henderson, J. R. and Fyhn, H. J. (1995). Physiological energetics of developing embryos and yolk-sac larvae of Atlantic cod (*Gadus morhua*). II. Lipid metabolism and enthalpy balance. *Marine Biology* **124**, 371– 379.

Folch, J. M., Lees, M. and Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *The Journal of Biological Chemistry* **226**, 497–509.

Fraser, A. J., Gamble, J. C. and Sargent, J. R. (1988). Changes in lipid content, lipid class composition and fatty acid composition of developing eggs and unfed larvae of cod (*Gadus morhua*). *Marine Biology* **99**, 307–313.

Fraulob, J. C., Ogg-Diamantino, R., Fernandes-Santos, C., Aguila, M. B. and De-Lacerda, C. A. M.- (2010). A mouse model of metabolic syndrome: insulin resistance, fatty liver and non-alcoholic fatty pancreas disease (NAFPD) in C57BL/6 mice fed a high fat diet. *Journal of Clinical Biochemistry and Nutrition* **46**, 212–223.

Freshney, R. I. (2010). *Culture of animal cells: a manual of basic techniques and specialized applications*. 6th edition (Ed. by R.I. Freshney), Wiley-Blackwell, New York (USA). 796 pp.

Freshney, R. I. and Freshney, M. G. (2002). *Culture of epithelial cells*. 2nd edition (Ed. by R.I. Freshney, M.G. Freshney), Wiley-Liss, New York (USA). 461 pp.

Frøyland, L., Madsen, L., Eckhoff, K. M., Lie, Ø. and Berge, R. (1998). Carnitine palmitoyltransferase I, carnitine palmitoyltransferase II, and acyl-CoA oxidase activities in Atlantic salmon (*Salmo salar*). *Lipids* **33**, 923–930.

Fryer, J. L., Yusha, A. and Pilcher, K. S. (1965). The *in vitro* cultivation of tissue and cells of pacific salmon and steelhead trout. *Annals of the New York Academy of Sciences* **126**, 566–586.

Funk, C. D. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* **294**, 1871–1875.

Furth, E. E., Sprecher, H., Fisher, E. A., Fleishman, H. D. and Laposata, M. (1992). An *in vitro* model for essential fatty acid deficiency: HepG2 cells permanently maintained in lipid-free medium. *Journal of Lipid Research* **33**, 1719–1726.

Gatlin, D. (2002). Nutrition and fish health. In: *Fish Nutrition* (Eds. Halver, J. E. and Hardy, R. W.), pp. 671–702. Academic Press, London (UK).

Geyer, R. P. (1967). Uptake and retension of fatty acids by tissue culture cells. In: *Lipid metabolism in tissue culture cells* (Eds. Rothblat, G. H. and Kritchevsky, D.), pp. 33–44. Wistar, Philadelphia (USA).

Ghioni, C., Tocher, D. R., Bell, M. V., Dick, J. R. and Sargent, J. R. (1999). Low C_{18} to C_{20} fatty acid elongase activity and limited conversion of stearidonic acid, 18:4(n-3), to eicosapentaenoic acid, 20:5(n-3), in a cell line from the turbot, *Scophthalmus maximus*. *Biochimica et Biophysica Acta* **1437**, 170–181.

Givens, D. I. and Gibbs, R. A. (2006). Very long chain n-3 polyunsaturated fatty acids in the food chain in the UK and the potential of animal-derived foods to increase intake. *Nutrition Bulletin* **31**, 104–110.

Gregory, M. K., See, V. H. L., Gibson, R. A. and Schuller, K. A. (2010). Cloning and functional characterisation of a fatty acyl elongase from southern bluefin tuna (*Thunnus maccoyii*). *Comparative Biochemistry and Physiology* **155**, 178–185.

Gregory, M. K., King, H. W., Bain, P. A., Gibson, R. A., Tocher, D. R. and Schuller, K. A. (2011). Development of a fish cell culture model to investigate the impact of fish oil replacement on lipid peroxidation. *Lipids* **46**, 753–764.

Grønn, M., Christensen, E., Hagve, T.-A. and Christophersen, B. O. (1991). Peroxisomal retroconversion of docosahexaenoic acid (22:6(n−3)) to eicosapentaenoic acid (20:5(n−3)) studied in isolated rat liver cells. *Biochimica et Biophysica Acta* **1081**, 85–91.

Gurr, M., Harwood, J. and Frayn, K. (2002). *Lipid biochemistry - An Introduction*. Blackwell Science, Oxford (UK), pp. 320.

Gustone, F. and Norris, F. A. (1983). *Lipids in food, chemistry, biochemistry and technology*. Pergamon Press, London (UK), pp. 148.

Hardy, R. (2010). Utilization of plant proteins in fish diet: effects of global demand and supplies of fishmeal. *Aquaculture Research* **41**, 770–776.

Harrison, R. G. (1907). Observations on the living developing nerve fiber. *The Anatomical Record* **1**, 116–128.

Hastings, N., Agaba, M. K., Tocher, D. R., Zheng, X., Dickson, C. a., Dick, J. R. and Teale, A. J. (2005). Molecular cloning and functional characterization of fatty acyl desaturase and elongase cDNAs involved in the production of eicosapentaenoic and docosahexaenoic acids from α-linolenic acid in Atlantic salmon (*Salmo salar*). *Marine Biotechnology* **6**, 463–474.

Haunerland, N. H. and Spener, F. (2004). Fatty acid-binding proteins--insights from genetic manipulations. *Progress in Lipid Research* **43**, 328–349.

Hemre, G.-I. I. and Sandnes, K. (1999). Effect of dietary lipid level on muscle composition in Atlantic salmon *Salmo salar*. *Aquaculture Nutrition* **5**, 9–16.

Hemre, G.-I., Sandnes, K., Lie, Ø., Torrisen, O. and Waagbø, R. (1995). Carbohydrate nutrition in Atlantic salmon. Growth and feed utilisation. *Aquaculture Research* **26**, 149–154.

Henderson, R. J. (1996). Fatty acid metabolism in freshwater fish with particular reference to polyunsaturated fatty acids. *Archives of Animal Nutrition* **49**, 5–22.

Henderson, R. J. and Tocher, D. R. (1987). The lipid composition and biochemistry of freshwater fish. *Progress in Lipid Research* **26**, 281–347.

Henderson, R. J. and Tocher, D. R. (1992). Thin-layer chromatography. In *Lipid Analysis: A Practical Approach* (ed. Hamilton, R.J., Hamilton, S.), pp. 65–111. Oxford University, Oxford, UK.

Herath, T., Costa, J., Thompson, K., Adams, A. and Richards, R. (2009). Alternative cell line for the isolation of salmonid alphavirus-1. *Icelandic Agricultural Sciences* **22**, 19–27.

Hertrampf, J. and Piedad-Pascual, F. (2000). *Handbook on Ingredients for Aquaculture Feeds*. Kluwer Academic, The Netherlands.

Holman, R. T. (1986). Control of polyunsaturated acids in tissue lipids. *Journal of the American College of Nutrition* **5**, 183–211.

Horton, J. D., Shah, N. A., Warrington, J. A., Anderson, N. N., Park, S. W., Brown, M. S. and Goldstein, J. L. (2003). Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. *Proceedings of the National Academy of Sciences* **100**, 12027– 12032.

Izquierdo, M. (1996). Essential Fatty Acid Requirements of Cultured Marine Fish Larvae. *Aquaculture Nutrition* 183–191.

Jensen, I., Larsen, R. and Robertson, B. (2002). An antiviral state induced in Chinook salmon embryo cells (CHSE-214) by transfection with double-stranded RNA poly I:C. *Fish and Shellfish Immunology* **13**, 367–378.

Jobling, M. (1993). Nutrition, diet formulation and feeding practices. In *Salmon Aquaculture* (ed. Heen, K., Monanhan, R., and Utter, F.), pp. 83–102. Fishing News books, Oxford, UK.

Jobling, M. (2004). On-growing to market size. In *Culture of Cold- water Marine Fish* (ed. Moksness, E., Kjorsvik, E., and Olsen, Y.), pp. 363–432. Blackwell Publishing, Oxford, UK.

Jørgensen, J. B., Johansen, A., Hegseth, M. N., Zou, J., Robertsen, B., Collet, B. and Secombes, C. J. (2007). A recombinant CHSE-214 cell line expressing an Mx1 promoter-reporter system responds to both interferon type I and type II from salmonids and represents a versatile tool to study the IFN-system in teleost fish. *Fish and Shellfish Immunology* **23**, 1294–1303.

Jump, D. and Clarke, S. (1999). Regulation of gene expression by dietary fat. *Annual Revision Nutrition* **19**, 63–90.

Jung, U. J., Millman, P. N., Tall, A. R. and Deckelbaum, R. J. (2011). n-3 fatty acids ameliorate hepatic steatosis and dysfunction after LXR agonist ingestion in mice. *Biochimica et Biophysica Acta* **1811**, 491–497.

Kajikawa, S., Imada, K., Takeuchi, T., Shimizu, Y., Kawashima, A., Harada, T. and Mizuguchi, K. (2011). Eicosapentaenoic acid attenuates progression of hepatic fibrosis with inhibition of reactive oxygen species production in rats fed methionine- and choline-deficient diet. *Digestive Diseases and Sciences* **56**, 1065–1074.

Kaur, G., Sinclair, A. J., Cameron-Smith, D., Barr, D. P., Molero-Navajas, J. C. and Konstantopoulos, N. (2011). Docosapentaenoic acid (22:5n-3) down-regulates the expression of genes involved in fat synthesis in liver cells. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **85**, 155–161.

Kennedy, S. R., Leaver, M. J., Campbell, P. J., Zheng, X., Dick, J. R. and Tocher, D. R. (2006). Influence of dietary oil content and conjugated linoleic acid (CLA) on lipid metabolism enzyme activities and gene expression in tissues of Atlantic salmon (*Salmo salar* L.). *Lipids* **41**, 423–436.

Kleveland, E. J., Ruyter, B., Vegusdal, A., Sundvold, H., Berge, R. K. and Gjøen, T. (2006). Effects of 3-thia fatty acids on expression of some lipid related genes in Atlantic salmon (*Salmo salar* L.). *Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology* **145**, 239–248.

Lakra, W. S., Swaminathan, T. R. and Joy, K. P. (2011). Development, characterization, conservation and storage of fish cell lines: a review. *Fish Physiology and Biochemistry* **37**, 1–20.

Lall, S. P. (2000). Nutrition and health of fish. In: *Avances en Nutrición Acuícola V. Memorias del V Simposium Internacional de Nutrición Acuícola*, 19th-22nd November 2000, Mérida, Yucatán (Mexico). (Eds. Cruz-Suárez, L. E., Ricque-Marie, D., Tapia- Salazar, M., Olvera-Novoa, M. A. and Civera-Cerecedo, R.), pp. 13–23.

Lannan, C. N., Winton, J. R. and Fryer, J. L. (1984). Fish cell lines: establishment and characterization of nine cell lines from salmonids. *In Vitro* **20**, 671–676.

Lazarow, P. B. and de Duve, C. (1976). An Acyl-CoA oxidizing system in rat liver peroxisomes; enhancement by clofibrate, a hypolipidemic drug. *Proceedings of the National Academy of Sciences* **73**, 2043–2046.

Leaver, M. J., Tocher, D. R., Obach, A., Jensen, L., Henderson, R. J., Porter, A. R. and Krey, G. (2006). Effect of dietary conjugated linoleic acid (CLA) on lipid composition, metabolism and gene expression in Atlantic salmon (*Salmo salar*) tissues. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* **145**, 258–267.

Leaver, M. J., Villeneuve, L. A. N., Obach, A., Jensen, L., Bron, J. E., Tocher, D. R. and Taggart, J. B. (2008). Functional genomics reveals increases in cholesterol biosynthetic genes and highly unsaturated fatty acid biosynthesis after dietary substitution of fish oil with vegetable oils in Atlantic salmon (*Salmo salar*). *BMC Genomics* **9**, 299.

Lee, K. (2010). The Advantages and Limitation of Tissue Culture towards Medical Advancement in the Future Lecturer.

Lee, D., Kim, M. S., Nam, Y. K., Kim, D. S. and Gong, S. P. (2013). Establishment and characterization of permanent cell lines from oryzias dancena embryos. *Fisheries and Aquatic Sciences* **16**, 177–185.

Leger, C. (1985). Digestion, absorption and transport of lipids. In: *Nutrition and feeding fish* (Eds. Cowey, C., Mackie, J. and Bell, J.), pp. 299–331. Academic Press, London (UK).

Li, M., Kong, Z. M. and Liu, Z. L. (2006). Antioxidant enzyme activities and lipid peroxidation induced by eicosapentaenoic acid (EPA) in PC12 cells. *Cell Biology and Toxicology* **22**, 331–337.

Lie, Ø., Lied, E. and Lambertsen, G. (1987). Lipid digestion in cod (Gadus morua). *Comparative Biochemistry and Physiology - Part B: Comparative Biochemistry* **88**, 697–700.

Liland, N. S., Rosenlund, G., Berntssen, M. H. G., Brattelid, T., Madsen, L. and Torstensen, B. E. (2013). Net production of Atlantic salmon (FIFO, Fish in Fish out) with dietary plant proteins and vegetable oils. *Aquaculture Nutrition* **19**, 289–300.

Lin, J., Yang, R., Tarr, P. T., Wu, P. H., Handschin, C., Li, S., Yang, W., Pei, L., Uldry, M., Tontonoz, P., Newgard, C. B. and Spiegelman, B. M. (2005). Hyperlipidemic effects of dietary saturated fats mediated through PGC-1beta coactivation of SREBP. *Cell* **120**, 261–273.

Lorenzen, E., Carstensen, B. and Olesen, N. J. (1999). Inter-laboratory comparison of cell lines for susceptibility to three viruses: VHSV, IHNV and IPNV. *Diseases of Aquatic Organisms* **37**, 81–88.

Manickam, E., Sinclair, A. J. and Cameron-Smith, D. (2010). Suppressive actions of eicosapentaenoic acid on lipid droplet formation in 3T3-L1 adipocytes. *Lipids in Health and Disease* **9**, 57.

Martinez-Rubio, L., Morais, S., Evensen, Ø., Wadsworth, S., Ruohonen, K., Vecino, J. L. G., Bell, J. G. and Tocher, D. R. (2012). Functional feeds reduce heart inflammation and pathology in Atlantic Salmon (*Salmo salar* L.) following experimental challenge with Atlantic salmon reovirus (ASRV). *PLoS One* **7**, e40266.

Martinez-Rubio, L., Wadsworth, S., González Vecino, J. L., Bell, J. G. and Tocher, D. R. (2013). Effect of dietary digestible energy content on expression of genes of lipid metabolism and LC-PUFA biosynthesis in liver of Atlantic salmon (*Salmo salar* L.). *Aquaculture* **384-387**, 94–103.

McGarry, J. D. and Brown, N. F. (1997). The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *European Journal of Biochemistry* **244**, 1–14.

McKenney, J. and Sica, M. D. (2007). Role of prescription omega-3 fatty acids in the treatment of hypertriglyceridemia. *Pharmacotherapy* **27**, 715–728.

McLoughlin, M. F. and Graham, D. A. (2007). Alphavirus infection in salmonids - a review. *Journal of Fish Diseases* **30**, 511–531.

Menoyo, D., López-Bote, C. J., Obach, A. and Bautista, J. M. (2005). Effect of dietary fish oil substitution with lindseed oil on the performance, tissue fatty acid profile, metabolism, and oxidative stability of Atlantic salmon. *Journal of Animal Science* **83**, 2853–2862.

Miller, M. R., Nichols, P. D. and Carter, C. G. (2008). n-3 Oil sources for use in aquaculture--alternatives to the unsustainable harvest of wild fish. *Nutrition Research Reviews* **21**, 85–96.

Minghetti, M., Leaver, M. J. and Tocher, D. R. (2011). Transcriptional control mechanisms of genes of lipid and fatty acid metabolism in the Atlantic salmon (*Salmo salar* L.) established cell line, SHK-1. *Biochimica et Biophysica Acta* **1811**, 194–202.

Monroig, Ó., Rotllant, J., Sánchez, E., Cerdá-Reverter, J. M. and Tocher, D. R. (2009). Expression of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis genes during zebrafish *Danio rerio* early embryogenesis. *Biochimica et Biophysica Acta* **1791**, 1093–1101.

Monroig, Ó., Zheng, X., Morais, S., Leaver, M. J., Taggart, J. B. and Tocher, D. R. (2010). Multiple genes for functional Δ6 fatty acyl desaturases (Fad) in Atlantic salmon (*Salmo salar* L.): Gene and cDNA characterization, functional expression, tissue distribution and nutritional regulation. *Biochimica et Biophysica Acta* **1801**, 1072–1081.

Monroig, O., Li, Y. and Tocher, D. R. (2011a). Delta-8 desaturation activity varies among fatty acyl desaturases of teleost fish: high activity in delta-6 desaturases of marine species. *Comparative Biochemistry and Physiology B Biochemistry & Molecular Biology* **159**, 206–213.

Monroig, Ó., Navarro, J. C. and Tocher, D. R. (2011b). Long-chain polyunsaturated fatty acids in fish : recent advances on desaturases and elongases involved in their biosynthesis. In: *Avances en Nutrición Acuícola XI – Memorias del Décimo Primer Simposio Internacional de Nutrición Acuícola*, 23rd-25th November 2011, San Nicolás de los Garza, Nuevo León (Mexico). (Eds. Cruz-Suárez, L. E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M. G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J. and Hernández-Hernández, L.), pp. 257–283.

Montero, D., Kalinowski, T., Obach, A., Robaina, L., Tort, L., Caballero, M. J. and Izquierdo, M. S. (2003). Vegetable lipid sources for gilthead seabream (*Sparus aurata*): effects on fish health. *Aquaculture* **225**, 353–370.

Morais, S., Monroig, O., Zheng, X. Z., Leaver, M. J. and Tocher, D. R. (2009). Highly unsaturated fatty acid synthesis in Atlantic salmon: characterization of ELOVL5-and ELOVL2-like elongases. *Marine Biotechnology* **11**, 627–639.

Morais, S., Mourente, G., Ortega, A., Tocher, J. A. and Tocher, D. R. (2011a). Expression of fatty acyl desaturase and elongase genes, and evolution of DHA:EPA ratio during development of unfed larvae of Atlantic bluefin tuna (*Thunnus thynnus* L.). *Aquaculture* **313**, 129–139.

Morais, S., Pratoomyot, J., Taggart, J. B., Bron, J. E., Guy, D. R., Bell, J. G. and Tocher, D. R. (2011b). Genotype-specific responses in Atlantic salmon (*Salmo salar*) subject to dietary fish oil replacement by vegetable oil: a liver transcriptomic analysis. *BMC Genomics* **12**, 255.

Moskowitz, M. S. (1967). Fatty acid-induced steatosis in monolayer cell cultures. In: *Lipid metabolism in tissue culture cells* (Eds. Rothblat, G. H. and Kritchevsky, D.), pp. 49–59. Wistar, Philadelphia (USA).

Mourente, G. and Vazquez, R. (1996). Changes in the content of total lipid, lipid classes and fatty acids of developing eggs and unfed larvae of the Senegal sole, *Solea senegalensis* Kaup. *Fish Physiology and Biochemistry* **15**, 221–235.

Mourente, G., Tocher, D. R., Díaz, E., Grau, A. and Pastor, E. (1999). Relationships between antioxidant enzyme activities and lipid peroxidation products during early development in *Dentex dentex* eggs and larvae. *Aquaculture* **179**, 309–324.

Mourente, G., Bell, J. G. and Tocher, D. R. (2007). Does dietary tocopherol level affect fatty acid metabolism in fish? *Fish Physiology and Biochemistry* **33**, 269–280.

Naylor, R. L., Goldburg, R. J., Primavera, J. H., Kautsky, N., Beveridge, M. C., Clay, J., Folke, C., Lubchenco, J., Mooney, H. and Troell, M. (2000). Effect of aquaculture on world fish supplies. *Nature* **405**, 1017–1024.

Naylor, R. L., Hardy, R. W., Bureau, D. P., Chiu, A., Elliott, M., Farrell, A. P., Forster, I., Gatlin, D. M., Goldburg, R. J., Hua, K. and Nichols, P. D. (2009). Feeding aquaculture in an era of finite resources. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 15103–15110.

Nelson, D. and Cox, M. (2008). *Principles of Biochemistry*. Freeman W.H. & Co. Ltd., New York (USA), pp. 1100.

Ng, W. K., Sigholt, T. and Bell, J. G. (2004). The influence of environmental temperature on the apparent nutrient and fatty acid digestibility in Atlantic salmon (*Salmo salar* L.) fed finishing diets containing different blends of fish oil, rapeseed oil and palm oil. *Aquaculture Research* **35**, 1228–1237.

Okuyama, H., Kobayashi, T. and Watanabe, S. (1997). Dietary fatty acids - the n-6/n-3 balance and chronic elderly diseases. Excess linoleic acid and relative n-3 deficiency syndrome seen in Japan. *Progress in Lipid Research* **35**, 409–457.

Olsen, R. E., Henderson, R. J. and Ringø, E. (1998). The digestion and selective absorption of dietary fatty acids in Arctic charr, *Salvelinus alpinus*. *Aquaculture Nutrition* **4**, 13–21.

Oosterveer, M. H., Dijk, T. H. van, Tietge, U. J., Boer, T., Havinga, R., Stellaard, F., Groen, A. K., Kuipers, F. and Reijngoud, D. (2009). High fat feeding induces hepatic fatty acid elongation in mice. *PLoS One* **4**, e6066.

Ostrowski, A. C. and Divakaran, S. (1991). Energy substrates for eggs and prefeeding larvae of the dolphin Coryphaena hippurus. *Marine Biology* **109**, 149–155.

Owen, J. M., Adron, J. A., Middleton, C. and Cowey, C. B. (1975). Elongation and desaturation of dietary fatty acids in turbot (*Scophthalmus maximus*) and rainbow trout (*Salmo gairdneri*). *Lipids* **10**, 528–531.

Page, R. D. (1996). TREEVIEW: An application to display phylogenetic tress on personal computers. *Computer Applications in the Biosciences* **12**, 357–358.

Peña, A. A., Bols, N. C. and Marshall, S. H. (2010). An evaluation of potential reference genes for stability of expression in two salmonid cell lines after infection with either *Piscirickettsia salmonis* or IPNV. *BMC Research Notes* **3**, 101.

Peng, S., Chen, L., Qin, J. G., Hou, J., Yu, N., Long, Z., Ye, J. and Sun, X. (2008). Effects of replacement of dietary fish oil by soybean oil on growth performance and liver biochemical composition in juvenile Black seabream, *Acanthopagrus schlegeli*. *Aquaculture* **276**, 154–161.

Pérez, E., Bourguet, W., Gronemeyer, H. and de Lera, A. R. (2012). Modulation of RXR function through ligand design. *Biochimica et Biophysica Acta* **1821**, 57–69.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**, e45.

Polvi, S. M. and Ackman, R. G. (1992). Atlantic salmon (*Salmo salar*) muscle lipids and their response to alternative dietary fatty acid sources. *Journal of Agricultural and Food Chemistry* **40**, 1001–1007.

Pratoomyot, J., Bendiksen, E. A., Bell, J. G. and Tocher, D. R. (2008). Comparison of effects of vegetable oils blended with southern hemisphere fish oil and decontaminated northern hemisphere fish oil on growth performance, composition and gene expression in Atlantic salmon (*Salmo salar* L.). *Aquaculture* **280**, 170–178.

Rainuzzo, J. R., Reitan, K. I. and Joergensen, L. (1992). Comparative study on the fatty acid and lipid composition of four marine fish larvae. *Comparative Biochemistry and Physiology - Part B: Comparative Biochemistry* **103**, 21–26.

Repa, J. J., Turley, S. D., Lobaccaro, J. A., Medina, J., Li, L., Lustig, K., Shan, B. and Heyman, R. A. (2000). Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science* **289**, 1524–1529.

Repa, J. J., Berge, K. E., Pomajzl, C., Richardson, J. A., Hobbs, H. and Mangelsdorf, D. J. (2002). Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors alpha and beta. *Journal of Biology Chemistry* **277**, 18793–18800.

Ronnestad, I., Koven, W. M., Tandler, A., Harel, M. and Fyhn, H. J. (1994). Energy metabolism during development of eggs and larvae of gilthead sea bream (*Sparus aurata*). *Marine Biology* **120**, 187–196.

Ronnestad, I., Finn, R. N., Lein, I. and Lie, O. (1995). Compartmental changes in the contents of total lipid, lipid classes and their associated fatty acids in developing yolk-sac larvae of Atlantic halibut, *Hippoglossus hippoglossus* (L.). *Aquaculture Nutrition* **1**, 119–130.

Ronnestad, I., Koven, W. M., Tandler, A., Harel, M. and Fyhn, H. J. (1998). Utilisation of yolk fuels in developing eggs and larvae of European sea bass (*Dicentrarchus labrax*). *Aquaculture* **162**, 157–178.

Rora, A., Birkeland, S., Hultmann, L., Rustad, T., Skara, T. and Bjerkeng, B. (2005). Quality characteristics of farmed Atlantic salmon (*Salmo salar*) fed diets high in soybean or fish oil as affected by cold-smoking temperature. *Food Science and Technology* 201–211.

Rosenlund, G., Obach, A., Sandberg, M. G., Standal, H. and Tveit, K. (2001). Effect of alternative lipid sources on long-term growth performance and quality of Atlantic salmon (*Salmo salar*). *Aquaculture Research* **32**, 323–328.

Rosenthal, M. . (1981). Accumulation of neutral lipids by human skin fibroblasts: Differential effects of saturated and unsaturated fatty acids. *Lipids* **16**, 173–182.

Rozen, S. and Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods in Molecular Biology* **132**, 365– 386.

Rustan, A. C., Nossen, J. O., Christiansen, E. N. and Drevon, C. A. (1988). Eicosapentaenoic acid reduces hepatic synthesis and secretion of triacylglycerol by decreasing the activity of acyl-coenzyme A:1,2-diacylglycerol acyltransferase. *Journal of Lipid Research* **29**, 1417–1426.

Ruxton, C. H. S., Calder, P. C., Reed, S. C. and Simpson, M. J. A. (2005). The impact of long-chain n-3 poliunsaturated fatty acids on human health. *Nutrition Research Reviews* **18**, 113–129.

Salem, J. N., Litman, B., Kim, H.-Y. and Gawrisch, K. (2001). Mechanisms of action of docosahexaenoic acid in the nervous system. *Lipids* **36**, 945–949.

Sanford, K. K., Earle, W. R. and Likely, G. D. (1948). The growth in vitro of single isolated tissue cells. *Journal of the National Cancer Institute* **9**, 229–246.

Sargent, J. R. (1976). The structure, metabolism and function of lipids in marine organisms. In: *Biochemical and biophysical perspectives in marine biology* (Eds. Malins, D. C. and Sargent, J. R.), pp. 149–212. Academic Press, New York (USA).

Sargent, J. R., Henderson, R. J. and Tocher, D. R. (1989). The lipids. In: *Fish Nutrition* (Eds. Halver, J. E. and Hardy, R. W.), pp. 154–218. Academic Press, London (UK).

Sargent, J. R., Bell, M. V., Bell, J. G., Henderson, R. J. and Tocher, D. R. (1995a). Evolution and roles of (n-3) polyunsaturated fatty acids in marine organisms. In: *Phospholipids: Characterization, Metabolism and Novel Biological Applications* (Eds. Cevc, G. and Paltauf, F.), pp. 248–259. American Oil Chemists' Society Press, Champaign (USA).

Sargent, J. R., Bell, J. G., Bell, M. V., Henderson, R. J. and Tocher, D. R. (1995b). Requirement criteria for essential fatty acids. *Journal of Applied Ichthyology* **11**, 183–198.

Sargent, J. R., McEvoy, L. A. and Bell, J. G. (1997). Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. *Aquaculture* **155**, 117–127.

Sargent, J. R., Tocher, D. R. and Bell, J. G. (2002). The lipids. In: *Fish Nutrition* (Eds. Halver, J. E. and Hardy, R. W.), pp. 181–257. Academic Press, London (UK).

Schirmer, K. (2006). Proposal to improve vertebrate cell cultures to establish them as substitutes for the regulatory testing of chemicals and effluents using fish. *Toxicology* **224**, 163–183.

Schultz, J. R., Tu, H., Luk, A., Pepa, J. J., Medina, J. C., Li, L., Schwendner, S., Wang, S., Thoolen, M., Mangelsdorf, D. J., Lustig, K. D. and Shan, B. (2000). Role of LXRs in control of lipogenesis. *Genes and Development* **14**, 2831–2838.

Seierstad, S. L., Poppe, T. T., Koppang, E. O., Svindland, A., Rosenlund, G., Frøyland, L. and Larsen, S. (2005). Influence of dietary lipid composition on cardiac pathology in farmed Atlantic salmon, *Salmo salar* L . *Journal of Fish Diseases* **28**, 677–690.

Sekiya, M., Yahagi, N., Matsuzaka, T., Najima, Y., Nakakuki, M., Nagai, R., Ishibashi, S., Osuga, J., Yamada, N. and Shimano, H. (2003). Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression. *Hepatology* **38**, 1529–1539.

Serhan, C. N., Chiang, N. and Van Dyke, T. E. (2008). Resolving inflammation: dual anti- inflammatory and pro-resolution lipid mediators. *Nature Reviews Immunology* **8**, 349–361.

Shirota, T., Haji, S., Yamasaki, M., Iwasaki, T., Hidaka, T., Takeyama, Y., Shiozaki, H. and Ohyanagi, H. (2005). Apoptosis in human pancreatic cancer cells induced by eicosapentaenoic acid. *Nutrition* **21**, 1010–1017.

Siddiqui, R. A., Harvey, K. and Stillwell, W. (2008). Anticancer properties of oxidation products of docosahexaenoic acid. *Chemistry and Physics of Lipids* **153**, 47–56.

Simopoulos, A. P. (2008). The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Experimental Biology and Medicine* **233**, 674–688.

Smith, R. R. (1989). Fish Nutrition. In: *Fish Nutrition* (Ed. Halver, J. E.), pp. 2-29. Academic Press, San Diego (USA).

Smith, A. (2000). *Oxford dictionary of biochemistry and molecular biology*. 2nd edition. Oxford University Press, Oxford, (UK), pp. 720.

Smith, P. A., Reveco, F., Contreras, J., Rojas, M. E., Venegas, C. and Guajardo, A. (2010). Infectivity study of *Piscirickettsia salmonis* in CHSE-214 cells by confocal and transmission electron microscopy. *Bulletin of the European Association of Fish Pathologists* **30**, 128–136.

Sokal, R. R. and Rohlf, F. J. (1998). *Biometry*. Freeman W.H. & Co. Ltd., New York (USA), pp. 672.

Spector, A. A. and Hoak, J. C. (1969). An improved method for the addition of long-chain free fatty acids to protein solutions. *Analytical Biochemistry* **32**, 297–302.

Spector, A. A., Mathur, S. N., Kaduce, T. L. and Hyman, B. T. (1981). Lipid nutrition and metabolism of cultured mammalian cells. *Progress in Lipid Research* **19**, 155–186.

Stubbs, C. D. and Smith, A. D. (1984). The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. *Biochimica et Biophysica Acta* **779**, 89–137.

Tacon, A. G. J. (1996). Lipid nutritional pathology in farmed fish. *Archives of Animal Nutrition* **49**, 33–39.

Tacon, A. G. J. and Metian, M. (2008). Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: trends and future prospects. *Aquaculture* **285**, 146–158.

Taggart, J. B., Bron, J. E., Martin, S. A. M., Seear, P. J., Hoyheim, B., Talbot, R., Villeneuve, L., Sweeney, G. E., Houlihan, D. F., Secombes, C. J., Tocher, D. R. and Teale, A. J. (2008). A description of the origins, design and performance of the TRAITS/SGP Atlantic salmon (*Salmo salar* L.) cDNA microarray. *Journal of Fish Biology* **72**, 2071–2094.

Takeuchi, T., Shiina, Y., Watanabe, T., Sekiya, S. and Imaizumi, K. (1992). Suitable levels of n−3 highly unsaturated fatty acids in diet for fingerlings of yellowtail. *Nippon Suisan Gakkaishi* **58**, 509–514.

Tang, Z., Sun, C., Yan, A., Wu, S., Qin, C., Zhang, Y. and Li, W. (2013). Genes involved in fatty acid metabolism: molecular characterization and hypothalamic mRNA response to energy status and neuropeptide Y treatment in the orange-spotted grouper *Epinephelus coioides*. *Molecular and Cellular Endocrinology* **376**, 114–124.

Tinoco, J. (1982). Dietary requirements and functions of alpha-linolenic acid in animals. *Progress in Lipid Research* **21**, 1–45.

Tocher, D. R. (1990). Incorporation and metabolism of (n-3) and (n-6) polyunsaturated fatty acids in phospholipid classes in cultured rainbow trout (*Salmo gairdneri*). *Fish Physiology and Biochemistry* **8**, 239–249.

Tocher, D. R. (1995). Glycerophospholipid metabolism. In: *Biochemistry and Molecular Biology of Fishes, Vol. IV* (Eds. Hochachka, P. W. and Mommsen, T. P.), pp. 119–157. Elsevier Press, Amsterdam (The Netherlands).

Tocher, D. R. (2003). Metabolism and functions of lipids and fatty acids in teleost fish. *Reviews in Fisheries Science* **11**, 1–78.

Tocher, D. R. (2009). Issues surrounding fish as a source of omega-3 long-chain polyunsaturated fatty acids. *Lipid Technology* **21**, 13–16.

Tocher, D. R. (2010). Fatty acid requirements in ontogeny of marine and freshwater fish. *Aquaculture Research* **41**, 717–732.

Tocher, D. R. (2015). Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. *Aquaculture*, In Press.

Tocher, D. R. and Dick, J. R. (1990). Polyunsaturated fatty acid metabolism in cultured fish cells: Incorporation and metabolism of (n-3) and (n-6) series acids by Atlantic salmon (*Salmo salar*) cells. *Fish Physiology and Biochemistry* **8**, 311–319.

Tocher, D. R. and Dick, J. R. (1991). Effect of polyunsaturated fatty acids on the growth of fish cells in culture. *Comparative Biochemistry and Physiology - Part A: Physiology* **100**, 461–466.

Tocher, D. R. and Dick, J. R. (1999). Polyunsaturated fatty acid metabolism in a cell culture model of essential fatty acid deficiency in a freshwater fish, carp (*Cyprinus carpio*). *Fish Physiology and Biochemistry* **21**, 257–267.

Tocher, D. R. and Ghioni, C. (1999). Fatty acid metabolism in marine fish: low activity of fatty acyl D5 desaturation in gilthead sea bream (*Sparus aurata*) cells. *Lipids* **34**, 433–440.

Tocher, D. R. and Sargent, J. R. (1987). The effects of calcium ionophore A23187 on the metabolism of arachidonic and eicosapentaenoic acids in neutrophils from a marine teleost fish rich in (n-3) polyunsaturated fatty acids. *Comparative Biochemistry and Physiology - Part B: Comparative Biochemistry* **87**, 733–739.

Tocher, D. R. and Sargent, J. R. (1990). Effect of temperature on the incorporation into phospholipid classes and metabolism via desaturation and elongation of n-3 and n-6 polyunsaturated fatty acids in fish cells in culture. *Lipids* **25**, 435–442.

Tocher, D. R., Sargent, J. R. and Frerichs, G. N. (1988). The fatty acid compositions of established fish cell lines after long-term culture in mammalian sera. *Fish Physiology and Biochemistry* **5**, 219–227.

Tocher, D. R., Carr, J. and Sargent, J. R. (1989). Polyunsaturated fatty acid metabolism in fish cells: Differential metabolism of (n-3) and (n-6) series acids by culture cells originating from a freshwater teleost fish and from a marine teleost fish. *Comparative Biochemistry and Physiology - Part B: Comparative Biochemistry* **94**, 367–374.

Tocher, D. R., Mourente, G. and Sargent, J. R. (1992). Metabolism of [1-14C] docosahexaenoate (22:6n-3), [1-14C] eicosapentaenoate (20:5n-3) and [1-14C] linolenate (18:3n-3) in brain cells from juvenile turbot *Scophthalmus maximus*. *Lipids* **27**, 494–499.

Tocher, D. R., Dick, J. R. and Sargent, J. R. (1995). Development of an in vitro model of essential fatty acid deficiency in fish cells. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **53**, 365–375.

Tocher, D. R., Dick, J. R. and Sargent, J. R. (1996). Stimulation of proliferation of an essential fatty acid-deficient fish cell line by C_{20} and C_{22} polyunsaturated fatty acids and effects on fatty acid composition. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **55**, 345–356.

Tocher, D. R., Bell, J. G., Dick, J. R. and Sargent, J. R. (1997). Fatty acyl desaturation in isolated hepatocytes from Atlantic salmon (*Salmo salar*): stimulation by dietary borage oil containing γ-linolenic acid. *Lipids* **32**, 1237–1247.

Tocher, D. R., Leaver, M. J. and Hodgson, P. A. (1998). Recent advances in the biochemistry and molecular biology of fatty acyl desaturases. *Progress in Lipid Research* **37**, 73–117.

Tocher, D. R., Bell, J. G., Dick, J. R., Henderson, R. J., McGhee, F., Michell, D. and Morris, P. C. (2000). Polyunsaturated fatty acid metabolism in Atlantic salmon (*Salmo salar*) undergoing parr-smolt transformation and the effects of dietary linseed and rapeseed oils. *Fish Physiology and Biochemistry* **23**, 59–73.

Tocher, D. R., Agaba, M., Hastings, N., Bell, J. G., Dick, J. R. and Teale, A. J. (2002). Nutritional regulation of hepatocyte fatty acid desaturation and polyunsaturated fatty acid composition in zebrafish (*Danio rerio*) and tilapia (*Oreochromis niloticus*). *Fish Physiology and Biochemistry* **24**, 309–320.

Tocher, D. R., Bell, J. G., Dick, J. R. and Crampton, V. O. (2003a). Effects of dietary vegetable oil on Atlantic salmon hepatocyte fatty acid desaturation and liver fatty acid compositions. *Lipids* **38**, 723–732.

Tocher, D. R., Bell, J. G., McGhee, F., Dick, J. R. and Fonseca-Madrigal, J. (2003b). Effects of dietary lipid level and vegetable oil on fatty acid metabolism in Atlantic salmon (*Salmo salar* L.) over the whole production cycle. *Fish Physiology and Biochemistry* **29**, 193–209.

Tocher, D. R., Agaba, M., Hastings, N. and Teale, A. (2003c). Biochemical and molecular studies of the polyunsaturated fatty acid desaturation pathway in fish. In: *The Big Fish Bang: Proceedings of the 26th Annual Larval Fish Conference* (Eds. Browman, H.I. and Skiftesvik, A.B.), Norwegian Institute of Marine Research, Bergen (Norway), pp. 211–228.

Tocher, D. R., Zheng, X., Schlechtriem, C., Hastings, N., Dick, J. . and Teale, A. J. (2006). Highly unsaturated fatty acid synthesis in marine fish; cloning, functional characterization and nutritional regulation of fatty acid Δ6 desaturase of Atlantic cod (*Gadus morhua* L.). *Lipids* **41**, 1003–1016.

Toit-Kohn, J. L., Louw, L. and Engelbrecht, A. M. (2009). Docosahexaenoic acid induces apoptosis in colorectal carcinoma cells by modulating the PI3 kinase and p38 MAPK pathways. *The Journal of Nutritional Biochemistry* **20**, 106–114.

Torstensen, B. E., Lie, O. and Froyland, L. (2000). Lipid metabolism and tissue composition in Atlantic salmon (*Salmo salar* L.) – effects of capelin oil, palm oil, and oleic acid-enriched sunflower oil as dietary lipid sources. *Lipids* **35**, 653–664.

Torstensen, B., Froyland, L. and Lie, O. (2004). Replacing dietary fish oil with increasing levels of rapeseed oil and olive oil effects on Atlantic salmon (*Salmo salar* L.) tissue and lipoprotein lipid composition and lipogenic enzyme activities. *Aquaculture Nutrition* 175–192.

Torstensen, B. E., Bell, J. G., Rosenlund, G., Henderson, R. J., Graff, I. E., Tocher, D. R., Øyvind, L., Sargent, J. R., Lie, Ø. and Sargent, J. R. (2005). Tailoring of Atlantic salmon (*Salmo salar* L.) flesh lipid composition and sensory quality by replacing fish oil with a vegetable oil blend. *Journal of Agricultural and Food Chemistry* **53**, 10166–10178.

Trushensk, J. T. and Boesenberg, J. (2009). Influence of dietary fish oil concentration and finishing duration on beneficial fatty acid profile restoration in sunshine bass *Morone chrysops* ♀ and *M. Saxatilis* ♂. *Aquaculture* **296**, 277–283.

Turchini, G. M., Torstensen, B. E. and Ng, W.-K. (2009). Fish oil replacement in finfish nutrition. *Reviews in Aquaculture* **1**, 10–57.

Turchini, G. M., Ng, W.-K. and Tocher, D. R. (2011). *Fish oil replacement and alternative lipid sources in aquaculture feeds*. Taylor and Francis group, Boca Raton (USA), pp. 533.

Vazquez, R., Gonzalez, S., Rodriguez, A. and Mourente, G. (1994). Biochemical composition and fatty acid content of fertilized eggs, yolk sac stage larvae and first-feeding larvae of the Senegal sole (*Solea senegalensis* Kaup). *Aquaculture* **119**, 273–286.

Viegas, M. N., Dias, J., Cancela, M. L. and Laizé, V. (2012). Polyunsaturated fatty acids regulate cell proliferation, extracellular matrix mineralization and gene expression in a gilthead seabream skeletal cell line. *Journal of Applied Ichthyology* **28**, 427–432.

Villeneuve, L., Leaver, M., Obach, A. and Tocher, D. R. (2007). Effects of dietary vegetable oils on gene expression and lipid metabolism in Atlantic salmon (*Salmo salar*). *Proceedings of Aquaculture Europe 2007*, Istanbul (Turkey), 24th-27th October 2007, European Aquaculture Society, p.588.

Visoottiviseth, P. and Chanwanna, N. (2001). A newly stablished cell culture from hybrid catfish (*Clarias gariepinus* × *Clarias macrocephalus*) for screening toxicity of triphenyltin hydroxide. *Applied Organometallic Chemistry* **15**, 463–472.

Waagbø, R. (1994). The impact of nutritional factors on the immune system in Atlantic salmon, *Salmo salar* L.: a review. *Aquaculture Research* **25**, 175–197.

Watanabe, T. (1982). Lipid nutrition in fish. *Comparative Biochemistry and Physiology - Part B: Comparative Biochemistry* **73**, 3–15.

Weiss, K., Mihály, J., Liebisch, G., Marosvölgyi, T., Schmitz, G., Decsi, T. and Rühl, R. (2011). Effect of synthetic ligands of PPAR α, β/δ, γ, RAR, RXR and LXR on the fatty acid composition of phospholipids in mice. *Lipids* **46**, 1013–1020.

Weylandt, K. H., Chiua, C.-Y., Gomolkaa, B., Waechtera, S. F. and Wiedenmann, B. (2012). Omega-3 fatty acids and their lipid mediators: towards an understanding of resolvin and protectin formation.Omega-3 fatty acids and their resolvin/protectin mediators. *Prostaglandins and Other Lipid Mediators* **97**, 73–82.

Whelan, J. (1996). Antagonistic effects of dietary arachidonic acid and n-3 polyunsaturated fatty acids. *The Journal of Nutrition* **126**, 1086S–1091S.

Whelan, J., Surette, M. E., Li-Stiles, B.-Y. and Bailey, J. W. (1995). Evidence that dietary arachidonic acid increases circulating triglycerides. *Lipids* **30**, 425–429.

Wiegand, M. D. (1996). Composition, accumulation and utilization of yolk lipids in teleost fi sh. *Reviews in Fish Biology and Fisheries* **6**, 259–286.

Wolf, K. (1988). *Fish viruses and fish viral diseases*. Cornell University Press, Ithaca (USA), pp. 476.

Wolf, K. and Quimby, M. C. (1962). Established eurythermic line of fish cells in vitro. *Science* **135**, 1065–1066.

Wolf, K. and Quimby, C. (1976). Primary monolayer culture of fish cells initiated from trypsinized tissues. *Tissue Culture Association Manual* **2**, 453–456.

Xu, H., Dong, X., Ai, Q., Mai, K., Xu, W., Zhang, Y. and Zuo, R. (2014). Regulation of tissue LC-PUFA contents, Δ6 Fatty Acyl Desaturase (FADS2) gene expression and the methylation of the putative FADS2 gene promoter by different dietary fatty acid profiles in Japanese seabass (Lateolabrax japonicus). *PLoS One* **9**, e87726.

Yaqoob, P. (2004). Fatty acid and the immune system: from basic science to clinical applications. *Proceedings of the Nutrition Society* **63**, 89–104.

Zheng, X., Tocher, D. R., Dickson, C. a., Bell, J. G. and Teale, A. J. (2004). Effects of diets containing vegetable oil on expression of genes involved in highly unsaturated fatty acid biosynthesis in liver of Atlantic salmon (*Salmo salar*). *Aquaculture* **236**, 467–483.

Zheng, X., Torstensen, B. E., Tocher, D. R., Dick, J. R., Henderson, R. J. and Bell, J. G. (2005). Environmental and dietary influences on highly unsaturated fatty acid biosynthesis and expression of fatty acyl desaturase and elongase genes in liver of Atlantic salmon (*Salmo salar*). *Biochimica et Biophysica Acta* **8**, 13–24.

Zheng, X., Ding, Z., Xu, Y., Monroig, O., Morais, S. and Tocher, D. R. (2009a). Physiological roles of fatty acyl desaturases and elongases in marine fish: characterisation of cDNAs of fatty acyl Δ6 desaturase and elovl5 elongase of cobia (*Rachycentron canadum*). *Aquaculture* **290**, 122–131.

Zheng, X., Leaver, M. J. and Tocher, D. R. (2009b). Long-chain polyunsaturated fatty acid synthesis in fish: Comparative analysis of Atlantic salmon (*Salmo salar* L.) and Atlantic cod (*Gadus morhua* L.) Delta6 fatty acyl desaturase gene promoters. *Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology* **154**, 255–263.

Research Outputs

LIST OF PUBLISHED PAPERS DURING THE PHD

Paladini, G., Hansen, H., Williams, C.F., Taylor, N.G.H., **Rubio-Mejía, O.L.**, Denholm, S.J., Hytterød, S., Bron J.E. and Shinn, A.P. (2014). Reservoir hosts for *Gyrodactylus salaris* may play a more significant role in epidemics than previously thought. *Parasites & Vectors* **7**, 576.

Almaida-Pagán, P.F., De Santis, C., **Rubio-Mejía, O.L.** and Tocher, D.R. (2015). Dietary fatty acids affect mitochondrial phospholipid compositions and mitochondrial gene expression of rainbow trout liver at different ages. *Journal of Comparative Physiology B* **185**, 73–86.

LIST OF PAPERS IN PREPARATION

Rubio-Mejía, O.L., Betancor, M.B. and Tocher, D.R. (*in prep.*). Effects of interaction and competition between supplemented PUFA on lipid and fatty acid compositions of CHSE-214 cells.

Rubio-Mejía, O.L., Dick, J.R. and Tocher, D.R. (*in prep.*). Effects of interaction between C_{18} PUFA and saturated/monounsaturated fatty acids on lipid and fatty acid compositions of CHSE-214 cells.

COLLABORATIVE POSTERS AND ORAL COMMUNICATIONS DELIVERED AT CONFERENCES DURING THE PHD

Rubio-Mejía, O.L., Leaver, M.J. and Tocher, D.R. (2012). Development and utilisation of *in vitro* cell culture model for an integrated approach to study the biochemical and molecular regulation of lipid metabolism in fish. *Proceedings of the 3rd Institute of Aquaculture PhD Research Conference, Stirling (UK),* 24th October *(poster).*

Rubio-Mejía, O.L., Betancor, M.B. and Tocher, D.R. (2014). Utilisation of CHSE-214 cell line for an integrated approach to study the biochemical and molecular regulation of lipid metabolism in fish. *Proceedings of Aquaculture Europe*, Donostia-San Sebastian (Spain), 14th–17th October, abstract 659 *(talk).*

Rubio-Mejía, O.L., Betancor, M.B. and Tocher, D.R. (2015). The potential of the CHSE-214 cell line as a model to study molecular regulation of EPA synthesis in salmonids. *Proceedings of the 4th Institute of Aquaculture PhD Research Conference, Stirling (UK),* 18th February (*talk*).